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# Detection of Influenza Virus Neuraminidase-Specific Antibodies by an Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay was developed for the titration of antibodies in human sera to influenza virus neuraminidase, employing partially purified N1 neuraminidase. Specificity of the test was demonstrated, and the test was more sensitive than either the conventional neuraminidase inhibition or plaque size reduction tests in detecting anti-neuraminidase antibody.

Antibodies to the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), of influenza A viruses confer immunity upon the host to the inducing virus subtype (21–23). The conventional hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests are not adequately sensitive to detect low concentrations of these antibodies, because protection has sometimes been noted in the absence of these antibodies as detected by the HI and NI tests (17, 20, 26). However, with the recent development of the enzyme-linked immunosorbent assay (ELISA) (6, 24) low levels of antibodies to the HA can now be accurately measured (17).

Detection of antibodies to NA has relied mainly on the comparatively insensitive NI test. Antibodies detected by this test may not represent the complete population of NA antibodies, and therefore, this test gives an underestimation of the contribution of NA antibodies in immunity to influenza virus. The mechanism by which these antibodies function in immunity probably is variable and complex (15) and includes their ability to aggregate virus particles, as well as any role they may have in inactivating enzymatic activity (11, 12). Antibodies detected by the NI test are not directed against the active site of the enzyme but inhibit by sterically blocking access to large substrates (7). By extrapolation, therefore, it seems reasonable that antibodies directed against the entire molecule (as detected by ELISA) would be more efficient at aggregation or inhibiting viral diffusion than those antibodies responsible only for enzymatic inhibition.

A further limitation in the NI test stems from the fact that an active enzymatic preparation in the form of whole virus is routinely used for antibody detection. Antibodies directed against viral surface components, e.g., the HA, can cause nonspecific enzymatic inhibition due to steric inhibition (13). In this report we describe an ELISA system utilizing a partially purified NA for the detection of NA-specific antibodies. This procedure can be adapted to large-scale screening of NA response, as in vaccine studies.

## MATERIALS AND METHODS

**ELISA.** The procedure used was a modification of that used by Voller et al. (25). Antigen was diluted in a 0.015 M $Na_2CO_3$–0.035 M $NaHCO_3$ (pH 9.6) carbonate buffer, and the optimal concentration was obtained by checkerboard titration of the antigen and a known positive serum. Immulon flat-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were sensitized with 100 μl of diluted antigen per well and incubated for 18 h (overnight) in a moist chamber at 4°C. Wells were washed three times in a solution of 0.15 M phosphate-buffered saline (pH 7.4) containing 0.05% polyoxyethylene sorbitan monolaurate (PBS-Tween), and 100 μl of a solution of PBS-Tween containing 0.5% bovine serum albumin was added to each well. Plates were placed in the refrigerator for 1 h, 100 μl of a 1:50 serum dilution (made in PBS-Tween containing 2% allantoic fluid) was then added to each plate, and twofold dilutions were made directly in the wells. Control wells in which there was no antigen or no antiserum, and others in which normal allantoic fluid was used as antigen at a protein concentration equivalent to that of the viral antigen, were also included in each test. Plates were again incubated overnight as before and washed three times with PBS-Tween, and 100 μl of an optimal dilution (in PBS-Tween plus 0.5% bovine serum albumin) of goat antiserum to human immunoglobulin G Fab fraction, conjugated with alkaline phosphatase (Dynatech Diagnostics, Inc., South Windham, Maine), was added to each well. Plates were incubated and washed as before, and 100 μl of a solution of *p*-nitrophenyl phosphate substrate (Sigma Chemical Co., St. Louis, Mo.) (1 mg/ml dissolved in 10% diethanolamine buffer) was added to each well. After 45 min of incubation at room temperature, or when positive control sera reached a predetermined standard titer for each serum, the reaction was immediately stopped with 25 μl of 3 M NaOH

per well, and the color reaction was read directly with a Microelisa 590 colorimeter (Dynatech).

