Increased Sensitivity and Reduced Specificity of Hemagglutination Inhibition Tests with Ether-Treated Influenza B/Singapore/222/79

A. P. KENDAL1 AND T. R. CATE2*

World Health Organization Collaborating Center for Influenza, Influenza Branch, Division of Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333,1 and lnfluenza Research Center, Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

Received 3 February 1983/Accepted 5 July 1983

Hemagglutination inhibition (HI) tests against whole virus (WV) influenza B/Singapore/222/79 antigen detected prevaccination serum antibody in only 15 (20%) of 50 predominantly elderly volunteers and fourfold or greater titer rises in only three (6%) after they received 1981-1982 trivalent influenza vaccine containing antigens of this virus. HI titters against ether-treated (ET) B/Singapore/222/79 were about eightfold higher than those against WV antigen and were comparable to microneutralization titters against this virus. The ET HI detected prevaccination antibody in 84%, a postvaccination titer rise in 32%, and a final titer of 80 or higher in 66%. Among 51 additional persons with known or presumed influenza B virus infections early in 1982, ET B/Singapore/222/79 was also more sensitive than WV for serodiagnosis (69 versus 49%), but eight persons with both WV and ET B/Singapore/222/79 HI responses also had an HI titer rise to WV A/Brazil/11/78 (H1N1) antigen. Conversely, among 14 college students with febrile, culture-proven influenza A (H1N1) infections early in 1982, 6 (43%) developed HI titer rises to ET B/Singapore/222/79 with no other serological evidence of influenza B virus infection. Moreover, young adult volunteers with mild experimental influenza A (H1N1) infections also exhibited a 17% (3 of 18) incidence of ET B/Singapore/222/79 HI titer rises, versus none in matched, uninfected volunteers. These data indicate that ET B/Singapore/222/79 virus has increased sensitivity but reduced specificity compared to WV as an HI antigen and that caution is needed in interpretation of a single HI test for serodiagnosis, whether with WV or ET antigen.

The relatively inexpensive and easily performed influenza virus hemagglutination inhibition (HI) test has been widely used for serodiagnosis of infection, measurement of serum antibody prevalence, and determination of vaccine-induced antibody responses (16). However, the existence of virus strains which are poorly reactive in HI tests has been recognized with all influenza A virus subtypes that have caused epidemics in humans (6, 7, 13; unpublished observations), and many investigators have observed the same problem with influenza B viruses in recent years (2, 5, 9, 15, 17, 18, 20). A means for increasing the reactivity of many influenza virus antigens in HI tests is ether treatment of the virus; this effect of ether treatment has been described for H1N1 viruses (11, 13), H2N2 viruses (19), one of the equine influenza viruses (1), and recently prevalent influenza B viruses (15). However, modifications of an antibody assay that increase its sensitivity also risk increasing the occurrence of nonspecific reactions (14).

The present studies were initiated when an HI test with whole virus (WV) influenza B/Singapore/222/79 antigen detected very little antibody and few serological responses among a group of predominantly elderly volunteers before and after receipt of standard influenza vaccine containing this virus. A sensitive microneutralization test and a repeat HI test against ether-treated (ET) virus both revealed widely prevalent antibody before vaccination and more frequent serological responses afterwards. However, additional studies of the utility of the HI with ET B/Singapore/222/79 for serodiagnosis of
infection suggested that the increased sensitivity of the test was associated with decreased specificity for antibody to the virus.

**MATERIALS AND METHODS**

Five groups of paired sera were tested. The first group was collected late in 1981 from 50 volunteers who donated blood samples before and 1 month after vaccination with the 1981-1982 trivalent influenza virus vaccine (15 μg each of A/Bangkok/1/79, A/Brazil/11/78, and B/Singapore/222/79 hemagglutinin per dose). Most of these volunteers were elderly adults (median age, 70.5 years) and all lived independently in the community. The second group consisted of acute- and convalescent-phase sera from 38 elderly nursing home residents who became ill during outbreaks of influenza B virus infection early in 1982 (3, 4). The third group was acute- and convalescent-phase sera from 13 children who had been diagnosed by virus isolation or complement-fixation (CF) testing at state health laboratories as having influenza B virus infection during local outbreaks in institutions or schools early in 1982. The fourth group was acute- and convalescent-phase sera from 14 college students whose nasal wash specimens yielded an H1N1 influenza A virus during naturally acquired febrile, influenza-like illness early in 1982. The fifth group consisted of paired sera from young adults who participated in several different influenza virus volunteer studies performed when no influenza virus other than the one being studied was active in the community; included were sera from 12 people experimentally infected with an influenza B virus representative of 1973, 1976, or 1977 strains (4 people with each strain), sera from 18 persons experimentally infected with A/USSR/77-like (15 people) or A/Brazil/78-like (3 people) H1N1 virus, and sera from 30 people who were matched for participation in the same studies as the preceding volunteers but who had no virus isolates or serological responses detected in their initial laboratory evaluation.

