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Serum Antibody Responses in Naturally Occurring Influenza A Virus Infection Determined by Enzyme-Linked Immunosorbent Assay, Hemagglutination Inhibition, and Complement Fixation

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Serum antibody responses to influenza A virus infection were examined in 388 normal subjects during a trial of chemoprophylaxis in an outbreak of influenza A in 1980–1981 in which both A/H1N1 and A/H3N2 viruses circulated. Paired serum specimens obtained over a 6-week period were tested for antibodies to both A/H1N1 and A/H3N2 viruses by conventional hemagglutination inhibition, complement fixation, and an enzyme-linked immunosorbent assay (ELISA). Antibody responses detected by ELISA were determined by calculation of the area generated between titration curves of paired sera (area method), as well as by a conventional endpoint dilution method (endpoint method).

Forty-two significant antibody rises were detected; 42 by ELISA (area method), 33 by ELISA (endpoint method), 32 by hemagglutination inhibition, and 13 by complement fixation. ELISA (area method) detected rises more frequently than either ELISA (endpoint method) ($P < 0.01$), hemagglutination inhibition ($P < 0.005$), or complement fixation ($P < 0.001$). Another sensitive assay, the microneutralization test, detected significantly fewer rises (33, $P < 0.025$) than the ELISA (area method). In the 42 subjects with ELISA (area method) rises, corroborating evidence of influenza A infection by other techniques (virus isolation, microneutralization, hemagglutination inhibition, or complement fixation tests) were available for 39 (93%). ELISA (area method) rises were subtype specific in all serum pairs in which other documentation of subtype-specific infection was available (38 of 38). Thus, ELISA (area method) was the single most sensitive assay for detection of serum antibody rises in this setting and possessed a high degree of subtype specificity.

A variety of serological methods are available for detecting antibody rises in influenza A infection. These include the hemagglutination inhibition (HAI) test, the complement fixation (CF) test, the microneutralization (Nt) test, and the enzyme-linked immunosorbent assay (ELISA). In previous studies, the more recently developed ELISA has been shown to be either similar to (1, 6, 8) or in one study less sensitive than (6) HAI for detecting significant antibody rises in influenza A infection. ELISA has been shown to be more sensitive than HAI for detecting seroconversion in volunteers receiving attenuated temperature-sensitive reassortant vaccines (8). The ELISA sensitivity in this latter study was primarily attributable to quantitation of antibody rises between pre- and postinfection sera by the area generated between the titration curves of the sera, rather than by antibody quantitation by differences in the dilution endpoints of the sera (8).

A placebo-controlled field trail of amantadine and rimantadine in the chemoprophylaxis of influenza A infection, conducted during an outbreak of A/H1N1 and A/H3N2 (2), provided an opportunity to compare seroresponses by HAI, CF, and ELISA to the more recently circulating influenza A viruses. In particular, the ELISA methodology developed by Murphy et al. (8) of detecting antibody rises by the areas generated between paired sera was applied and compared to ELISA rises determined by endpoint dilution and to other conventional methods for detection of antibody rises to influenza A virus.

MATERIALS AND METHODS

Influenza A outbreak and specimen collection. The 1980–1981 influenza A outbreak and chemoprophylac-

tic trial in Burlington, Vt., has been described previously (2). Serum samples were taken at the beginning and the end (6 weeks) of the study period from all individuals in the study. In addition, throat swabs for virus isolation were taken from individuals with evidence of respiratory illness during the study period.

Virus isolation and identification. Throat swabs were placed in veal infusion broth containing 0.5% bovine serum albumin, and 0.2-ml volumes of the sample were inoculated into tubes of primary Rhesus monkey kidney, $LLCMK_2$, MDCK, HEp-2, and WI-38 cells. The cultures were incubated at 33°C and examined for cytopathic effect and for hemadsorption with a 0.5% suspension of guinea pig erythrocytes. Hemadsorption-positive cultures were subcultured, and virus was typed by HAI (3).

Serological procedures. The microtiter HAI (3) assay employed A/Brazil/11/78 (H1N1) and A/Bangkok/1/79 (H3N2) antigens in allantoic fluid kindly provided by Alan Kendal, Centers for Disease Control, Atlanta, Ga. Human O-type erythrocytes were used in the assay. The Laboratory Branch CF Test was employed with 5 CH_{50} units of complement (9) and influenza A-soluble antigen (Medical Technology Corporation, Hackensack, N.J.). The Nt test was performed as described by Wulff et al. (10). Virus isolates obtained from volunteers in the study were identified as H1N1 or H3N2 subtypes by HAI and were grown in Rhesus monkey kidney cells for the Nt test. Human O cells were used to identify virus-specific HAI in the wells.

