Serological Diagnosis of Influenza B Virus Infection: Comparison of an Enzyme-Linked Immunosorbent Assay and the Hemagglutination Inhibition Test

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The sensitivity of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibody to influenza B virus was compared with that of the hemagglutination inhibition test on acute- and convalescent-phase sera obtained from adults and children infected with influenza B virus. Two whole virus, tissue culture-grown antigen preparations were used in the ELISA, influenza B/West Virginia/81 and influenza B/Hong Kong/72. Four antigens were used in the hemagglutination inhibition test. These included the tissue culture-grown whole virus antigens that were used in the ELISA. In addition the standard egg-grown antigens, influenza B/Singapore/79 and influenza B/Hong Kong/72, were included for comparison. The ELISA antibody titer was significantly correlated to the hemagglutination inhibition antibody titer, and 10 of 10 adults and 17 of 21 children infected with influenza B had fourfold antibody increases as detected by ELISA with either antigenic type of tissue culture-grown whole virus. Increases in geometric mean antibody titers of 16- to 71-fold were detected by ELISA. Increases in geometric mean antibody titers of 3- to 10-fold were detected by hemagglutination inhibition depending on the type of antigen utilized. We found that ELISA with whole virus antigens was a sensitive and specific test for the detection of antibody to influenza B virus.

Although the hemagglutination inhibition (HAI) test is the standard assay for detecting antibodies against influenza A and B viruses, in some instances it does not detect antibody increases in patients naturally infected with influenza viruses. This may be due to the antigenic drift manifested by influenza A viruses from year to year, so that use of standardized hemagglutinin reagents may not detect antibody after infection with heterologous influenza A viruses (3, 7, 8, 18). In the case of influenza B virus, less frequent and less extensive antigenic changes occur (16, 17), yet not all infected persons develop HAI antibody increases. Recently, enzyme-linked immunosorbent assays (ELISA) utilizing various antigen preparations of influenza viruses have been developed for the detection of antibodies to influenza A or B viruses (2, 4, 5, 8, 9, 11, 12; C. C. Dacso, J. Quarles, M. W. Harmon, B. Baxter, and R. B. Couch, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 19th, Boston, Mass. abstr. no. 46, 1979). These enzyme immunoassays have been shown to be more sensitive than either complement fixation or HAI for detection and quantitation of antibody to influenza viruses.

During our studies on nosocomial infections due to influenza B virus in 1980, we observed that 3 of 10 patients who shed influenza B virus in their respiratory secretions failed to develop HAI antibody increases to either Singapore or Hong Kong hemagglutinin antigen (17a). For this reason we sought to develop a more sensitive assay to detect antibody to influenza B virus. In this report, we compare the sensitivity of the ELISA to that of the HAI for the detection of antibody to influenza B virus. We also compare a tissue culture-grown HAI antigen prepared in our laboratory to the commercially available egg-grown antigen for the detection of HAI antibody to influenza B virus.

MATERIALS AND METHODS

Sera. Sera were obtained from 10 adults and 21 children who became naturally infected in the 1979 to 1980 influenza B virus epidemic in Huntington, W. V. Sera were obtained from 10 adult inpatients from the Huntington Veterans Administration Medical Center
infected when a nosocomial outbreak of influenza B virus occurred (17a). Preinfection and postinfection sera were obtained from the 21 children (mean age, 28.1 months) as part of ongoing seroepidemiological surveillance in the community of Huntington, W. V. (1). Influenza B/West Virginia/80 virus (an influenza B/ Illinois/79-like virus) was isolated from the respiratory secretions of each adult and child to confirm infection. In addition, paired sera were obtained from 20 adults who were suspected of having influenza B virus infection because they were hospitalized with an influenza-like illness at the time of a nosocomial influenza B virus outbreak, but from whom influenza B virus was not isolated. Preinfection and postinfection serum pairs from 28 children (mean age, 28.7 months) infected with other viruses (documented by isolation of a virus from their respiratory secretions) were included as control sera to determine the specificity of the ELISA.

**ELISA procedure.** Antigens for the ELISA were produced in our laboratory. Briefly, African green monkey kidney cells (AGMK) were infected with a wild-type strain of influenza B virus (influenza B/West Virginia/1/80 [B/WV-tc]; Table 1) isolated from a patient during the 1979 to 1980 influenza B virus epidemic that occurred in Huntington, W. V. The virus was characterized by the World Health Organization (WHO) Influenza Center, Centers for Disease Control, Atlanta, Ga., as similar to influenza B/Illinois/79. Four days after inoculation, the AGMK tissue culture was frozen and thawed to disrupt the cell monolayer. The resulting suspension was centrifuged at 1,500 rpm, and the supernatant fluid was used as the viral antigen. A second viral antigen was prepared in an identical manner by using influenza B/Hong Kong/5/72 (B/HK-tc) virus which was obtained from the WHO Influenza Center. Uninfected AGMK cells with tissue culture medium were treated in a similar manner, and the supernatant fluid was used as control antigen in the ELISA. Antigens were stored frozen at −70°C until used.