**Whole-virus antigen.** Initially, whole recombinant viruses having an irrelevant HA and/or NA surface components(s) (Table 1) were used in the ELISA test for sensitizing the test plates. These viruses were grown in 11-day embryonated chicken eggs, pelleted by ultracentrifugation, and purified in 30 to 60% sucrose gradients. The viruses were diluted to contain 2 hemagglutination units per 100 μl for the ELISA test and were stored in multiple aliquots at −70°C.

**NASTA.** NA-specific testing antigen (NASTA) was prepared as follows. Purified, pelleted virus was suspended at room temperature in 50 mM sodium acetate–2 mM calcium chloride–0.2 mM EDTA (pH 7.0) (Buffer A) to a final protein concentration of 3 to 7 mg/ml. An equal volume of 15% 1-*O*-*n*-octyl-β-D-glucopyranoside (octylglucoside) in Buffer A was then added, and the mixture was mixed on a Vortex apparatus and immediately centrifuged for 1 h at 11,000 rpm in an SW50.1 rotor (14,000 × *g*) at 4°C. The supernatant fluid was reserved as the HA-NA-rich fraction, and the pellet was resuspended with 7.5% octylglucoside-Buffer A to the volume of the sample before centrifugation.

NA was further purified by DEAE-Sephadex chromatography of the HA-NA-rich fraction. This fraction was adjusted to 0.1% cetyltrimethyl ammonium bromide and passed through DEAE-Sephadex equilibrated with 50 mM Tris (pH 7.5)–0.1% octylglucoside; NA was eluted with the same buffer. HA was subsequently eluted by raising the salt concentration to 0.5 M NaCl and changing the detergent to 0.1% Triton-X. The NA-rich pool was dialyzed against water for 72 h and then used in ELISA as NASTA.

**Biochemical characterization of NASTA.** Protein determinations were made by the method of Lowry et al. (16). NA activity and HA titers were obtained by methods already described (2, 9, 19).

**Preparation of M and NP.** The influenza virus matrix (M) protein and the nucleoprotein (NP) were prepared as described by Bucher et al. (4, 5).

**Serum samples.** Human sera were obtained from a vaccine study (conducted in collaboration with Lederle Laboratories, Pearl River, N.Y.) in which volunteers were bled before inoculation (serum sample no. 1) with an NA-specific, Formalin-inactivated whole virus vaccine (H7N1) (Table 1), with an H1N1 experimental vaccine, or with a placebo (vaccine diluent only). Serum sample no. 2 was taken 1 month later, and then a second vaccine dose was administered. A third serum sample was taken 2 weeks after this second vaccine dose. In addition to these sera from vaccinees, three pairs of infants' sera, taken in the acute and convalescent phases of their illness in February 1976 during an outbreak of the Victoria strain of H3N2 influenza strain, were also tested.

**HI and NI assays.** The conventional techniques for determining HA and NA antibodies have been described (2, 9, 19). The recombinant virus used for determining HA antibodies contained an irrelevant NA and was ether-treated by standard protocols. For the NI test, H6N1 was grown in embryonated eggs, and the allantoic fluid was harvested and stored at −70°C for use directly in the NI test.

**PSR.** Plaque size reduction (PSR) titrations with H6N1 virus were done by previously published methods (10, 14).

**ELISA specificity.** To show that antibodies measured by the ELISA test when NASTA was used to sensitize the wells were actually NA-specific antibodies, cross-absorption experiments were performed with selected sera and the whole viruses H6N1 and H6N4. Pelleted, purified virus was suspended in PBS to a protein concentration of 0.16 mg/ml (equivalent to an HA titer of 2,048). Equal volumes of serum and virus were mixed, incubated at 37°C for 30 min, and refrigerated overnight, and the immune complexes were pelleted at 150,000 × *g* for 45 min. This absorbed serum is referred to as 1×; 2× represents a reabsorption of the 1× material. All samples were finally diluted to the same initial serum dilution in the ELISA test. The infants' sera were further absorbed with the Victoria strain (Table 1) of H3N2 virus.