ELISA. The ELISA employed was modified from that designed by Murphy et al. (8). Influenza whole virus vaccines A/Brazil/78 (H1N1) and A/Bangkok/79 (H3N2) were employed as antigens in the assay and were kindly provided by F. Marilyn Bozeman of the Bureau of Biologics, Bethesda, Md. The viral antigen was diluted in carbonate buffer (pH 9.6), and 100 μl (8 hemagglutinating units) was added to duplicate wells of the 96-well polystyrene plate (Dynatech Laboratories, Inc., Alexandria, Va.). Normal allantoic fluid at the same dilution as the viral antigen (1/1600) was added at 100 μl per control well. The plates were covered, incubated overnight in a moist container at 4°C, then washed six times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween). Human serum was diluted 1/100 in PBS-Tween containing 1% fetal bovine serum and 1% allantoic fluid, and 200 μl of the serum was added to antigen and control wells. Serial twofold dilutions to 1/1600 were made with the Titertek multichannel pipettor (Flow Laboratories, Inc., Rockville, Md.). The plates were covered, incubated in a moist container for 2 h at 25°C, and then washed six times with PBS-Tween. Affinity column-purified goat anti-human immunoglobulin G (IgG) or IgM conjugated with alkaline phosphatase (Dynatek Diagnostics, Inc., South Windham, Maine) was diluted to 1/1000 in PBS-Tween and added to all wells at 100 μl per well. The plates were covered, incubated in a moist container overnight at 4°C, and then washed six times with PBS-Tween. Fresh enzyme substrate, *p*-nitrophenylphosphate (Sigma 104; Sigma Chemical Co., St. Louis, Mo.) dissolved in 10% diethanolamine buffer, was added at 100 μl per well, the plate was incubated for 1 h at 25°C, and the reaction was stopped with 50 μl per well of 3 M NaOH. The reaction product was measured at an optical density at 410 in the Microelisa Minireader (Dynatek Instruments, Inc., Santa Monica, Calif.).

Calculation of ELISA rises by area method. The area between the curves generated by twofold dilutions of paired sera was calculated as described previously (8), using the trapezoid rule and the formula $1/2\ (a+b)xw$ for the area between the curves at each dilution. Calculations were performed with the Hewlett Packard 97 programmable calculator (Hewlett Packard, Cornwallis, Oreg.) with a program kindly prepared by David W. Alling, National Institute of Allergy and Infectious Diseases, Bethesda, Md. In preliminary experiments, the area plus two standard deviations between two dilution curves that could be generated by assay variability alone was determined by first generating 31 separate dilution curves on duplicate samples of a serum with an HAI titer of 1/160 and on duplicate samples of a serum with an HAI titer of <1/10. In addition, 31 separate dilution curves were generated on a serum pair with a fourfold rise in HAI titer (<1/10 to 1/20). The mean areas ± 2 standard deviations generated by the dilution curves were 6.02 ± 21.44 for the duplicate samples of the HAI 1/160 serum, 1.73 ± 8.64 for the duplicate samples of the <1/10 serum, and 43.20 ± 26.84 for the serum pair with the fourfold rise in HAI titer (<1/10 to 1/20). A value representing assay variability was then calculated from the mean areas generated by the dilution curves of the two sera with duplicate samples (6.02 + 1.73/2 = 3.88) plus the average of 2 standard deviations generated by the dilution curves of the two sera with duplicate samples and the serum pair with a fourfold rise (21.44 + 8.64 + 26.84/2 = 18.97). The value obtained was 3.88 + 18.97 = 22.85.

In the subsequent assays of serum pairs, areas generated between dilution curves ≥23 were considered positive, i.e., representing a significant antibody rise. Serum pairs with areas between 15 and 30 were reassayed, to confirm that areas ≥23 were present. Employing this two-step process and modeling probabilities by using a mixed Gaussian and Gaussian distribution for negative and positive data, respectively, the statistical probability of detection of a true negative was calculated to be 99% and that of a true positive was 97%.

Calculation of ELISA by endpoint method. Endpoint titers of the sera were determined to be the reciprocal of the highest twofold dilution with an optical density at 410 ≥0.2 for IgG and ≥0.1 for IgM. These values were calculated from ELISA dilution curves on sera with HAI titers of ≤10 for A/Brazil and A/Bangkok and CF titers of ≤4 for influenza A-soluble antigen (12 sera for IgG, 12 sera for IgM).

Statistics. The frequencies of serum antibody rises as detected by HAI, CF, Nt, and ELISA were compared by the McNemar test of significance (5).