The ELISA was modified from the procedure of Murphy et al. (12). The optimal dilutions of each antigen were determined by checkerboard titration in carbonate buffer. The optimum dilutions corresponded to 13 to 32 hemagglutinating units of influenza B virus per microtiter well. Viral antigen and control antigen was adsorbed to flat-bottom, 96-well microtiter plates (micro-ELISA M129A; Dynatech Laboratories, Inc., Alexandria, Va.) for a minimum of 18 h at 4°C before use. After washing three times with phosphate-buffered saline, sera in fourfold dilutions, 1:100 to 1:25,600, were then added to duplicate wells. Dilutions of sera less than 1:100 frequently manifested reactivity with control antigens, and therefore they were not tested (14). After 18 h of incubation at 4°C, the excess serum was washed from each well, the optimal dilution of alkaline phosphatase conjugated anti-human immunoglobulin G prepared in goats (Miles Laboratories, Elkhart, Ind.) was added, and the plates were incubated at 35°C for 2 h. After washing to remove excess conjugate, substrate (p-nitrophenyl phosphate; Sigma 104; Sigma Chemical Co., St. Louis, Mo.), diluted to 1 mg/ml in diethanolamine buffer, was added. After 30 min of incubation at 37°C, the absorbance (405 nm) of each well was determined by an automated reader (Multiskan; Flow Laboratories, McLean, Va.).

A single reference serum, in fourfold dilutions of 1:100 to 1:25,600, was included in each group of tests as a standard. This reference serum has been used many times in our laboratory with the antibody titer set at 1:1,600. The 1:1,600 dilution was considered the endpoint since this dilution had an absorbance value of approximately 0.40, whereas the absorbance value of the serum at 1:6,400 dilution was less than 0.10. The reference serum, at a 1:1,600 dilution, was included on each plate to monitor the development of the enzyme reaction.

The antibody titer for each test serum was calculated as follows. A calibration line was fitted by least squares to a series of log dilutions and the corresponding logit absorbances of the reference serum. The fitted logit absorbance, designated y*, of the preselected dilution (1:1,600) was derived from the slope and intercept of the calibration line. For each experimental serum, a line was fitted by least squares to a series of log dilutions and the corresponding logit absorbances. The titer of the experimental serum was taken to be the antilog of the fitted log dilution on the line in question which corresponded to y*. Fourfold or greater rises in ELISA titers between acute and convalescent sera were considered diagnostic of infection.

**HAI procedure.** The HAI procedure was that of the WHO Influenza Center (19). The initial serum dilution used was 1:10. The antigens used in the ELISA and HAI are summarized in Table 1. The influenza B/Singapore/79 (B/Sing-egg) and influenza B/Hong Kong/72 (B/HK-egg) HAI antigens were egg-grown antigens obtained from the WHO Influenza Center. The tissue culture-grown antigens were the same antigen preparations used in the ELISA.

### Table 1. Antigen preparations used in this study

<table>
<thead>
<tr>
<th>Antigen determination method</th>
<th>Antigen</th>
<th>System of production</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>Influenza B/West Virginia/1/80</td>
<td>Tissue culture&lt;sup&gt;a&lt;/sup&gt;, &lt;sup&gt;b&lt;/sup&gt;</td>
<td>B/WV-tc</td>
</tr>
<tr>
<td>ELISA</td>
<td>Influenza B/Hong Kong/72</td>
<td>Tissue culture&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B/HK-tc</td>
</tr>
<tr>
<td>HAI</td>
<td>Influenza B/West Virginia/1/80</td>
<td>Tissue culture&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B/WV-tc</td>
</tr>
<tr>
<td>HAI</td>
<td>Influenza B/Hong Kong/72</td>
<td>Tissue culture&lt;sup&gt;c&lt;/sup&gt;</td>
<td>B/HK-tc</td>
</tr>
<tr>
<td>HAI</td>
<td>Influenza B/Singapore/79</td>
<td>Egg</td>
<td>B/Sing-egg</td>
</tr>
<tr>
<td>HAI</td>
<td>Influenza B/Hong Kong/72</td>
<td>Egg</td>
<td>B/HK-egg</td>
</tr>
</tbody>
</table>

<sup>a</sup> The same antigen preparation was used by both test methods.

<sup>b</sup> Similar to influenza B/Illinois/79/
4.1, and the corresponding coefficients of variation were 3.2, 3.4, and 7.3%, respectively. The log$_{10}$ mean titers using the B/HK-tc antigen were 3.2, 2.9, and 4.0, and the corresponding coefficients of variation were 6.3, 3.9, and 2.5%, respectively. There was less than a threefold difference in titer in replicate determinations comparing the maximum and minimum values obtained at any time for any of the three sera tested.