WV antigens for HI tests were grown in embryonated chicken eggs. Virus for preparing ET B/Singapore/222/79 was similarly grown and then treated with ether as described (15). This treatment resulted in an approximately 16-fold increase in hemagglutinating titer. The HI tests were performed by standard microtiter procedure (8), using sera pretreated with receptor-destroying enzyme and an automatic serum diluter (Cooke Laboratory Products, Alexandria Va.). Samples (25 μl) of sera were reacted at room temperature for 30 min with 4 hemagglutinating units of each virus antigen (in 25 μl) and then reacted with 50 μl of a 0.5% suspension of chicken erythrocytes. Microneutralization tests were done with fresh serum for enhancement as previously described (9); the assays were performed with Madin-Darby canine kidney cells against 3.2 to 32 50% tissue culture infectious doses of Madin-Darby canine kidney-grown B/Singapore/222/79. CF tests were performed by standard microtiter procedure (12), using egg-grown influenza A/Hong Kong/8/68 (H3N2) and B/Hong Kong/5/72 antigens which had been treated with ether and adsorbed with chicken erythrocytes to remove hemagglutinin. Titers were expressed reciprocally.

**RESULTS**

Comparison of HI and microneutralization tests for measurement of pre- and postvaccination antibody. An HI test against WV B/Singapore/222/79 antigen failed to detect antibody (titer of less than 10) in 35 (70%) of the prevac
cination sera and in 18 (36%) of the postvacci
tination sera from group 1. A repeat HI test with ET virus and a microneutralization test against B/Singapore/222/79 both suggested that most or all of the sera that failed to inhibit hemagglutina
tion by WV antigen nevertheless possessed antibody (Fig. 1, panels A and B). For sera with no detectable antibody in the WV HI test, the geometric mean titer (GMT) in the ET HI test was 21.6 (range, less than 10 to 80), and the neutralizing GMT was 12.8 (range, 3 to 96). Sera with barely detectable antibody in the WV HI test (titer of 10) had ET HI and neutralizing GMTs which were severalfold higher (ET HI, 76.3; neutralizing, 54.1), and titers in the WV HI

![Graph](https://example.com/graph.png)

**FIG. 1.** Relationship of serum antibody titers to B/Singapore/222/79 measured by HI against WV antigen (WV HI), HI against ET virus antigen (ET HI), and neutralization (NEUT.). Open circles represent prevaccination sera from 50 predominantly elderly volunteers and closed circles represent sera 1 month after receipt of trivalent influenza vaccine containing B/Singapore/222/79 antigens.
TABLE 1. Pre- and postvaccination serum antibody titers to B/Singapore/222/79 for a group of 50 elderly volunteers

<table>
<thead>
<tr>
<th>Test</th>
<th>% With titer of:</th>
<th>GMT*a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥10</td>
<td>≥20</td>
</tr>
<tr>
<td>WV HI</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>ET virus HI</td>
<td>84</td>
<td>72</td>
</tr>
<tr>
<td>Neutralization</td>
<td>68</td>
<td>54</td>
</tr>
</tbody>
</table>

*a Sera were collected before and 1 month after vaccination in the fall of 1981 with 15 μg each of A/Brazil/11/78 (H1N1), A/Bangkok/1/79 (H3N2), and B/Singapore/222/79 hemagglutinin per dose.

*b For calculation of GMT, HI titers less than 10 were assigned a value of 5, and neutralizing titers less than 2 were assigned a value of 1.

c Fourfold or greater increase in titer.

increased from that point more or less in parallel with those in the other two tests. In contrast to the relationship between WV HI and neutralizing titers for sera at the lower end of the spectrum, no difference existed between the neutralizing titers of sera with an ET HI titer of less than 10 and those with an ET HI titer of 10 (neutralizing GMTs of 5.2 and 6.0, respectively). Neutralizing titers were higher for sera with an ET HI titer of 20 (GMT, 14.7; range, 4 to 48), and titers in the ET HI and neutralizing tests increased more or less in parallel from that point (Fig. 1C).