TABLE 1. Viruses used in this study

| Virus nomenclature | | | Specific use |
|---|---|---|---|
| Old | New[a] | Strain or recombinant designation | |
| $Heq1N1_{USSR}$(X-68) | H7N1 | A/equine/Prague/1/56-USSR/92/77 | NA-specific vaccine |
| $H1_{USSR}N1_{USSR}$(X-67) | H1N1 | A/USSR/92/77-PR/8/34 | Experimental conventional vaccine |
| | H3N2 | | Commercial conventional |
| | H1N1 | | triple vaccine |
| | B | | |
| Hav6N1(USSR) | H6N1 | A/duck/France/MA42/76-USSR/92/77 | NI testing and homologous absorbing antigen |
| H1(Brazil)Neq1 | H1N7 | A/Brazil/11/78-equine/Prague/1/56 | HI testing antigen |
| Heq1Neq1 | H7N7 | A/equine/Prague/1/56 | HI testing antigen |
| Hav6Nav4 | H6N4 | A/duck/France/MB42/76 | Heterologous absorbing antigen |
| Hsw1Nsw1(X-53a) | H1N1 | A/Fort Dix/741/76-PR/8/34 | Source of M protein and NP |
| Heq1N2(X-48) | H7N2 | A/Prague/1/56-Victoria/3/75 | ELISA testing antigen for infants' sera |
| | H3N2 | A/Victoria/3/75 | Homologous absorbing and HI testing antigens for infants' sera |
| | H3N2 | A/Port Chalmers/1/73 | HI testing antigen for infants' sera |

[a] World Health Organization revised nomenclature (1980) (1).

**Calculation of titers.** HI titers were recorded as the reciprocal of the last dilution of serum giving complete inhibition of hemagglutination. The NI titer was the reciprocal of the dilution of antiserum, in a 3.2-fold dilution series, that inhibited 50% of viral NA activity. Endpoint titers were obtained by interpolation between the appropriate dilutions to obtain exactly a 50% inhibition of enzyme activity (2). ELISA titers were obtained similarly by interpolation except that instead of 50% inhibition of enzyme activity, the endpoint titer was calculated at an optical density (OD) reading of 0.3. This endpoint OD value was chosen by using two criteria as follows. (i) On a graph of OD versus serum dilution on semilog paper, this OD value was in the linear range of the S-shaped curve and, therefore, still in the range of reliability. (ii) Since the microreader was zeroed in using the antigen control wells, the serum control wells had a "background" reading of <0.10. The titer at an endpoint of 0.3 would then always have a P/N ratio of >2 (3).

## RESULTS

Measurement of antibody by ELISA in a pair of pre- and post-immunization sera is shown in Fig. 1. This graph illustrates both the response to immunization and the increasing reduction in background to a level of <0.05 OD with increasing serum dilution. Based on results of this and similar experiments, endpoint titers were calculated at 0.3 OD (see above).

Data obtained with whole virus as the antigen for sensitizing plates are shown in Table 2. The specificity of the N1-NA system can be demonstrated by examination of the results for subject no. 79. Serum for this subject, who received one dose of immunogen H7N1, had a high ELISA titer when tested on antigen H6N1. Absorption

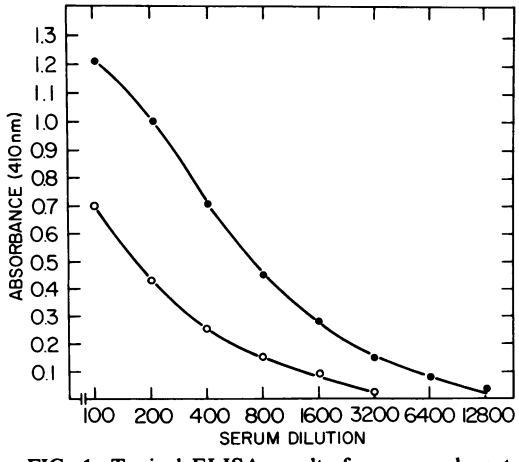


FIG. 1. Typical ELISA results for pre- and post-immunization serum specimens are plotted as OD values versus serum dilution. Symbols: (○) Pre-immunization serum sample; (●) post-immunization serum sample.

