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# Improved Colorimetric Assay for Detecting Influenza B Virus Neutralizing Antibody Responses to Vaccination and Infection

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**An automated neutralization test for influenza B virus is described in which antibody titers are determined according to the release of neutral red from infected or uninfected cells of the Madin-Darby canine kidney line. Endpoints are determined in a standard enzyme-linked immunosorbent assay reader. The test requires no expensive immunologic reagents and was used to evaluate responses to both vaccination and natural infection against influenza B virus. Overall responses to vaccination were comparable with those obtained by hemagglutination inhibition, using Tween-ether-split influenza B/Ann Arbor/1/86 virus as the antigen (the HI-TE test). The sensitivities of neutralization responses compared with those obtained by the HI-TE test for two vaccines were 88 and 89%; the specificities were lower at 61 and 60%, respectively. Responses to vaccination, measured by hemagglutination inhibition, were significantly higher with split virus compared with whole virus. However, seroconversion by both the HI-TE and neutralization tests was observed in 5 of 10 individuals from whom virus was detected by either culture of nasal or throat washings or the presence of antigen from immunofluorescence in cells from nasal washings.**

Conventional hemagglutinin inhibition (HI) tests are relatively insensitive for determining serologic responses to influenza B viruses. Sensitivity may be increased by the use of virus disrupted with Tween 20 and ether (6) or by the use of viruses propagated in cells of the Madin-Darby canine kidney (MDCK) line (8). However, tests in which disrupted viruses are used are less specific than neutralization tests (6). Neutralizing antibody levels generally provide a more functional indication of immunity for viruses than do other types of antibody, but neutralization tests are more laborious to set up and require a longer time to obtain results. A rapid neutralization procedure has been described for influenza B virus in which susceptible cells were inoculated with virus-serum mixtures; endpoints were determined in an enzyme-linked immunosorbent assay reader according to the expression of group-specific influenza virus antigen after the addition of conjugated monoclonal antibody (4). In this paper we describe an automated neutralization procedure in which the endpoint is measured by the release of neutral red from cells of the MDCK line that had been inoculated with virus-serum mixtures. The test has been used to determine responses to the influenza B component of two subunit vaccines and to natural infection with influenza B virus. The results were compared with those obtained with HI, using whole or disrupted virus (the HI-W and HI-TE tests, respectively).

## MATERIALS AND METHODS

**Virus.** Influenza virus strain B/Ann Arbor/1/86 was obtained from I. M. Cheyne of the Commonwealth Serum Laboratories, Parkville, Victoria, Australia. Stocks were prepared after growth in the allantoic cavities of 10-day-old chicken embryos for 2 to 3 days at 33°C. Allantoic fluids were pooled, placed in an ampoule, rapidly frozen, and stored at −80°C.

**Neutralization assay.** The neutral red release assay was based on previously described cytotoxicity T-cell assays (9, 10). Ninety-six-well, flat-bottomed enzyme-linked immunosorbent assay plates (Linbro; Flow Laboratories, Inc., McLean, Va.) were seeded with MDCK cells ($3 \times 10^4$ per well) in a growth medium consisting of Eagle minimal essential medium with 10% fetal calf serum, 0.0375% $NaHCO_3$, 100 U of penicillin $ml^{-1}$, 100 μg of streptomycin $ml^{-1}$, and 5 μg of amphotericin B (Fungizone) $ml^{-1}$. The plates were incubated for 1 to 2 days at 34°C in a 5% $CO_2$ incubator until confluent cell growth was obtained. Fourfold dilutions of each serum in serum-free medium from an initial dilution of 1:4 were incubated with an equal volume of a preparation of influenza B virus, diluted to contain 200 50% tissue culture infective doses 100 $\mu l^{-1}$. The mixtures were incubated at 37°C for 1 h, and 100-μl samples of each mixture were inoculated into four wells. After adsorption for 30 min, each well was washed with 200 μl of serum-free medium to remove trypsin inhibitors present in serum and then 200 μl of a 1:1 mixture of Leibovitz L-15 medium and Eagle minimal essential medium with 0.0375% $NaHCO_3$, 100 U of penicillin $ml^{-1}$, 100 μg of streptomycin $ml^{-1}$, 100 μg of gentamicin $ml^{-1}$, and 5 mg of amphotericin B $ml^{-1}$ with 1 μg of crystalline trypsin (catalog no. 3740; Worthington Diagnostics, Freehold, N.J.) $ml^{-1}$ was added. The plates were incubated for a further 4 days at 37°C, and medium was removed from each well and replaced with 200 μl of 0.036% (wt/vol) neutral red. After incubation at 34°C for 30 min, neutral red was removed and the cells were washed three times with phosphate-buffered saline. Two hundred microliters of a 1:1 mixture of ethanol and phosphate-buffered saline was then added to each well, and the plates were shaken for 3 min until all cells were suspended and the dye was released. Released neutral red had a yellow color of

