

Ether Treatment of Type B Influenza Virus Antigen for the Hemagglutination Inhibition Test

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Ether treatment was studied as a method of increasing the ability of type B influenza antigen to detect antibody by hemagglutination inhibition. Comparisons were made with the untreated antigen, with an eluate made from the same virus, and with a standard type B antigen of an earlier virus. Results were evaluated based on the comparative ability to detect rises in antibody titer, as well as the relative frequency of antibody prevalence determined by each method. The ether-treated antigen was far superior to the untreated antigen in both respects; it was also superior to the eluate, although the difference was less pronounced. The treated antigen performed better than the standard type B antigen in detecting antibody in children, but there was little difference in adults. This pattern was felt to be a result of the closer relation of the treated antigen to the infecting strain. The method is, therefore, proposed as a means of producing more reactive antigens of currently circulating strains of type B influenza virus.

The hemagglutination inhibition (HAI) test has been for many years the standard method for detecting the presence of influenza virus antibodies (5). The test is valuable because it is relatively simple to perform and endpoints are clear and easy to read. In fact, protection from infection has frequently been correlated with certain levels of HAI antibodies (1, 13). However, it has always been recognized that problems do exist, especially involving nonspecific inhibitors in serum and differences in the ability of virus strains to detect antibody, sometimes termed avidity (7, 9). Difficulty in detecting rises in antibody titer has been a particular problem with type B influenza strains (3). In the current study, comparisons were made between serological results obtained in HAI tests employing four different type B antigens.

MATERIALS AND METHODS

Virus antigens. B/Tecumseh/2/77 was isolated in this laboratory from a clinical specimen collected in Tecumseh, Mich. The virus was originally recovered in primary rhesus monkey kidney cells and passed five times in embryonated eggs. Pools of the virus were made from this seed by one further passage in eggs inoculated allantoically and incubated for 72 h at 34°C. Virus antigens consisted of (i) nontreated infected allantoic fluids (NT), (ii) ether-treated fluids (ET) and (iii) an erythrocyte eluate of the virus. The fourth antigen used was a B/Hong Kong/5/72 recombinant (BX1-HP) supplied by the World Health Organization Collaborating Center for Influenza, Center for Disease Control, Atlanta, Ga. The ether treatment method was that described by Berlin et al. (2). The allantoic fluid harvests were first clarified by centrifugation.

They were then exposed to an equal volume of cold anesthetic-grade ether. After mixing under pressure between two syringes interconnected with fused 15 and 18 gauge needles, the material was allowed to layer at 4°C for approximately 15 min. The uppermost, ether-containing layer was removed, leaving the lower layer and the interface. Nitrogen gas was then bubbled through the remaining fluid to remove residual ether. The virus erythrocyte eluate was prepared by releasing erythrocytes from the embryo while harvesting the fluids. The erythrocyte-containing fluid was placed at 4°C for 1 h. The erythrocytes were removed by centrifugation from the supernatant, which was discarded; the cells were suspended in 1/10 the original volume of phosphate-buffered saline and placed at 37°C for 2 h to elute the virus. The erythrocytes were sedimented by centrifugation and discarded; the supernatant fluid represented the final eluate. The fourth antigen, B/Hong Kong/5/72 (BX1-HP), has been used extensively for serological testing because of its known high avidity for antibody.

Sera tested. In the course of the study of respiratory illness in Tecumseh, Mich., serum specimens were collected on a regular, systematic basis from all participating individuals (11) and stored in the Tecumseh serum bank. Specimens for testing were selected from a listing arrayed in random order by families. They had been collected from 1976 through early 1978, thus including the type B influenza outbreak of 1977. All specimens from the same individual were run at one time. The first half of families in this randomized list were tested against three antigens, B/Tecumseh NT, B/Tecumseh ET, and B/Tecumseh eluate. The second half were run against the NT and ET antigens plus the B/Hong Kong antigen.