TABLE 2. ELISA NA specificity with whole virus antigen

| Plate-sensitizing antigen | Absorbing virus | ELISA titer[a] (anti-H7N1) |
|---|---|---|
| H6N1 | None | 17,216 |
| | H6N1 | 335 |
| | H6N4 | 8,842 |
| H6N4 | None | 15,732 |
| | H6N1 | 1,669 |
| | H6N4 | 207 |

[a] Subject no. 79-2.

of this antiserum with H6N1 removed most of these antibodies, whereas heterologous absorption with H6N4 did not significantly reduce this titer. Because the H6N4 absorption was efficient (as can be seen in the lower portion of Table 2) and NA was the only variable, we concluded that antibodies to N1 NA were being measured. These data indicate the specificity of the N1-NA system.

However, whole viruses (even if they possess one irrelevant surface antigen) are an inappropriate choice in the ELISA system for detecting specific antibodies to a single surface glycoprotein, because apparently antibodies to shared internal antigens, such as M and NP, may also be detected (3, 8, 18).

Detergent-extracted surface glycoproteins of a recombinant virus having an irrelevant HA would seem feasible for use in the ELISA system for detection of NA-specific antibodies. Accordingly, viral preparations were treated with octylglucoside (see Materials and Methods). The supernatant fraction obtained by such treatment showed an HA band in addition to NA on polyacrylamide gel electrophoresis (M. Gallagher, manuscript in preparation). The results of this NA purification process after octylglucoside extraction are shown in Table 3. The recovery of NA in the NASTA preparation, further purified by column chromatography, was about 50%, and this preparation showed no recognizable viral protein band on polyacrylamide gel elecrophoresis other than the NA component.

Analysis of the octylglucoside preparation by immunodiffusion using human anti-H7N1 serum revealed two major antigens and at least one minor component (data not shown). The unidentified (non-NA) major antigen was later identified as a host contaminant in the viral preparation. The minor component probably represented shared internal viral antigens (3, 8, 18).

The NASTA preparation was seen to contain a single sharp precipitin line when reacting with human sera of high NI titers to N1 NA. A less distinct band that was observed was presumed to be due to minor contaminating host antigens

TABLE 3. Recovery of NA activity and enhancement of specific activity during extraction of enzyme from H6N1 virus

| Virus sample | Activity (nmol/min) | Recovery (%) | Sp Act (nmol/min per mg) |
|---|---|---|---|
| Octylglucoside-treated virus, unspun | 7,755 | 100 | 194 |
| Octylglucoside-treated virus, supernatant | 6,179 | 80 | 277 |
| Octylglucoside-treated virus, pellet | 790 | 10 | |
| DEAE-Sephadex (NASTA) dialyzed vs water | 3,889 | 50 | 1,080 (5.6×) |

still present after column chromatography, and was demonstrated only with antiserum known to contain large amounts of antibodies to egg (host) proteins. Most of the host antigens (as assayed by immunodiffusion) eluted with the HA fraction (data not shown).

ELISA data obtained with the octylglucoside preparation, NASTA, M, and NP are presented in Tables 4 and 5. Sequential absorptions were carried out (Table 4) to demonstrate that whereas antibodies were decreasing at each successive homologous (H6N1) absorption step, this decrease was not seen with the heterologous (H7N4) absorption step. It should be noted further that antibodies to M and NP were significantly decreased by this heterologous absorption.

Evidence for ELISA specificity for N1 NA is summarized in Table 5. No antibody response to host antigens was seen in subject no. 581, as assayed by response against allantoic fluid, but antibody was demonstrated in both pre- and post-inoculation sera when tested against the octylglucoside extract. However, when NASTA was used as the sensitizing antigen, a definite NA-specific antibody response was seen with these paired specimens which was eliminated by homologous absorption, but not by heterologous absorption. The antibody responses to M and NP are also included in Table 5 for comparison with the anti-NA response.

The HI and ELISA results on three pairs of sera obtained from infants early in illness and in convalescence during an H3N2 (Victoria) influenza outbreak are presented in Table 6. Although there was serological confirmation of H3N2 infection in all subjects, as shown by the HI results, none of these subjects had measurable increases in N1-NA antibody by ELISA or the NI test. However, anti-N2-NA responses were noted by both ELISA and NI tests. The specificity of the N2-NA response was again demonstrated by cross-absorption experiments as performed for N1 NA (previously described).

