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Use of the Enzyme-Linked Immunosorbent Assay to Detect Serum Antibody Responses of Volunteers Who Received Attenuated Influenza A Virus Vaccines

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Sera from volunteers who received live influenza A wild-type or *ts* recombinant virus were tested by hemagglutination inhibition (HI) assay, neuraminidase inhibition (NI) assay, and the enzyme-linked immunosorbent assay (ELISA) to determine which assay system was the most sensitive in detecting an immunological response to infection. The ELISA was performed with inactivated whole virus antigen, and the optical density at each of five serial twofold dilutions of pre- and postimmunization sera was measured. The difference in the amount of ELISA antibody in pre- and postinoculation serum specimens was taken to be proportional to the area between the respective titration curves. The ELISA was more sensitive than the HI or NI test in detecting a seroresponse in volunteers infected with A/Hong Kong/123/77 (H1N1), A/New Jersey/8/76 (Hswine N1), or A/Alaska/6/77 (H3N2) *ts* recombinant virus. These results suggest that the ELISA should be used to determine the frequency of infection with attenuated viruses as well as the 50% human infectious dose of candidate live influenza A vaccine viruses.

Attenuated, temperature-sensitive (*ts*) recombinants bearing the H3N2 surface antigens were administered to seronegative adults or children, and infection was readily documented in 70 to 90% of the vaccinees by measuring a rise in serum antibody to the hemagglutinin or neuraminidase antigens (4, 6-9, 14, 15, 23). However, when *ts* recombinants bearing the Hswine N1 or H1N1 antigens were administered to susceptible vaccinees, a seroresponse was detected in less than 50% of the vaccinees (10; L. J. Markoff et al., unpublished observations). The latter results indicated the need to develop more sensitive tests to detect immunological responses to such attenuated viruses. Recently, radioimmunoassays and enzyme-linked immunosorbent assays (ELISA) have been shown to be more sensitive than conventional serological tests for the detection of serum antibody to influenza virus (1, 5, 17). In this study, we adapted the simpler ELISA for the detection of a seroresponse in vaccinees who were given an influenza A wild-type or vaccine virus. The ELISA proved to be significantly more efficient in detecting serological evidence of infection than the hemagglutination inhibition (HI) or neuraminidase inhibition (NI) assay.

MATERIALS AND METHODS

Clinical specimens. Live wild-type and attenuated influenza A viruses were given to volunteers as part of a vaccine development program (12). Pre- and postinfection sera were collected approximately 1 month apart. The serum pairs tested were obtained from volunteers or patients who were infected with one of the following agents: (i) A/Hong Kong/123/77 (A/HK/123/77) (H1N1) wild-type virus, $10^{4.0}$ 50% tissue culture infective doses ($TCID_{50}$) (adults); (ii) A/HK/123/77-*ts*-1A2 (H1N1) recombinant, $10^{6.5}$ $TCID_{50}$ (adults); (iii) A/Alaska/6/77 (H3N2) wild-type virus, $10^{4.2}$ $TCID_{50}$ (adults); (iv) A/Alaska/6/77-*ts*-1A2 (H3N2) recombinant, $10^{6.8}$ $TCID_{50}$ (adults); (v) A/New Jersey/8/76 (A/NJ/8/76)-*ts*-1[E] (Hswine N1) recombinant virus, $10^{6.5}$ $TCID_{50}$ (adults); (vi) parainfluenza type I wild-type virus, $10^{5.0}$ $TCID_{50}$ (adults) (11); (vii) *Escherichia coli* or cholera vaccine as part of the program to develop vaccines against these agents at the University of Maryland, School of Medicine, Baltimore; and (viii) influenza B wild-type virus antigenically similar to B/HK/15/72 virus (natural infection of children). Natural infection of children with influenza B virus was documented by recovery of virus and a rise in serum complement-fixing antibody. Influenza B virus circulated in Washington, D.C., at a time when influenza A (H3N2) virus was absent and before influenza A (H1N1) virus was first recovered. The sera were kindly supplied by Hyun-Wha Kim, Children's

Hospital, National Medical Center, Washington, D.C. The clinical, virological, and serological response of the volunteers who received a wild-type virus or *ts* recombinant has been presented in detail in other publications except for the A/NJ/8/76-*ts*-1[E] recombinant and the A/HK/123/77 wild-type virus and its *ts*-1A2 recombinant (7, 11). The former virus was produced and characterized like other genetically similar *ts*-1[E] recombinants (6, 9, 14, 16, 18), and the A/Hong Kong/123/77 *ts*-1A2 recombinant was produced and evaluated in volunteers in a manner similar to other *ts*-1A2 recombinants (7).