Sera to be tested were treated with receptor-destroying enzyme as previously described (10). An equal volume of this enzyme was added to 0.2 ml of serum.

After overnight incubation at 37°C, 2 volumes of citrate saline were added, and the resultant solution was inactivated at 56°C for 0.5 h. The treated sera were serially diluted with phosphate-buffered saline in U-bottomed microplates from a starting dilution of 1:8. An equal volume of phosphate-buffered saline containing 4 hemagglutinating units of the particular antigen was added, and the mixture was incubated for 1 h at room temperature. Thereafter, 2 volumes of a 0.5% suspension of chick erythrocytes was added to each well, and patterns of hemagglutination were read 45 min later, after cells had settled.

RESULTS

All sera were tested against NT and ET antigens of B/Tecumseh/2/77. Results were calculated in terms of the number of fourfold rises in titer per series of collected sera from an individual; this indicated the ability to detect infection. Table 1 presents the infection data for 420 individuals, divided into broad age groups. The superiority of the ET antigen was clear for all ages. Only rarely did the NT antigen detect a rise in titer that was not also observed with the ET antigen. The fall-off in infection rate with increasing age is a reflection of the pattern of occurrence of type B influenza, which most heavily involves school-age children. Rises in antibody titer correlated significantly with typical influenza illness during the period of type B

virus isolation. Viewed another way, of the 73 rises in titer detected by either method, 95.9% were found by using the ET antigen, and 30.1% were found with the NT antigen.

Results were also examined in terms of presence of antibody at a 1:8 titer in the first serum collected from an individual (Table 2). The ET antigen was far superior to the NT antigen here in detecting antibody prevalence at a 1:8 dilution. This was especially the case in children, where practically no antibody was detected with the NT antigen. The poor reactivity of the antigen before exposure to ether suggests that it was particularly nonavid; variability in avidity of recently isolated strains has always been a problem in comparing results in HAI tests (7, 9). Viewed in terms of the 200 sera with antibody detected by either or both tests, 99% had antibody present against the ET antigen, and 22% had antibody against the NT antigen. Overall, in contrast to the situation with the infection data, in which children showed the highest rates of antibody increases, antibody prevalence was at similar levels in the different age groups.

Comparison with other type B antigens.

Two additional antigens were used to evaluate further the relative advantages of using the ET preparation; the sera examined were those described above. An eluate was prepared from egg fluid harvests of B/Tecumseh/2/77. In its preparation, the process of absorption and elution from erythrocytes produced an antigen which was more purified with removal of allantoic fluid components. In this characteristic, the antigen was similar to the ET antigen. The eluate was tested against sera from approximately half of the families. We compared results obtained with this antigen with those obtained with the ET and NT antigens, again in terms of rise in antibody titer (Table 3) and antibody prevalence at 1:8 serum dilution in the first specimen (Table 4). For ease in description of the three-way comparison, the results are presented based on the proportion of overall positives detected by any single procedure; this type of calculation has

TABLE 1. Rises in HAI antibody titer detected with NT and ET antigens of B/Tecumseh

Age group (yr)	n	Frequency ^a of fourfold titer rises with indicated antigens			
		B/Tecumseh (ET) only	B/Tecumseh (NT) only	Both ET and NT	Total
5-19	120	22 (18.3)	0 (0)	12 (10.0)	34 (28.3)
20-39	111	12 (10.8)	1 (0.9)	4 (3.6)	17 (15.3)
40+	189	17 (9.0)	2 (1.1)	3 (1.6)	22 (11.6)
Total	420	51 (12.1)	3 (0.7)	19 (4.5)	73 (17.4)

^a Number with rise (percent) in indicated age group.