For subjects whose sera did not contain antibodies to host (chicken embryo) antigens, such as subject no. 581 and the infected infants, use of the NASTA preparation permitted specific measurement of N1-NA antibodies by ELISA. However, since the NASTA contained small amounts of host antigens, it was found advisable to include 0.5% allantoic fluid in the serum diluent to block antibodies to host antigens, thus eliminating their reaction with those antigens bound to the solid phase (i.e., the sensitized microtiter plates). Therefore, with the addition of this modification to the testing protocol, the NASTA preparation could be used to test vaccines for N1-NA-specific antibodies by ELISA.

Table 7 shows the HI, NI, and ELISA results on sera from subjects who were vaccinated in the fall of 1978 with H7N1 (N-specific vaccine) or with a placebo. In all vaccinated subjects and one control subject, anti-NA antibody was measured also by the biological test of PSR (10, 14). Approximately 6 months after vaccination, influenza virus infection with an H1N1 strain (A/Brazil/11/78) was documented by HI tests (Table 8). Hence, in this situation, NA antibody responses were seen to both vaccine induction and a natural infection. Response to both stimuli is summarized in Table 8, in which the geometric

TABLE 4. ELISA NA specificity with octylglucoside-extracted NA (from H6N1): comparison with M and NP antigens

| Sensitizing Antigen | ELISA titers on serum 79-2 (anti-H7N1) | | | | | |
|---|---|---|---|---|---|---|
| | Unabsorbed | Absorbed with H6N1 | | | Absorbed with H6N4 | |
| | | 1× | 2× | 3× | 1× | 2× | 3× |
| Octylglucoside NA (from H6N1) | 5,941 | 4,277 | 3,337 | 815 | 1,803 | 1,193 | 1,160 |
| M protein[a] | 4,087 | 2,816 | 2,146 | 1,020 | 977 | 294 | 186 |
| NP[a] | 2,469 | 1,086 | 523 | 275 | 354 | 115 | 97 |

[a] Proteins from recombinant virus X-53a. Coded by genes from A/PR/8/34 (H1N1) virus.

TABLE 5. ELISA NA specificity with NASTA (from H6N1): comparison with M and NP antigens from recombinant X-53a

| Sensitizing antigen | Absorbing antigen | ELISA titers on serum no. 581 | |
|---|---|---|---|
| | | 581-1 (pre-inoculation) | 581-2 (post-inoculation) |
| Allantoic fluid (concentrated) | None | <100 | <100 |
| Octylglucoside-extracted NA | None | 2,844 | 3,913 |
| NASTA | None | <100 | 611 |
| | H6N1 virus | <100 | <100 |
| | H6N4 virus | <100 | 592 |
| M protein | None | 492 | 927 |
| | H6N1 virus | 190 | 344 |
| | H6N4 virus | 195 | 240 |
| NP | None | 342 | 706 |
| | H6N1 virus | <100 | 170 |
| | H6N4 virus | <100 | 131 |

mean antibody titers for the NA-specific vaccine and placebo groups are compared.

Seven of nine subjects showed a response to NA-specific immunization by ELISA, whereas only three of nine manifested detectable responses in a standard NI test. That the ELISA results are relevant to inhibition of viral replication is suggested by the concordance of ELISA and PSR response in six of nine subjects. No antibody increases were demonstrated in nine control subjects by any test. It is clear, however, that there was no positive correlation between individual ELISA OD readings and NI titers. The titer of PSR antibody was highest in subjects 167 and 206, two of the three subjects in whom NI response was demonstrated. Infection, as indicated by significant HI antibody response between the third and fourth or fourth and fifth bleedings in eight subjects whose vaccine test results are shown in Table 7, was associated with only slight increases in ELISA/NA antibody in two of these eight cases. However, significant NI antibody responses also occurred only in two of eight cases.

## DISCUSSION

The efficacy of influenza vaccines has been correlated with antibodies to the HA and NA glycoproteins on the surface of the influenza virus (23). Antibodies to HA and NA have been measured by the HI and NI tests, respectively. However, the sensitivities of these two tests have often been questioned (17, 20, 26), and sometimes protection has been noted in the absence of detectable antibodies to either or both of these antigens (17, 20, 26). Those results suggest the need for more sensitive tests for the detection of HA- and NA-specific antibodies.