RESULTS

Significant antibody rises detected by ELISA, HAI, and CF, tests. Serum pairs from 388 volunteers were tested for significant antibody rises to influenza A by ELISA, HAI, and CF tests (Table 1). Of the 42 significant antibody rises to influenza A, the ELISA (area method) detected the greatest number of rises (42), followed by the ELISA (endpoint method) (33) and then the HAI (32) and CF (13) tests. The frequency of detection of seroresponses was significantly greater

TABLE 1. Serum antibody responses to influenza A viruses as determined by ELISA, HAI, and CF methods

No. of serum pairs tested	No. of serum pairs with significant antibody rises by indicated methods				
	ELISA (area)[a]	ELISA (endpoint)[b]	HAI[c]	CF[d]	All methods
388	42	33	32	13	42

[a] Tested with A/Brazil and A/Bangkok antigens for IgG and IgM; an area ≥23 was considered significant.
[b] Tested with A/Brazil and A/Bangkok antigens for IgG and IgM; a ≥ fourfold rise was considered significant.
[c] Tested with A/Brazil and A/Bangkok antigens; a ≥ fourfold rise was considered significant.
[d] Tested with influenza A-soluble protein antigens; a ≥ fourfold rise was considered significant.

by ELISA (area method) than by ELISA (endpoint method, $P < 0.01$), by HAI ($P < 0.005$), or by CF ($P < 0.001$) tests. The frequency of detection of seroresponses by ELISA (endpoint method) was comparable (33 rises) to that obtained by the HAI test but was significantly greater than that obtained by the CF test ($P < 0.001$).

A direct comparison of the ELISA (area method) to other assays is depicted in Table 2. All serum pairs which were positive by HAI were also positive by ELISA (area method). However, 10 pairs which were positive by ELISA (area method) were HAI negative. The possibility that the increased number of seroresponses observed employing ELISA (area method) represent false positives is unlikely. Corroborating evidence of influenza A infection by other techniques (HAI, CF, Nt, or virus isolation) was present in 39 of 42 of the subjects (93%) in whom the ELISA (area method) rises were noted. This includes 7 of the 10 pairs positive by ELISA (area method) and negative by HAI. In the remaining ELISA (area method) positive pairs for whom no other evidence of influenza A infection was available, one serum pair was obtained from a subject with an influenza-like illness for which no etiological agent was detected, whereas the other two subjects did not have acute respiratory illnesses.

A comparison of the relative sensitivity of ELISA (area method), ELISA (endpoint method), HAI, and another serological assay, the Nt test, is tabulated in Table 3. Because of its cumbersome nature, the Nt test was performed only on the 42 serum pairs which had antibody rises by ELISA (area method) on the serum pair of an individual from whom virus was isolated, but who did not show antibody rise by any of the serological tests employed, and on an additional 58 serum pairs from volunteers with influenza-like illness but without laboratory documented evidence (virus isolation, HAI, CF, or ELISA) of influenza A infection. The Nt test (Table 3) detected significantly fewer rises (33, $P < 0.025$) than the ELISA (area method). The individual from whom virus was isolated was positive by the Nt test but negative by ELISA (area method). No significant rises were detected by the Nt test in the additional 58 serum pairs from volunteers with influenza-like illness but without laboratory-documented evidence of influenza A infection. Both the ELISA (endpoint method) and HAI were comparable in sensitivity to the Nt test.

Classes of antibody rises detected by ELISA (area method). Among the serum antibody rises detected by the ELISA (area method), 35 rises were in IgG antibody, 4 were both in IgG and IgM, and 3 were in IgM alone.

ELISA subtype specificity. To determine the subtype specificity of this assay, we compared ELISA (area method) responses to subtype-specific responses detected by HAI, Nt test, and virus isolation (Table 4). Of the 42 ELISA (area method) seroresponses, 38 were to a single subtype and 4 were to both subtypes with the area for one subtype being greater than the other. Thirty-five of the ELISA (area method) rises to a single subtype, e.g., A/Brazil (H1N1) or A/Bangkok (H3N2), correlated completely with the corresponding HAI rise, Nt rise, or virus isolation. For three serum pairs in which rises to both A/Brazil (H1N1) and A/Bangkok (H3N2) were detected by ELISA (area method), the greater area corresponded to the homologous subtype determined by HAI serology or virus isolation. Thus, 38 of the ELISA (area method) rises were subtype specific. Corroborating evidence for subtype specificity was not available for the remaining four ELISA (area method) rises. One pair had a CF rise, and the

TABLE 2. Comparison of serum antibody responses to influenza A virus as determined by ELISA, HAI, and CF techniques

Method	ELISA (endpoint)[a]		HAI[a]		CF[a]	
	Positive (n = 33)	Negative (n = 355)	Positive (n = 32)	Negative (n = 356)	Positive (n = 13)	Negative (n = 375)
ELISA (area)[a]						
Positive (n = 42)	33	9	32	10	13	29
Negative (n = 346)	0	346	0	346	0	346

[a] See Table 1 for assay description.