HI and NI assays. The techniques for determining HI and NI antibody titers to the A/Alaska/6/77 virus have been described, and the specific antigens used have been indicated (7). For evaluating the HI antibody response to the A/NJ/8/76-*ts*-1[E] virus, the A/NJ/8/76 virus was used in the HI assay. This antigen preparation permitted detection of a seroresponse in most primed individuals who received inactivated vaccine (i.e., approximately 90% had a fourfold or greater rise in HI antibody), so the lack of detection of an HI response in the NJ/76-*ts*-1[E] vaccinees was not a function of a non-avid test antigen. The virus used to detect an NI antibody response in NJ/76-*ts*-1[E] vaccines was a recombinant containing the Hequi 1 hemagglutinin derived from the A/equine 1/Prague/56 virus and the N1 neuraminidase from the A/NJ/76 (Hswine H1) virus. The A/USSR/90/77 split-virus vaccine (Parke, Davis & Co., Detroit, Mich.) was used as antigen to assay HI antibody in individuals who were given the influenza A/HK/123/77 (H1N1) virus, whereas a recombinant virus bearing the Hequi 1 hemagglutinin and the N1 (A/USSR/92/77) antigens was used in the NI tests. The recombinant viruses and antisera to them were kindly provided by Alan Kendal, Center for Disease Control, Atlanta, Ga.

ELISA. The ELISA was modified from that of Voller et al. (19) and Yolken et al. (21). The optimal concentration of reagents was determined by checkerboard titration (20). Inactivated whole virus vaccine was diluted in carbonate buffer (pH 9.8) to yield 8 hemagglutinating units. Then 100 μl of virus was added to the wells of a microtiter plate (Cooke Micro-ELISA plate), which was then incubated at 4°C for at least 14 h. To a series of control wells, 100 μl of a dilution of normal allantoic fluid comparable to that of the virus suspension was added instead of virus. The outermost rows of wells of each plate were not used. Plates were stored at 4°C in a moist container and before use were washed three times in a solution of phosphate-buffered saline containing polysorbate (Tween 20) at a concentration of 0.5 ml/liter (PBS-Tween).

Serum was diluted in PBS-Tween containing 1% allantoic fluid and 1% fetal calf serum. Then 100 μl of serum was added in duplicate to antigen-containing wells and singly to control wells. The starting dilution for H3N2 antigen-coated plates was 1:200, whereas it was 1:20 for Hswine N1, H1N1, and influenza B virus-coated plates. Serum was diluted in twofold steps, using a Titertek multichannel pipette (Flow Laboratories, Inc., Rockville, Md.). The plate was then incubated at 4°C overnight, after which it was washed again three times with PBS-Tween. Then 100 μl of a predetermined dilution of goat anti-human immunoglobulin G (IgG) or IgM (Antibodies Inc., Davis, Calif.) conjugated with alkaline phosphatase (2) was added. The sensitivity and specificity of these conjugates were described previously (22). After incubation for 2 h at 37°C, the plates were again washed three times in PBS-Tween, and 100 μl of *p*-nitrophenyl phosphate substrate (Sigma 104; Sigma Chemical Co., St. Louis, Mo.) dissolved in 10% diethanolamine was added. After 30 min at 37°C, the amount of yellow color produced by the substrate as a result of the action of the enzyme bound to the solid phase was determined by a colorimeter which detected the optical density of the solution through the bottom of the microtiter plates (21).

The viruses used to coat the ELISA plates were the following inactivated whole virus zonally purified vaccines: (i) A/Fort Dix/741/76 (Hswine N1); (ii) A/USSR/90/77 (H1N1); (iii) A/Texas/X-47/77 (H3N2); or (iv) B/HK/15/72.