The relationships of titers on group 1 sera before and after vaccination are summarized in Table 1. The proportion of volunteers with a fourfold or greater titer rise was only 6% in the WV HI, 32% in the ET HI, and 44% in the neutralization test. Altogether, 26 (52%) of the volunteers had one or more serological responses to vaccination as follows: 3 in all three tests, 9 in both ET HI and neutralization tests, 10 in the neutralization test alone, and 4 in the ET HI test alone. Examining the serological responses to vaccination in another way, the proportion of volunteers with an ET HI titer of 80, a neutralizing titer of 32, or both increased from about 25% before vaccination to 66% afterwards. In contrast, WV HI titers as high as 20 were present in only 8% before vaccination and in 28% afterwards.

Serodiagnostic testing. The usefulness of HI tests with ET B/Singapore/222/79 for serodiagnosis of infection was compared to that with the WV antigen by using sera from groups 2, 3, 4, and 5. Among elderly persons who became ill during nursing home outbreaks of influenza B virus infection, 58% exhibited serum HI responses with ET antigen compared to 42% with WV B/Singapore/222/79 (Table 2). It is not known whether the other individuals did not develop an HI response to infection or were not actually infected with influenza B virus, as they were included in the study exclusively on the basis of clinical and epidemiological criteria. The majority of children who donated group 3 sera were initially diagnosed as having influenza B infection early in 1982 by CF testing in a state health department laboratory. All exhibited an HI response with ET antigen, whereas only 69% had significant responses detected with WV B/Singapore/222/79. No influenza A viruses were recognized in association with the influenza B outbreaks from which the group 2 and group 3 sera were derived, but, nevertheless, eight (16%) of the donors exhibited fourfold or greater antibody responses to influenza A/Brazil/11/78 (H1N1) WV antigen. All of the latter persons also exhibited HI titer rises with both ET and WV B/Singapore/222/79 antigens.

Among group 4 sera from college students with naturally acquired culture-proven H1N1 influenza A virus infection, 12 (86%) of 14 pairs exhibited a fourfold or greater titer rise in CF tests against influenza A antigen, 11 (79%) in HI tests against A/Brazil/11/78 (H1N1), 7 (50%) in neutralization tests against A/Brazil/11/78, and 13 (93%) in one or more of these tests. An influenza B/Singapore/79-like virus was active on campus just before and somewhat overlapping the H1N1 virus, but none of the nasal wash specimens from the group 4 donors yielded influenza B virus on culture. Moreover, none of their serum pairs exhibited a titer rise in HI tests against B/Singapore/222/79 WV antigen or in neutralization tests against B/Singapore/222/79, and none of their acute- or convalescent-phase sera had detectable CF antibody against influenza B antigen. Nevertheless, six (43%) of the serum pairs exhibited fourfold or greater titer rises in HI tests against ET B/Singapore/222/79; all six of these serum pairs were among those with both HI and CF rises against influenza A virus.

To further assess the frequency of nonspecific HI titer rises, we selected paired sera from young adult volunteers who had been experimentally infected with an influenza B or A
(H1N1) virus and from matched volunteers who participated in the same studies but had no laboratory evidence of infection on initial evaluation. WV B/Singapore/222/79 detected only 1 of 12 previously known infections with earlier B variants, and no titer rises occurred among 18 volunteers infected with influenza A (H1N1) virus or 30 volunteers without prior evidence of infection. In contrast, the ET B/Singapore/222/79 HI detected 5 of 12 previously known influenza B virus infections and provided the only laboratory evidence of infection in two additional volunteers who had been inoculated with a 1976 strain of wild-type influenza B virus. Virus isolations and HI and neutralization tests against B/Hong Kong/72-like virus had been negative for the latter two volunteers, although one had developed an afebrile respiratory illness without other detected cause. None of the remaining 28 volunteers without prior evidence of infection (including 18 participants in H1N1 virus studies) developed an ET HI titer rise, but 3 (17%) of 18 infected with an A (H1N1) virus did so. WV A/Brazil/11/78 antigen detected 9 of 18 infections with earlier H1N1 variants. No rises in titer against A/Brazil/11/78 occurred among volunteers with evidence of influenza B virus infection, but one did occur among 28 volunteers with no other evidence of infection. The latter volunteer had been challenged with the 1976 strain of wild-type influenza B virus in August 1979, 3 months after intranasal vaccination with an attenuated influenza B virus.