TABLE 3. Comparison of serum antibody responses employing Nt, ELISA, and HAI procedures

Method	ELISA (area)[b]		ELISA (endpoint)[b]		HAI[b]	
	Positive (n = 42)	Negative (n = 1)	Positive (n = 33)	Negative (n = 10)	Positive (n = 32)	Negative (n = 11)
Nt[a]						
Positive (n = 33)	32	1	25	8	27	6
Negative (n = 10)	10	0	8	2	5	5

[a] Tested with A/Brazil and A/Bangkok isolates grown in Rhesus monkey kidney cells; a ≥ fourfold rise was considered significant.
[b] See Table 1 for assay description.

remaining three had no other evidence of influenza A infection. The ELISA (endpoint method) was less subtype specific. Of the 33 rises, 29 were subtype specific, 2 were heterologous rises, 1 had a CF antibody rise, and 1 had no other laboratory-documented evidence of influenza A infection.

ELISA (area method) rises were not observed in the four subjects who during the study shed viruses other than influenza A, including parainfluenza 2, parainfluenza 3, and herpes simplex virus.

Correlation of ELISA rises to HAI and CF titer rises. The magnitude of ELISA (area method) rises were compared to the corresponding HAI (Fig. 1) and CF (Fig. 2) titer rises of the paired sera. The magnitude of ELISA (area method) rises correlated closely with the rises in HAI titers ($r = 0.82$, $P < 0.001$), but not with the rises in CF titers ($r = 0.16$, $P < 0.5$).

DISCUSSION

This report compares different assays in the serodiagnosis of influenza A virus infection during an influenza A outbreak. The ELISA (area method) was significantly more sensitive than ELISA (endpoint method) ($P < 0.01$), HAI ($P < 0.005$), or CF ($P < 0.001$) tests in detection of seroresponse in 388 serum pairs. ELISA (area method) was also significantly more sensitive than Nt ($P < 0.025$) in a limited number of serum pairs. The increased sensitivity of the ELISA results from the determination of antibody rises by measuring the areas between the dilution curves, since ELISA rises determined by ≥ fourfold differences in titer (endpoint method) were no more frequent than antibody rises detected by HAI or Nt tests.

The ELISA (area method) developed by Murphy et al. (8) detected more seroresponses than the HAI or neuraminidase inhibition tests or both in volunteers who received temperature-sensitive vaccines derived from A/HK/123/77 (H1N1), A/Alaska/77 (H3N2), and A/NJ/76 (Hswine N1) but was comparable to these tests in detecting seroresponses in volunteers who received wild-type parent viruses. Our observations confirm and extend the usefulness of this method to naturally occurring influenza A infections where the ELISA (area method) detected significantly more seroresponses than the HAI or Nt tests.

Our HAI test employed whole virus rather than split virus antigen, and other workers have suggested that the HAI test is more sensitive when split virus antigen is used (6). Although not directly tested in our studies, it appears unlikely that using split virus antigen would raise the sensitivity of the HAI to that of the ELISA (area method). Murphy et al. used split virus antigen in the HAI test and found that the ELISA (area method) detected more seroresponses than the HAI (8). In addition, our ELISA (area method) detected significantly more serum antibody rises than the Nt test whose sensitivity is generally

TABLE 4. Comparison of subtype-specific serum antibody responses detected by ELISA (area method) and by HAI, Nt, and virus isolation

Method	HAI[a]		HAI + virus isolation		Virus isolation alone		Nt alone	
	H1N1 (n = 19)	H3N2 (n = 2)	H1N1 (n = 9)	H3N2 (n = 2)	H1N1 (n = 3)	N3N2 (n = 0)	H1N1 (n = 2)	H3N2 (n = 1)
ELISA (area)								
H1N1 (n = 31)	18	0	8	0	3	0	2	0
H3N2 (n = 4)	0	2	0	1	0	0	0	1
H1N1 + H3N2 (n = 3)	1[b]	0	1[b]	1[b]	0	0	0	0

[a] Nt rises detected in these individuals had the same subtype specificity.
[b] The ELISA area was greater for the homologous antigen.