Determination of a seroresponse by ELISA. A change in quantity of antibody in preinoculation (or acute) and postinoculation (or convalescent) sera was taken to be the area between the respective titration curves as detailed in Fig. 1. An area was considered to represent a significant rise in serum antibody if it met three criteria. First, the preinfection or acute-phase serum had to be the lower of the two curves in the figure. In those cases in which this was not the case, the area was assigned a negative value. Second, the difference between the pre- (or acute) and postinfection curves had to be significantly different. The significance of the difference was determined from the ratio t = area between the curves/standard error of area, with the use of a t-test entered in at $2k$ df. k is the number of levels in the series of dilutions; in all cases in this report it was 5. The standard error was calculated from the differences between duplicate determinations at the various dilutions. An ELISA response (i.e., the area between the pre- and postimmunization curves) was considered significant at a P value of less than or equal to 0.05 as indicated in the t-test table. Third, it was observed that by using the first two criteria, certain low areas (i.e., less than 50) could be significant by the t-test but were not reproducible. It was therefore important to identify those area values that could be generated by dilutional error or other test variables. To do this, a serum was selected such that at a starting dilution of 1:200 it gave an optical density reading of 0.70, using an H1N1 antigen-coated plate. An area was determined by two separate samplings of the same serum and by performing five twofold dilutions starting at 1:200. Twenty-three separate determinations of this type were made. The expected mean of the 23 areas was 0, and the mean of the measurements was 1.4 ± 5.4 standard error (SE). A similar analysis for another serum with an initial optical density reading of 1.5 at an initial serum dilution of 1:200 yielded a mean of −1.1. To further explore the significance of the area generated by serum pairs, a single serum pair that exhibited a "low positive" area was tested 29 times and had a mean area of 84 ± 5.4 (SE). The standard deviation determined from the pooled data of the two "negative" sera and the "low positive" serum pair was 24.1. This indicated that an area less than 50 (approximately 2 standard devia-

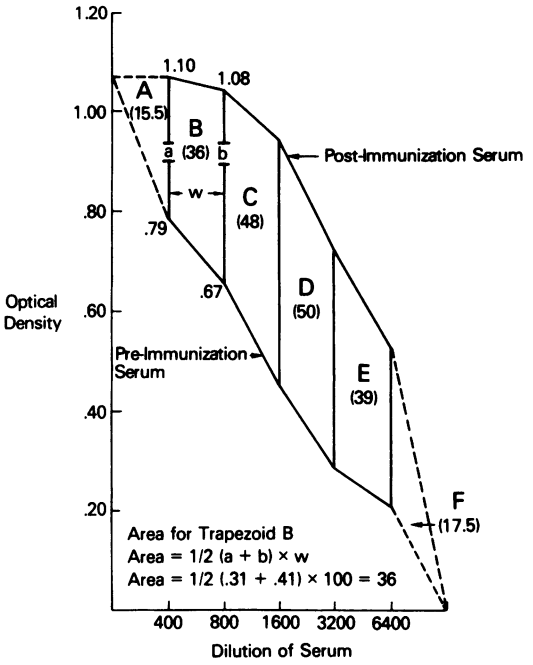

FIG. 1. *Determination of areas between the pre- and postimmunization sera, using an A/Texas/X-47/77 antigen, is illustrated. The areas were calculated by using the trapezoid rule (3) in which area is calculated according to the formula 1/2(a + b) × w as indicated. w, i.e., the serum dilution, is a constant and is arbitrarily assigned a value of 100. The area between the pre- and postimmunization area depicted in the graph is the sum of the individual areas A + B + C + D + E + F = 15.5 + 36 + 48 + 50 + 39 + 17.5 = 207. Areas B, C, D, and E are measured areas; i.e., they are described between the starting dilution of 1:400 and the last dilution of 1:6,400, whereas areas A and F are areas generated by the hypothetical closure of the two curves.*

tions) could be generated by dilutional error or other test variables. Thus, the third criterion was that an area had to exceed a value of 50 before it was considered significant. An area of 50 was exceeded in only one test of a set of 46 negative serum pairs. In the set of 29 low positives (average area of 84), only one determination was less than 50. All areas between 50 and 100 were confirmed by a repeat assay before being considered positive.

It is important to emphasize that quantitation of the ELISA area was more sensitive for detecting a rise in antibody level than for comparing titers in pre- and post-inoculated specimens by standard ELISA methodology. For example, 92% of serum pairs with an area of greater than 100 also exhibited a fourfold or greater rise in ELISA titer. The ELISA titers were calculated by the conventional positive-over-negative (P/N) method (22) in which the endpoint was the highest dilution that gave a P/N ratio of equal to or greater than 2. However, of 36 serum pairs with an area between 50 and 100, only 12 exhibited a fourfold or greater rise in ELISA titer by this method. For this reason the ELISA area was chosen as the method to identify a seroresponse by recipients of an attenuated influenza A virus.