Using the B/WV-tc antigen we compared the ELISA titer with the HAI titer in sera from adults and children (Fig. 1). There was good correlation between the log HAI and log ELISA titers ($r = 0.71$, $P < 0.01$). Nine sera (all from children) were antibody free as indicated by both ELISA and HAI. Only one serum contained antibody that was detected by HAI, but not by ELISA. In contrast, 15 sera had ELISA antibody, but did not have HAI antibody. ELISA detected antibody significantly more often than did HAI ($\chi^2 = 6.9; P < 0.01$).

We compared the sensitivity of ELISA to that of HAI for the detection of fourfold antibody increases among adults and children infected with influenza B virus (Table 2). Among 10 adults who were infected with influenza B virus, each had fourfold or greater increases in ELISA antibody to either the B/WV-tc or B/HK-tc antigens. In contrast, with the B/Sing-egg antigen only five adults ($P = 0.032$, Fisher exact test, two-tailed probability) had a fourfold or greater increase in HAI antibody; six had a fourfold or greater increase in HAI antibody to the B/HK-egg antigen ($P = 0.086$, Fisher exact test, two-tailed probability). Nine and 10 of the 10 adults had fourfold or greater increases in HAI antibody to the B/WV-tc antigen and B/ HK-tc antigen, respectively. Overall tissue culture-grown antigens were significantly more sensitive than egg-grown antigens in detecting HAI antibody increases (19 of 20 versus 11 of 20, $\chi^2$

![FIG. 1. Comparison of reciprocal ELISA and HAI titers in 102 sera from children (■) and adults (○) with B/WV-tc antigen ($r = 0.71$).](http://jcm.asm.org/)

### RESULTS

The reproducibility of the ELISA antibody titers was evaluated by testing three sera 10 times each. The log$_{10}$ mean titers of the three sera with the B/WV-tc antigen were 3.1, 2.9, and 4.1, and the corresponding coefficients of variation were 3.2, 3.4, and 7.3%, respectively. The log$_{10}$ mean titers using the B/HK-tc antigen were 3.2, 2.9, and 4.0, and the corresponding coefficients of variation were 6.3, 3.9, and 2.5%, respectively. There was less than a threefold difference in titer in replicate determinations comparing the maximum and minimum values obtained at any time for any of the three sera tested.

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### TABLE 2. Comparison of the sensitivity of ELISA and HAI using various antigens for the detection of antibody to influenza B virus

<table>
<thead>
<tr>
<th>Study group</th>
<th>Virus shed in respiratory secretions</th>
<th>Total no. in group</th>
<th>No. with fourfold antibody increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ELISA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B/WV-tc</td>
</tr>
<tr>
<td>Adults</td>
<td>Influenza B Virus</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Children</td>
<td>Influenza B Virus</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Parainfluenza 1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Parainfluenza 2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Parainfluenza 3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Adenovirus</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Respiratory syncytial virus</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Influenza A</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ See Table 1 for summary of antigens.

$^b$ NT, Not tested.
FIG. 2. Comparison of fold ELISA antibody increase to fold HAI antibody increase among 21 children (■) and 10 adults (○) with influenza B virus infection. B/WV-tc antigen was used in each assay. The line of unity is indicated.

= 6.5, P < 0.05). Among 21 children infected with influenza B virus, 17 had fourfold or greater increases in ELISA antibody to B/WV-tc or B/HK-tc antigens. Fifteen had fourfold increases in HAI antibody with the B/WV-tc antigen (17 of 21 versus 15 of 21, \( \chi^2 < 1, P = \text{not significant} \)).

We also evaluated the specificity of the B/WV-tc ELISA by testing paired sera from children infected with other respiratory viruses (Table 2). Sera from six patients infected with parainfluenza 1, four patients infected with parainfluenza 2, two patients infected with parainfluenza 3, five patients infected with adenovirus, six patients infected with respiratory syncytial virus, and five patients infected with influenza A virus were tested by ELISA. None of these 28 patients had fourfold antibody increases to influenza B virus as indicated by ELISA.

Using B/WV-tc antigen we compared the magnitude of the antibody response as measured by ELISA and HAI of 10 adults and 21 children who were infected with influenza B virus (Fig. 2). ELISA detected larger fold increases than did HAI in 14, whereas HAI detected larger fold increases than did ELISA in only 3 (14 of 31 versus 3 of 31, \( \chi^2 = 9.8, P < 0.01 \)).