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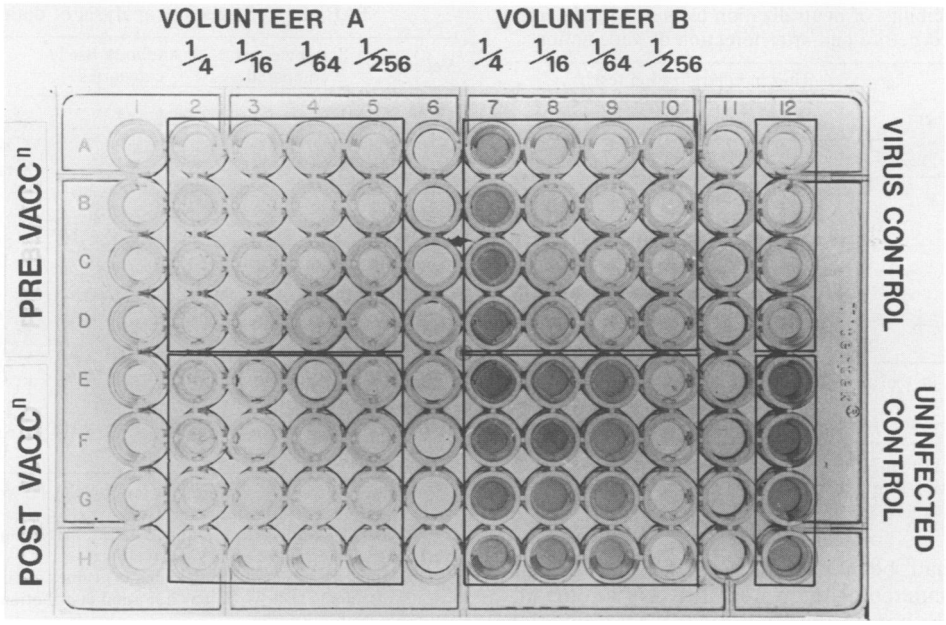


FIG. 1. Appearance of plate showing neutralization tests on sera collected from two volunteers before and after vaccination. Dilutions of serum for each virus-serum mixture are indicated; four wells were inoculated with samples of each mixture. Neutral red was released from uninfected cells in each well as described in Materials and Methods, and the endpoint was taken as the mean $A_{492}$ for the uninfected control wells minus three standard deviations. Pre- and postvaccination (PRE VACC$^n$ and POST VACC$^n$) titers for volunteer A were <4 and <4, and those for volunteer B were 4 and 64.

$A_{492}$; the $A_{492}$ of each well was determined in a Flow Titertek Multiscan reader. The mean and standard deviation of the absorbance for four uninfected control wells were also determined, and endpoints were determined according to the formula: net $OD_{492}$ = (mean $OD_{492}$ of uninfected control wells − 3SD) − $OD_{492}$ of test well, where a positive net optical density (OD) value denotes the presence of unneutralized virus. A serum endpoint was assigned to the serum dilution which yielded positive net $OD_{492}$ values for at least two of the four test wells. A typical pattern for two serum pairs is shown in Fig. 1. Infectivity titers of stock viruses used in virus-serum mixtures were similarly determined by inoculating 10-fold dilutions of each stock into confluent MDCK cells and determining the $A_{492}$ of released neutral red. Endpoints were determined by the method of Reed and Muench (11).