RESULTS

Virus specificity of ELISA area. To determine whether the areas generated by serum pairs were specific for the infecting virus, four groups of sera were tested (Table 1). Volunteers infected with A/HK/77 (H1N1) wild-type virus had a significant response when tested with H1N1 antigen but not with influenza B/HK/72 antigen. The reverse was true for children naturally infected with influenza B virus. Volun-

TABLE 1. *Specificity of ELISA for influenza A and B viruses*

Group	Subject no.	ELISA area defined by acute and convalescent sera, using:[a]	
		A (H1N1)	B
Infected with wild-type H1N1 influenza A virus intranasally	1	38	28
	2	90	1
	3	189	0
	4	208	43
	5	226	26
	6	245	23
Natural infection with influenza B virus	1	−1	55
	2	−55	71
	3	20	108
	4	−4	282
	5	4	435
	6	−9	457
	7	31	465
	8	−22	499
	9	24	532
	10	−27	649
Infected with parainfluenza type 1 wild-type virus intranasally	1	21	−2
	2	38	13
	3	−10	−5
	4	1	14
	5	9	35
	6	−18	−26
	7	14	9
	8	−36	15
	9	22	29
Infected with *E. coli* or cholera	1	10	14
	2	−2	19
	3	2	10
	4	52	−13
	5	0	23
	6	−7	4
	7	26	−7
	8	23	4
	9	12	32
	10	20	6
	11	−4	32
	12	4	30
	13	−9	13
	14	−3	−49

[a] Anti-human IgG conjugate was used in ELISA. Underlined value indicates a significant ELISA response.

teers administered parainfluenza type 1 virus, who had a serum HI rise to the parainfluenza A type 1 antigen (11), did not have an ELISA response to either H1N1 or influenza B antigen. Similarly, volunteers who received a bacterial vaccine failed to develop an ELISA response to H1N1 or influenza B antigen except for one low response to the H1N1 antigen.

ELISA areas in volunteers receiving wild-type or temperature-sensitive vaccine virus. The sera from volunteers who received A/HK/77 (H1N1) wild-type virus or its *ts*-1A2 recombinant were tested by ELISA, using goat anti-human IgG and IgM, and the results were compared with those observed with the standard HI and NI techniques (Table 2). Each of six volunteers who received H1N1 wild-type virus had a response detected by HI and ELISA. Volunteer no. 4 had an ELISA response detected after using anti-IgG but not anti-IgM, and the reverse was true for volunteer no. 1. Thus, each of the responses detected by ELISA was also detected by the HI assay in volunteers who received the wild-type virus, but this was not the case for recipients of the attenuated *ts*-1A2 recombinant. Whereas only four responses were detected by HI, eight were detected by ELISA. Importantly, it was necessary to use an anti-IgM conjugate in ELISA to detect a significant proportion of the responses developed by the vaccinees. Those vaccinees with the greatest HI responses also tended to have higher ELISA areas, although the correlation was not exact.

Volunteers who received an A/Alaska/77 (H3N2) or A/NJ/76 (Hswine N1) *ts* recombinant were analyzed in a similar fashion (Table 3). With these *ts* recombinants, a response was detected more often by ELISA than by HI or NI assay. However, a combination of the HI and NI assays detected as many responses in the volunteers given the A/Alaska/77 (H3N2)-*ts*-1A2 recombinant as did the ELISA. The correlation of seroresponses detected by the HI test and ELISA is presented in Table 4. All seroresponses detected by HI test were detected by the ELISA, but the ELISA detected more responses. Finally, the increased efficiency of ELISA for detection of a seroresponse compared with the HI assay was statistically significant (McNamar test of significance, $P < 0.01$) for the A/NJ/76 (Hswine N1) and A/Alaska/77 *ts* recombinants.