The magnitude of the antibody response as detected by ELISA and HAI with the different antigens on sera from 10 infected adults is summarized in Table 3. The increases were larger as detected by ELISA compared with HAI; 16-fold and 71-fold increases in geometric mean antibody titer occurred to B/WV-tc and B/HK-tc antigens, respectively. A 10-fold increase in geometric mean HAI antibody titer was detected with the B/WV-tc or B/HK-tc antigen. Sixfold and threefold increases in geometric mean antibody HAI titers were observed with the B/HK-egg and B/Sing-egg antigens, respectively.

DISCUSSION

Documentation of infection with influenza viruses is often not possible because some patients with clinical findings of influenza A or B virus infection do not have detectable influenza virus shedding (7), nor do all patients who shed virus manifest diagnostically significant HAI antibody rises (3, 6, 7, 13, 15, 18, 20; Dacso et al., 19th ICAAC, abstr. no. 46). A more sensitive antibody assay would be useful for epidemiological investigation of influenza B virus outbreaks, especially when virus isolation procedures cannot be used.

Previously reported ELISA techniques for determining influenza B virus antibodies have used either a solid-phase antigen preparation (2) or an extensively purified virus or hemagglutinin (11, 12; Dacso et al., 19th ICAAC, abstr. no. 46). Neither antigen preparation is generally available. In this report we have described an ELISA for influenza B antibody determination which utilized whole-virus antigen purified only by low-speed centrifugation. In addition to the ease of antigen production, the influenza B virus antigen readily adsorbed to microtiter plates. Other advantages of this ELISA as compared with HAI included the suitability to perform the assay en masse and the fact that ELISA did not

<table>
<thead>
<tr>
<th>Time blood specimen obtained</th>
<th>Reciprocal geometric mean titer</th>
<th>ELISA</th>
<th>HAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B/WV-tc</td>
<td>B/HK-tc</td>
<td>B/WV-tc</td>
</tr>
<tr>
<td>Acute illness</td>
<td>1,580</td>
<td>600</td>
<td>16</td>
</tr>
<tr>
<td>Convalescent</td>
<td>25,600</td>
<td>42,700</td>
<td>160</td>
</tr>
<tr>
<td>Increase (fold)</td>
<td>16</td>
<td>71</td>
<td>10</td>
</tr>
</tbody>
</table>

*See Table 1 for summary of antigens.*
require repeated titrations of the antigens or pretreatment of serum.

ELISA was a sensitive and specific assay for the determination of antibody increases among adults and children infected with influenza B virus. However, among 21 children infected with influenza B virus, 4 did not develop fourfold or greater antibody rises by ELISA or HAI, indicating that some infections with influenza B virus in children cannot be confirmed by either serological technique. Indeed, Wright et al. found that only 5 of 14 children with documented influenza B virus infection demonstrated diagnostically significant rises in HAI titers (20). Others also have found that children and young adults have not demonstrated good antibody responses to primary infection with influenza viruses. None of the children who failed to manifest an ELISA antibody response had detectable ELISA antibody (ELISA titer <50) before or after infection with influenza B virus (7, 18). An immunoglobulin M antibody response was the principal response that occurred in children who lacked prior experience with influenza A/H1N1 surface antigens (11). However, using an influenza B whole virus antigen which was more purified than ours, Murphy et al. were able to detect immunoglobulin G antibody rises in 10 of 10 children with influenza B virus infection (12). A more purified antigen may be necessary to measure small amounts of influenza B virus antibody in children, since lower dilutions of sera can be used without giving cross-reactions with other antigens in the preparations, as occurs in our ELISA with unpurified whole virus.

Avidity between antibody and influenza B virus has been reported to be more important than the antigenic type of influenza B virus used in the HAI test, and ether treatment of virus has been used to increase avidity (10). We also found that using different antigenic types of influenza B virus did not significantly alter the sensitivity of the HAI test or ELISA test. However, we found that uncloned tissue culture-grown antigens were superior to egg-grown antigens in the HAI test. Whether this phenomenon is due to the differing passage histories of the viruses or due to the cell system in which the virus was produced was not determined by these experiments. The ELISA described in this report used the whole virus and therefore contained the internal viral antigens as well as the surface antigens. Thus, this ELISA may not reflect a difference in serum antibody response to various hemagglutinins of influenza B virus. Similar enzyme immunoassays which use whole virus antigen preparations do not detect strain-specific antibody to influenza A virus. To detect strain-specific antibody to influenza A virus requires the use of purified hemagglutinin as the ELISA antigen (11). Murphy et al. have demonstrated that a hemagglutinin-specific ELISA was more sensitive than HAI in detecting antibody (11), but the HAI used by Hammond et al. was more sensitive than a whole-virus enzyme immunoassay to detect antibody to influenza A virus (5). Whether ELISA with purified influenza B virus hemagglutinin could detect antibody rises after primary infection of children remains an area for future investigation.

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LITERATURE CITED