**DISCUSSION**

The HI test with WV B/Singapore/222/79 antigen appeared to underestimate the prevalence of serum antibody and the frequency of titer rises after vaccination in the present studies. HI titers measured with ET virus were severalfold higher than those with WV antigen for sera from a group of predominantly elderly volunteers and were comparable to titers obtained in a sensitive microneutralization test. About half of the volunteers developed fourfold or greater rises in ET HI titer or neutralizing titer, or both after vaccination, but only 6% did so in the WV HI test. The proportion of volunteers with a neutralizing titer of 32 or greater, which should be protective based on data in other influenza virus systems (9; Frank, A. L., personal communication), increased from 28% before vaccination to 66% afterward. Similarly, the proportion with an ET HI titer of 80 or greater increased from 22 to 66%, although WV HI titers as high as 20 were found in only 8% before vaccination and 28% afterward.

HI tests with ET B/Singapore/222/79 were also more sensitive than those with the WV for detecting serological responses to presumed or known influenza B virus infections during the 1981-1982 season and experimental infections with earlier B variants. WV HI tests against neither a highly avid strain of influenza B/Hong Kong/5/72 nor an influenza B virus recovered during one of the outbreaks detected as many titer rises as ET B/Singapore/222/79 (data not shown). However, concern about nonspecific reactivity in the HI tests was raised by the occurrence of simultaneous titer rises to WV influenza A/Brazil/11/78 antigen in 16% of persons with presumed or known naturally acquired influenza B virus infections. Nonspecific A/Brazil/11/78 titer rises were not detected among volunteers with generally mild experimental influenza B virus infections, but similar HI titer

**TABLE 1—Continued**

<table>
<thead>
<tr>
<th>Postvaccination</th>
<th>% With titer of:</th>
<th>GMT*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥10</td>
<td>≥20</td>
</tr>
<tr>
<td>64</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>98</td>
<td>98</td>
<td>88</td>
</tr>
</tbody>
</table>

**TABLE 2.** HI responses to different influenza antigens measured with paired sera from persons involved in influenza B outbreaks

<table>
<thead>
<tr>
<th>Group</th>
<th>No. (%) of serological rises^a detected with:</th>
<th>No. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WV B/Singapore/79</td>
<td>ET B/Singapore/79</td>
</tr>
<tr>
<td>Nursing home residents</td>
<td>16 (42)</td>
<td>22 (58)</td>
</tr>
<tr>
<td>Children</td>
<td>9 (69)</td>
<td>13 (100)</td>
</tr>
</tbody>
</table>

^a Fourfold or greater increase in titer.
rises against WV influenza A (H1N1) antigens were reported after influenza B virus infections during the 1979-1980 season (10). Conversely, the sensitive ET B/Singapore/222/79 antigen detected HI titer rises in 43% of college students with naturally acquired febrile influenza A (H1N1) infection in the present studies, even though WV HI and neutralization tests against B/Singapore/222/79 and an influenza B CF test each revealed no rises. Similarly, ET B/Singapore/222/79 detected 3 (17%) HI titer rises among 18 volunteers with mild experimental influenza A (H1N1) infection, versus none among concomitantly studied volunteers without infection. It is not known whether nonspecific reactivity is as extensive with influenza A subtypes other than H1N1 and with influenza B viruses other than those like B/Singapore/222/79 or whether it occurs after receipt of inactivated influenza virus vaccine as frequently as after infection. However, caution is clearly needed in interpreting the results of any single HI test for serodiagnosis, whether with WV or ET antigen. Caution should also be used in extrapolating the findings obtained with ET B/Singapore/222/79 to HI tests with other influenza virus strains, which may not necessarily respond in a similar manner to either treatment (15). Although these studies have shown that ether treatment of B/Singapore/222/79 will increase the low reactivity of this virus as an antigen in HI tests, at least some of the increased reactivity after infection appears not to be specific for B/Singapore/222/79. Further work will be necessary to define the basis for the nonspecific reactions and the situations in which they occur.

ACKNOWLEDGMENTS

Expert technical assistance was provided by Barbara Baxter, Carol Stocksdale, Ruth Ann Tucker, and Herta Wolff. Supported in part by Public Health Service contract AI-92611 awarded by the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