**HI titrations.** The HI-W test was carried out by a standard procedure, using 4 hemagglutinating units per mixture (7). Titrations were carried out over a twofold-dilution range from an initial dilution of 1:10. A similar procedure was used for the HI-TE test. The method used to disrupt virus was that of Jennings et al. (5). Equal volumes of allantoic fluid and ether plus 0.01% Tween 20 were combined and left at 4°C overnight. Phases were separated by centrifugation, and the lower aqueous phase was extracted twice with ether before being dialyzed against phosphate-buffered saline and stored at −80°C.

**Vaccine study.** A double-blind study was carried out from April to May 1987 in the Newcastle region with 270 volunteers who were administered either one of two trivalent subunit vaccines (A or B) or a placebo containing isotonic saline. Each vaccine contained the surface antigens of A/Mississipi/1/85 (H3N2), A/Taiwan/1/86 (H1N1), and B/Ann Arbor/1/86 at concentrations of 15 μg dose$^{-1}$. Sera were collected at the time of vaccination and after 4 weeks. All serologic testing of samples was carried out under code.

**Acute-phase specimens.** From July to August of the 1987 Australian winter, a total of 454 unvaccinated volunteers in the Newcastle region were monitored for the presence of respiratory viruses. Influenza B virus was detected in the acute nasal or throat washings of 10 volunteers over an 8-week period by cultivation in cells of the MDCK line or identification of a viral group-specific antigen in cells from centrifuged nasal washings by a direct immunofluorescent-antibody procedure (1) or both. Sera were obtained before and after the sampling period, and the last sample was obtained at least 2 weeks after virus had been identified in the washings.

**Treatment of sera.** Sera for use in neutralization tests were incubated at 56°C for 30 min. Inhibitors were removed from sera used for HI titrations by treatment with receptor-destroying enzyme by a standard procedure (7).

**Statistical methods.** The increase in antibody titer by each test was calculated for individual volunteers in the vaccination study. Seronegative individuals were assigned a score of 1 for the neutralization test (i.e., $4 \times 4^{-1}$) and 5 for the HI-W and HI-TE tests ($10 \times 2^{-1}$). Tables were constructed showing increases in titer by the neutralization test versus the HI-W or the HI-TE test, and values of $\chi^2$ were determined for matched pairs. The McNemar test (2) used for 2 × 2 tables, and the Stuart-Maxwell test (2) was used for 3 × 3 tables. Kappa statistics (2) were applied as an intraclass correlation coefficient to assess the level of agreement beyond that expected due to chance alone. When rises were sufficiently large, kappa values were determined from a 2 × 2 table, using the antibody rise or categories of <4 and ≥4, or a 3 × 3 table, using categories of <4, 4 to <16, and ≥16.

Sensitivity and specificity for the neutralization test were determined against the standard by using the HI-TE test (2). A fourfold-or-greater rise in antibody was regarded as significant by either test, and sensitivity was calculated as the probability of detecting such a rise by the neutralization test,

TABLE 1. Reproducibility of neutralization tests in paired serum specimens obtained before and after infection or vaccination

| Type of sample[a] | Volunteer | Titer in neutralization test[b]: 1 Pre | 1 Post | 2 Pre | 2 Post | 3 Pre | 3 Post |
|---|---|---|---|---|---|---|---|
| Infection | 1 | 4[c] | 256 | <4 | 256 | <4 | 256 |
| | 2 | <4 | <4 | <4 | <4 | <4 | <4 |
| | 3 | 16 | 16 | 16 | 16 | 16 | 16 |
| Vaccination | 4 | 16 | 64 | 16 | 64 | 4 | 16 |
| | 5 | <4 | 256 | 4 | 256 | 4 | 256 |
| | 6 | 64 | 64 | 256 | 256 | 256 | 256 |

[a] Sera were collected at the times of infection or vaccination and 2 to 4 weeks later.
[b] Tests were carried out separately on three different occasions.
[c] Neutralization titers were determined from the dilution of serum that was sufficient to inhibit the growth of a final concentration of 100 50% tissue culture infective doses of B/Ann Arbor/1/86 in virus-serum mixtures.

if a similar rise had been detected by the HI-TE test. Specificity was calculated as the probability that a fourfold-or-greater rise could not be detected by the neutralization test if such a rise could not be detected by the HI-TE test.