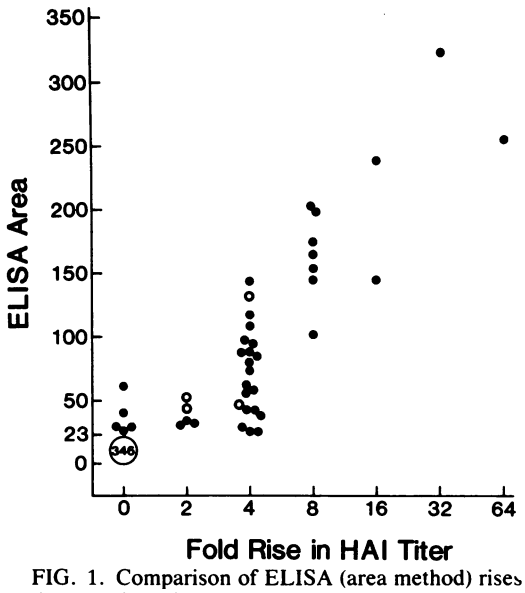

FIG. 1. Comparison of ELISA (area method) rises and HAI rises for paired sera. Symbols: ○, IgM values; ●, IgG values in ELISA (area method). Spearman rank (4) r for ELISA areas ≥23 and HAI rises ≥fourfold is 0.82 ($P < 0.001$).

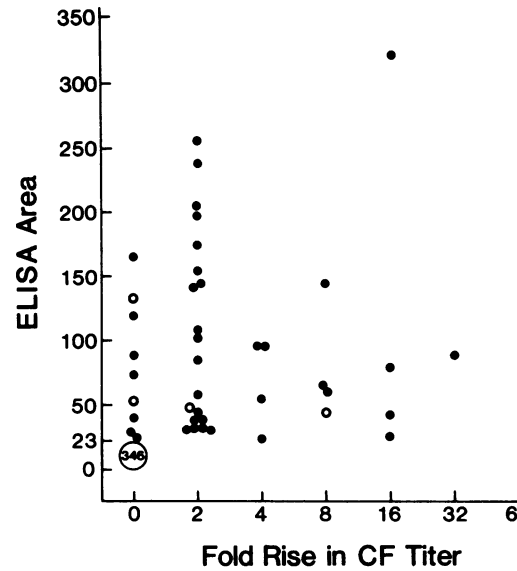

FIG. 2. Comparison of ELISA (area method) rises and CF rises for paired sera. Symbols: ○, IgM values; ●, IgG values in ELISA (area method). Spearman rank (4) r for ELISA areas ≥23 and CF rises ≥fourfold is 0.16 ($P < 0.5$).

considered to be equal to or greater than that of HAI. In our study (Table 3), the Nt and HAI tests had equal sensitivity by the McNemar test.

Antibody rises detected by the ELISA (area method) correlated well with influenza A infection, since 39 of the 42 individuals with ELISA (area method) rises had evidence by one or more other criteria (HAI, Nt, CF, or virus isolation) of influenza A infection, and none of the four subjects in the study from whom viruses other than influenza were isolated had ELISA (area method) rises. In addition, serum antibody rises by the ELISA (area method) were highly subtype specific, with 38 of 38 rises corresponding to rises for which there was independent confirmation of subtype-specific infection by HAI and Nt tests or by virus isolation. The ELISA (endpoint method) had less subtype specificity, with 29 of 33 of the serum pairs being subtype specific, 2 of 33 having heterologous rises, and the remaining 2 having no corroborative evidence of subtype specificity. The high correlation of the magnitude of serum antibody rises by the ELISA (area method) with the HAI rises (0.82, $P < 0.001$) but not with CF rises also suggests that antibodies with subtype specificities were being detected by the ELISA (area method).

An earlier study (6) reported that ELISA (endpoint method) serodiagnosis could not distinguish between influenza A H1N1 and H3N2 infections, and this lack of subtype specificity was attributed to the use of whole virus antigen in the ELISA. Our studies, in which subtype-specific responses were detected with whole virus antigen when the ELISA (area method) was used, suggest that the previously observed lack of subtype specificity may have been a result of the method employed (ELISA endpoint) rather than the use of whole virus antigen. Use of purified hemagglutinin as an antigen should also result in measurement of subtype-specific responses (7). However, this reagent may not be as readily available as whole virus antigen, and the relative advantages and disadvantages of using purified hemagglutinin in large scale seroepidemiological studies have not been evaluated.

In summary, our studies demonstrate that the ELISA (area method) is a convenient, sensitive, and subtype-specific test useful in serodiagnosis of influenza A infection.

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