TABLE 2. Prevalence of HAI antibody as detected with NT and ET antigens of B/Tecumseh

Age group (yr)	n	Frequency ^a of antibody at 1:8 serum dilution with indicated antigens			
		B/Tecumseh (ET) only	B/Tecumseh (NT) only	Both ET and NT	Total
5-19	120	50 (41.7)	0 (0)	4 (3.3)	54 (45.0)
20-39	111	38 (34.2)	0 (0)	12 (10.8)	50 (45.0)
40+	189	68 (36.0)	2 (1.1)	26 (13.8)	96 (50.8)
Total	420	156 (37.1)	2 (0.5)	42 (10.0)	200 (47.6)

^a Number with antibody in first specimen (percent) in indicated age group.

TABLE 3. Comparison of rises in antibody titers detected with three B/Tecumseh antigens

Age group (yr)	Total no. with rises detected	No. (%) of total rises detected using indicated antigen		
		B/Tecumseh (ET)	B/Tecumseh (NT)	B/Tecumseh (eluate)
5-19	14	14 (100.0)	4 (28.6)	4 (28.6)
20-39	13	10 (76.9)	3 (23.1)	9 (69.2)
40+	20	13 (65.0)	4 (20.0)	12 (60.0)
Total	47	37 (78.7)	11 (23.4)	25 (53.2)

TABLE 4. Comparison of antibody prevalence detected with three B/Tecumseh antigens

Age group (yrs)	Total no. with antibody detected ^a	No. (%) of total with antibody detected using indicated antigen		
		B/Tecumseh (ET)	B/Tecumseh (NT)	B/Tecumseh (Eluate)
5-19	22	22 (100.0)	3 (13.6)	6 (27.3)
20-39	23	21 (91.3)	7 (30.4)	10 (43.5)
40+	54	51 (94.4)	17 (31.5)	27 (50.0)
Total	99	94 (94.9)	27 (27.3)	43 (43.4)

^a At 1:8 titer in initial serum.

TABLE 5. Comparison of rises in antibody titer detected by two B/Tecumseh antigens and B/Hong Kong antigen

Age group (yrs)	No. of total rises in titer detected	No. (%) of total rises detected using indicated antigen		
		B/Tecumseh (ET)	B/Tecumseh (NT)	B/Hong Kong
5-19	21	20 (95.2)	8 (38.1)	14 (66.7)
20-39	9	6 (66.7)	2 (22.2)	5 (55.6)
40+	11	7 (63.6)	1 (9.1)	9 (81.8)
Total	41	33 (80.5)	11 (26.8)	28 (68.3)

TABLE 6. Comparison of prevalence of antibody detected by two B/Tecumseh antigens and B/Hong Kong antigen

Age group (yrs)	Total no. with antibody detected ^a	No. (%) of total with antibody detected using indicated antigen		
		B/Tecumseh (ET)	B/Tecumseh (NT)	B/Hong Kong
5-19	38	32 (84.2)	1 (2.6)	29 (76.3)
20-39	36	29 (80.6)	5 (13.9)	33 (91.7)
40+	53	43 (81.1)	11 (20.8)	45 (84.9)
Total	127	104 (81.9)	17 (13.4)	107 (84.3)

^a At titer of 1:8 in initial serum.

already been used above. Thus, it can be seen in Table 3 that of the 14 rises in titer detected by any method in the 5- to 19-year-olds, all were detected by the ET antigen and 4 each were detected with the NT antigen and eluate. The eluate outperformed the NT antigen in the older age group but was never as successful in detecting titer rises as was the ET antigen. In terms of antibody prevalence (Table 4), the eluate was, for all age groups, intermediate in sensitivity between the NT antigen and the ET preparation. Thus, the use of the eluate was an improvement over using the NT antigen, but it did not result in as sensitive a test as that achieved with ether treatment.