## RESULTS

**Reproducibility of neutralization titers in tests carried out on different occasions.** Paired serum specimens from three volunteers who experienced natural infection and three who had been vaccinated were assayed on three separate occasions. The results (Table 1) indicate good reproducibility between tests. Titers varied by as much as fourfold (a single dilution) from other values for the same serum. However, importantly, the patterns of significant antibody rises (i.e., fourfold or greater) remained the same between tests, and the procedure was, therefore, reliable and could be used for serodiagnosis.

**Responses to the influenza B component of trivalent subunit vaccines.** Table 2 summarizes responses to influenza B vaccine antigens when paired serum specimens from each group were tested, under code, for antibody by the neutralization test and by the HI-TE and HI-W tests. No rises in antibody were detected in the placebo group, and a statistical analysis for each vaccine group is shown in Table 3. Significantly more antibody rises for either vaccine occurred by the neutralization and HI-TE tests than by the HI-W test. However, differences in the numbers showing antibody rises by the neutralization and HI-TE tests were not significant. Volunteers receiving vaccine A recorded significantly more rises of 16-fold or greater by the neutralization test than by the HI-TE test, compared with others receiving vaccine B, which is reflected in the difference in the geometric mean titers between each group (Table 2).

Because increases in antibody that were detected by the HI-TE and neutralization tests were not significantly different, kappa statistics were calculated to measure the level of agreement. Although there was significantly higher agreement than could be attributable to chance alone, the level of agreement was relatively low, which was reflected in kappa values of 0.4968 to 0.5206, when using the presence or absence of seroconversion, and 0.4026 to 0.4307, when using categories of antibody increases of <4, 4 to <16, and ≥16

TABLE 3. Statistical analysis of data in Table 2

| Vaccine | Serologic tests compared | Antibody rise[a] categories | Value[b] | P[c] |
|---|---|---|---|---|
| A | HI-W vs Neut[d] | <4, ≥4 | $\chi^2_{21}$, 48.0200 | <0.001 |
| | HI-W vs HI-TE | <4, ≥4 | $\chi^2_{21}$, 43.0222 | <0.001 |
| | HI-TE vs Neut | <4, ≥4 | $\chi^2_{21}$, 0.8421 | <0.359 |
| | | | $\kappa_2$, 0.4968 | <0.001 |
| | | <16, ≥16 | $\chi^2_{21}$, 6.7222 | <0.010 |
| | | <4, 4–8, ≥16 | $\chi^2_{22}$, 8.7160 | <0.013 |
| | | | κ, 0.4026 | <0.001 |
| B | HI-W vs Neut | <4, ≥4 | $\chi^2_{21}$, 37.5319 | <0.001 |
| | HI-W vs HI-TE | <4, ≥4 | $\chi^2_{21}$, 37.0256 | <0.001 |
| | HI-TE vs Neut | <4, ≥4 | $\chi^2_{21}$, 0.5000 | <0.480 |
| | | | κ, 0.5206 | <0.001 |
| | | <16, ≥16 | $\chi^2_{21}$, 0.0500 | <0.823 |
| | | <4, 4–8, ≥16 | $\chi^2_{22}$, 0.9535 | <0.621 |
| | | | κ, 0.4307 | <0.001 |

[a] Antibody rises of >8 were too few by the HI-W test to allow further disaggregation of rises in the ≥4 class.
[b] $\chi^2$ values at 1 df were determined by the McNemar test; $\chi^2$ values at 2 df were determined by the Stuart-Maxwell test.
[c] Probabilities associated with kappa (κ) values were calculated using the Z test (2), assuming that kappa has a normal distribution.
[d] Neut, Neutralization.

TABLE 2. Responses to the B/Ann Arbor/1/86 component of trivalent subunit vaccines determined by different tests

| Vaccine | No. | Test | GMT[a] Pre | GMT[a] Post | % with indicated titer: Pre <4 | Pre >16 | Pre <10 | Pre >40 | Post <4 | Post >16 | Post <10 | Post >40 | % Antibody rise[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Placebo | 90 | Neut[c] | 5 | 5 | 42 | 16 | | | 42 | 16 | | | 0 |
| | | HI-TE | 21 | 21 | | | 33 | 22 | | | 33 | 22 | 0 |
| | | HI-W | <10 | <10 | | | 77 | 1 | | | 77 | 1 | 0 |
| Vaccine A | 87 | Neut | <4 | 18 | 53 | 9 | | | 12 | 38 | | | 71 |
| | | HI-TE | 13 | 71 | | | 45 | 10 | | | 6 | 59 | 66 |
| | | HI-W | <10 | <10 | | | 91 | 0 | | | 46 | 1 | 14 |
| Vaccine B | 93 | Neut | <4 | 30 | 50 | 15 | | | 10 | 58 | | | 74 |
| | | HI-TE | 15 | 144 | | | 44 | 22 | | | 2 | 80 | 70 |
| | | HI-W | <10 | 12 | | | 85 | 0 | | | 23 | 7 | 28 |