DISCUSSION

The present study demonstrates that the ELISA is more sensitive than the HI and NI techniques for detecting an antibody response in recipients of live attenuated influenza A virus belonging to different antigenic subtypes. Although conventional HI and NI tests are satisfactory for detecting an antibody rise after infection with wild-type virus, a more sensitive assay is needed for recipients of attenuated vaccines since the antigenic load is considerably less than that generated during infection with a wild-type virus. The present findings indicate that the

TABLE 2. *Comparison of the ELISA with the HI and NI assays for detection of a serum antibody response in volunteers who received A/HK/77 (H1N1) wild-type virus or ts-1A2 recombinant*

Influenza A virus administered	Volunteer no.	ELISA					Log₂ HI rise[a]	NI rise[b]
		Area		IgG response	IgM response	IgG or IgM response		
		IgG	IgM					
H1N1 wild type	1	38	78	0	+	+	3	0
	2	90	166	+	+	+	5	0
	3	189	72	+	+	+	3	0
	4	208	28	+	0	+	3	+
	5	226	134	+	+	+	4	0
	6	245	128	+	+	+	7	+
Total with response or rise				5	5	6	6	2
H1N1-*ts*-1A2 recombinant	1	3	3	0	0	0	0	0
	2	29	14	0	0	0	0	0
	3	40	30	0	0	0	0	0
	4	20	62	0	+	+	0	0
	5	86	121	+	+	+	0	0
	6	126	68	+	+	+	0	0
	7	−36	111	0	+	+	1	0
	8	−3	193	0	+	+	2	0
	9	13	596	0	+	+	2	0
	10	305	50	+	+	+	3	0
	11	46	376	0	+	+	4	0
Total with response or rise				3	8	8	4	0

[a] Fourfold rise considered significant.
[b] 1.5 $\log_2$ rise considered significant.

TABLE 3. *Comparison of ELISA with HI and NI assays for detection of seroresponse by volunteers who received wild-type virus or ts recombinant*

Virus received	No. of volunteers	No. with indicated response				Total no. with response
		ELISA[a]	HI	NI	HI and/or NI	
A/Alaska/77 wild type	8	7	6	6	7	7
A/Alaska/77-*ts*-1A2 recombinant	28	22	11	14[b]	22	24
A/NJ/8/76-*ts*-1[E] recombinant	14	10	1	0	1	10
A/HK/77-*ts*-1A2 recombinant	11	8	4	0	4	8

[a] Anti-human IgG conjugate used in ELISA.

[b] Two responses were detected by NI test that were not detected by ELISA or HI test; 21 rises were detected by a combination of the HI and NI assays.

TABLE 4. *Correlation of responses detected by ELISA and HI assays*

Volunteers infected with:	No. tested	No. of volunteers with indicated pattern of ELISA and HI antibody responses		
		ELISA	HI	
			Response	No response
A/HK/77-*ts*-1A2 recombinant (H1N1)	11	Response	4	4
		No response	0	3
A/NJ/76-*ts*-1[E] recombinant (Hswine N1)	14	Response	1	9
		No response	0	4
A/Alaska/76-*ts*-1A2 recombinant (H3N2)	22	Response	11	11
		No response	0	6

ELISA is a suitably sensitive assay for this purpose. It should prove useful in determining by serological assay the human $TCID_{50}$ for highly attenuated influenza A virus vaccines. The present study also indicates that an anti-human IgG conjugate is not sufficient to detect all responses by ELISA. For the vaccinees who received the H1N1-*ts*-1A2 recombinant, each of eight rises was detected by using an anti-IgM conjugate, whereas only three of eight were detected by using anti-IgG. Since these vaccinees were selected because they lacked prior experience with H1N1 surface antigens, a predominant IgM component in their primary response was not unexpected.

When whole virus is used as antigen, antibodies to viral components other than the surface glycoproteins (the hemagglutinin and neuraminidase) are probably also measured. For this reason, there is no assurance that the seroresponse detected by the ELISA would be correlated with protection. To focus upon that portion of the immune response directly related to resistance, it will be necessary to develop hemagglutinin- and neuraminidase-specific ELISAs. We have observed some volunteers who received attenuated influenza A viruses who were resistant to wild-type virus challenge in the absence of a serum HI antibody rise to the vaccine (6, 15). Hence, there is a need to develop glycoprotein antigen-specific tests sufficiently sensitive to detect low levels of serum and nasal wash antibody and to use such assays to determine the level of antibody associated with resistance to influenza A virus infection. Appropriately constructed glycoprotein specific ELISA appears well suited to attain these goals.

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