Recently, Murphy et al. (17) described an ELISA test system for the detection of HA-specific antibodies. The antigen used was an almost pure preparation of HA, obtained by a

TABLE 6. ELISA anti-NA titers of paired sera of infants with serological evidence of H3N2 infection

| Subject/age | Bleeding dates | Titers against indicated virus antigens | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HI titers | | | NI titers | | | ELISA titers | | | | | |
| | | H3N2 strain: | | H1N1, swine | N1, H6N1 | N2, H7N2 | N1, NASTA | H7N2[a] absorbed with: | | | H6N4[a] absorbed with: | | |
| | | Port Chalmers | Victoria | | | | | Unabsorbed | H6N4 | H3N2 | Unabsorbed | H6N4 | H3N2 |
| A/6 mo | 1/28/76 | <10 | <10 | <10 | <10 | 0 | 154 | 309 | <100 | <100 | 667 | 100 | 202 |
| | 2/16/76 | 10 | 80 | <10 | <10 | 20 | 167 | 5,245 | 2,949 | 444 | 3,074 | 147 | 492 |
| B/6 mo | 2/10/76 | <10 | 10 | <10 | <10 | <0 | 159 | 660 | 299 | <100 | 920 | <100 | 273 |
| | 2/20/76 | 20 | 160 | <10 | <10 | 14 | 117 | 4,036 | 3,055 | 390 | 4,616 | 255 | 1,251 |
| C/7 mo | 2/16/76 | <10 | 20 | <10 | <10 | <0 | <100 | 1,419 | 693 | <100 | 2,508 | 337 | 618 |
| | 2/25/76 | 10 | 160 | <10 | <10 | 24 | <100 | 5,440 | 3,418 | 270 | 4,234 | 453 | 811 |

[a] These antigens were whole-virus preparations.

TABLE 7. Measurement of NA antibody in NA-vaccinated and control subjects before and after immunization

| Subject no. | NA vaccine | ELISA with sera[a]: | | | NI test with sera[a] | | | Anti-NA increase by: | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 1 | 2 | 3 | ELISA | PSR | NI |
| 118 | + | 247 | 664 | 750 | 0 | 29 | 43 | + | + | + |
| 120 | + | 348 | 552 | 557 | 0 | 0 | 0 | + | + | 0 |
| 136 | + | 644 | 750 | 966 | 0 | 0 | 0 | + | + | 0 |
| 212 | + | 629 | 971 | 1,053 | 0 | 0 | 0 | + | 0 | 0 |
| 167 | + | 386 | 727 | 630 | 0 | 50 | 54 | + | + | + |
| 170 | + | 331 | 209 | 318 | 0 | 0 | 0 | 0 | + | 0 |
| 202 | + | 758 | 702 | 862 | 0 | 0 | 0 | ± | 0 | 0 |
| 205 | + | 245 | 427 | 415 | 0 | 0 | 0 | + | 0 | 0 |
| 206 | + | 527 | 1,545 | 1,379 | 0 | 59 | 71 | + | + | + |
| Mean | | 457 | | 770 | | | | 1.7 | | |
| 117 | 0 | 347 | 124 | 140 | 0 | 0 | 0 | 0 | | 0 |
| 132 | 0 | 753 | 750 | 740 | 0 | 0 | 0 | 0 | 0 | 0 |
| 133 | 0 | 344 | 333 | 385 | 0 | 0 | 0 | 0 | | 0 |
| 138 | 0 | 308 | 324 | 307 | 0 | 0 | 0 | 0 | | 0 |
| 144 | 0 | 583 | 600 | 650 | 0 | 0 | 0 | 0 | | 0 |
| 115 | 0 | 167 | 175 | 192 | 100 | 98 | 99 | 0 | | 0 |
| 124 | 0 | 369 | 344 | 287 | 0 | 0 | 0 | 0 | | 0 |
| 128 | 0 | 374 | 381 | 354 | 68 | 54 | 64 | 0 | | 0 |
| 142 | 0 | 121 | 184 | 200 | 0 | 0 | 4 | 0 | | 0 |
| Mean | | 374 | | 361 | | | | 0.0 | | |