Serological results obtained with the treated and NT antigens on the other half of the families

were compared with those determined with the antigen of B/Hong Kong provided by the Center for Disease Control, a reagent of known high avidity. Results (Tables 5 and 6) again compare the sensitivity of antigens in detecting rises in antibody titer and in detecting antibody prevalence at 1:8 dilution. The B/Tecumseh ET antigen detected, in the 5- to 19-year-old age group, all but 1 of the 21 rises found with any procedure (Table 5). The B/Hong Kong antigen showed a rise in titer in 66.7% of all infections detected, intermediate between the ET and NT B/Tecumseh antigens. In contrast, in the two older age groups, both the treated and the B/Hong Kong antigens gave similar results. Since the strain causing infection in 1977 was a B/Tecumseh-like virus, the difference in ability to detect titer rises in the children may be a reflection of the antigenic drift between this virus and the 1972 isolate. The lack of difference in the adults may be related to their past experience with B/Hong Kong-like strains, conditioning their antibody response (6).

When antibody prevalence was considered, the results for the ET antigen and the B/Hong Kong antigen were quite similar. Once more, the ET preparation was better in the children and the B/Hong Kong was superior in adults. Overall, the results were not significantly different. Again, the data may be a reflection of the differences between the 1977 strain and those previous strains with which older individuals had more experience.

DISCUSSION

The HAI test is the single most valuable procedure used to quantify serum antibody against influenza. The technique is simple and reproducible, and, most importantly, titers have been shown to correlate with protection against infection (1, 13). However, a major problem has been recognized over the years, involving variability in the reactivity of various influenza strains with antibody. The variation can be classified generally under the term "avidity"; the phenomenon has been studied in detail for the type A strains and has been shown to relate to factors such as passage history, whether in eggs or animals (9, 12). However, much also depends on the virus itself, and manipulation after isolation may have little effect on ability of a nonavid strain to detect antibody (4, 7). It has been recognized that, even with strains avid for antibody, the HAI test often is relatively insensitive in detecting rises in titer. This is particularly a problem with type B virus (3).

Ether treatment of an allantoic fluid harvest of influenza viruses represents a simple method

for increasing its ability to react with antibody. The technique itself has been used before, especially with some of the type A equine strains, and through cleavage of the virion probably makes more sites available to react with antibody (2, 14). It has been widely employed since 1977 in detection of antibodies to the H1N1 influenza A viruses (8). Untreated virus was not efficient in detecting rises in titer after infection of individuals under age 24 who had not previously been exposed to this subtype. An ether-split preparation originally prepared for use as a vaccine was extensively employed for this purpose (8). Similarly, in the current study, it was with children that the ET antigen showed the major improvement in detecting infection.

The ether treatment method is particularly valuable since it can be employed on newly isolated strains, which can then be used to test sera collected during the same period for changes in antibody titer against that virus. Thus it is not necessary to go back to an old and often different strain, such as B/Hong Kong/5/72, which has been extensively manipulated to make it more antibody sensitive. Type A H3N2 viruses also can be rendered more reactive by ether treatment. A recent H3N2 A/Texas-like isolate, A/Alaska/5/77, and a 1980 type B isolate have been similarly treated, and again there was nearly a doubling of the ability to detect rises in antibody titer; prevalence of antibody at 1:8 dilution was similarly affected (A. S. Monto and J. Trojanowski, unpublished data). However, not all influenza viruses increase in reactivity with ether treatment (Monto and Trojanowski, unpublished data). The method is probably most successful with relatively new isolates which appear to be nonavid, and results should be evaluated for sensitivity with known antigens before putting the method into operation. Actual antibody titers do increase with ET antigens, and protection from influenza has previously been defined in terms of a certain specific level, usually 1:32 (1, 13). Therefore, it may be necessary to redetermine the needed level of antibody when more sensitive, treated antigens are employed. The ET antigens will be most useful in studies in which complete determination of serological infection rates is needed, especially when children are involved. This will be of special value in describing the effects in population groups of newly emergent viruses, or viruses

with changed surface antigens. It may well be found that apparent sparing of children under age 5 years from type B influenza infection is an artifact produced by the inability of the usual test to detect rise in titer in this age group (11).

ACKNOWLEDGMENT

This study was supported in part by a Public Health Service contract (AI-62514) from the Development and Applications Branch, National Institute of Allergy and Infectious Diseases.

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