[a] Serum 1, Pre-immunization; serum 2, 30 days after first vaccine dose; serum 3, 44 days after first vaccine dose and 14 days after booster.

gel filtration technique. However, experience in our laboratory showed that such preparations contain a variable amount of host antigens, which although not detected by other criteria of purity were detected in the ELISA, especially with sera from subjects who responded to these host antigens on immunization. As an example, subject no. 581 in our study (results in Table 5) apparently did not respond to host antigens, and our NASTA preparation, therefore, detected only NA-specific antibodies. On the other hand, sera from subjects who did show antibody responses to host antigens had to be absorbed with allantoic fluid to insure assay only of NA-specific antibodies. Thus, with proper use of purified HA as reported by Murphy and co-workers (17) and partially purified NA (NASTA) (the present results), specific HA and NA antibodies could be detected with much greater sensitivity by ELISA systems.

The choice of the appropriate recombinant viruses having irrelevant HA for use in vaccines and as testing reagents makes it possible to perform cross-absorption, which eliminates heterologous antibodies while NA-specific antibodies remain unabsorbed. Since H7N1 was the immunogen in this study, the use of H6N4 as an absorbing reagent removed antibodies to shared viral non-surface antigens, especially M and NP.

Although whole recombinant viruses could be used to sensitize ELISA plates, and the antisera could be specifically absorbed with the appropri-

TABLE 8. Comparison of the ELISA and NI tests and definition of periods of vaccine response (sera 1 to 3) and infection response (sera 3 to 5) by mean HI titers

| Vaccine | No. of subjects | Tests | GMT on serum no.: | | | | | Ratios of sera: | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 2/1 | 3/1 | 5/3 |
| NA-specific | 9 | HI[a] | 8 | 12 | 11 | 32 | 52 | 1.5 | 1.4 | 4.7 |
| | | NI | 0 | 4 | 4 | 7 | 11 | — | — | 2.8 |
| | | ELISA | 427 | 657 | 722 | 685 | 780 | 1.5 | 1.7 | 1.1 |
| Placebo | 9 | HI | 16 | 18 | 20 | 26 | 50 | 1.1 | 1.3 | 2.5 |
| | | NI | 2 | 2 | 3 | 3 | 6 | 1.0 | 1.2 | 2.0 |
| | | ELISA | 377 | 333 | 322 | 398 | 433 | 0.9 | 0.8 | 1.3 |

[a] A/Brazil/11/78 (H1N1) virus. Fourteen of the eighteen subjects had significant (i.e., fourfold or greater) HI response in the interval bracketed by the third and fifth bleedings.

ate heterologous recombinant virus to detect NA-specific antibodies, this procedure would not be feasible in a large vaccine study. Nor could the unmodified octylglucoside preparation by itself be used, since variable amounts of internal viral proteins were also extracted. Although NASTA was itself neither entirely homogenous nor NA-specific, the absence of other viral constitutents warrants its use; efforts are now being made to further purify NA free of host antigens.

The greater sensitivity of ELISA, as compared to NI or PSR, for detection of NA-specific antibodies was demonstrated by the greater conversion rate, with respect to NA antibody, when the results of these three tests were compared (Tables 7 and 8). Conversion rates of vaccinated individuals were assessed by comparing these responses relative to the responses of the placebo group. The ratio of post-inoculation titer to prevaccine titer for the placebo group was $\leq 1.0$, whereas the NA-specific vaccine group showed ratios of 1.5 to 1.7 on the second and third bleedings. An arbitrary significant ratio for estimation of seroconversion can therefore be taken to be 1.3 (Tables 7 and 8). Results from the study of infected infants experiencing their initial immunization with influenza virus antigens demonstrated the specificity of anti-NA responses (Table 6).

Results of the NA-specific vaccine trial will be presented elsewhere. These preliminary studies of NA-specific antibody response suggest the feasibility of using the ELISA as a more sensitive and perhaps more appropriate test than the NI test for detecting NA-specific antibodies. Further studies are needed to correlate the ELISA NA antibody titers with protection.

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