[a] GMT, Geometric mean titer; Pre, prevaccination; Post, postvaccination.
[b] Percentage with a fourfold-or-greater increase in titer after vaccination.
[c] Neut, Neutralization.

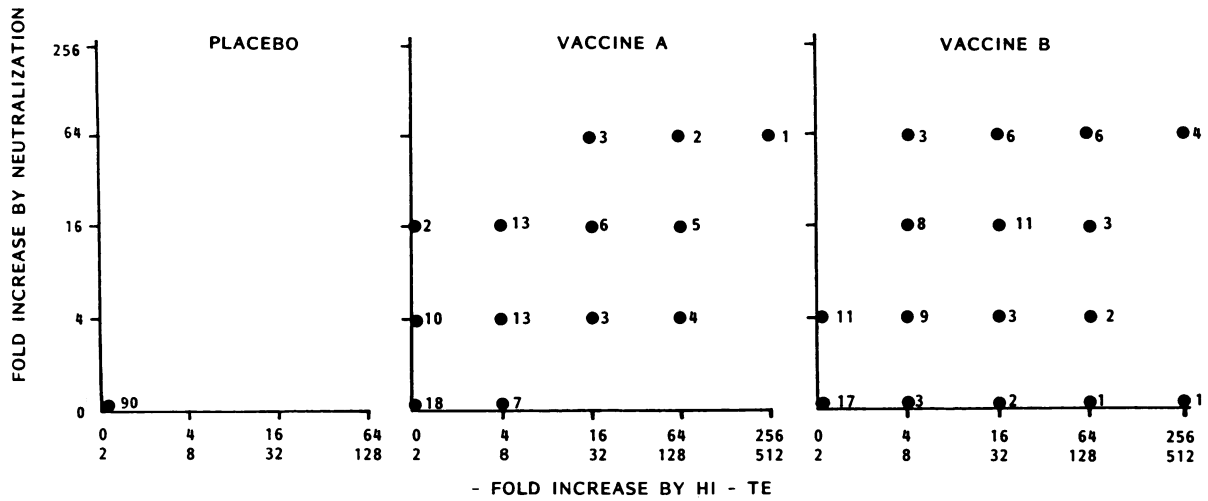


FIG. 2. Increases in antibody titer induced by vaccines A and B, as determined by the neutralization and HI-TE tests. Twofold serum dilutions were used for HI-TE tests, and fourfold dilutions were used for neutralization tests.

(Table 3). These trends are indicated in Fig. 2, which shows that 23 (13%) with an antibody increase by the neutralization test did not show an increase by the HI-TE test and 14 (8%) had antibody rises by the HI-TE test but not by the neutralization test.

**Responses to natural infection.** Pre- and postinfection sera from volunteers from whom virus was detected in acute-phase specimens were determined by the neutralization and HI-TE tests. Although the numbers were smaller than those for the vaccination study, the results (Table 4) indicate complete agreement between the tests. However, only five volunteers had antibody rises by both tests; the remaining five who had shown evidence of virus in nasal or throat washings did not have rises by either test. Importantly, four of five individuals from whom virus was detected by culture seroconverted by both the HI-TE and neutralization tests, suggesting that isolation in culture may be a better correlate of seroconversion than detection of antigen by a direct immunofluorescent-antibody procedure.

TABLE 4. Serologic responses in volunteers from whom influenza B virus was detected in nasal or throat washings

| Volunteer | Virus detected by[a]: | Neutralization response | | | HI-TE response | | |
|---|---|---|---|---|---|---|---|
| | | Pre | Post | Antibody rise[b] | Pre | Post | Antibody rise[b] |
| 1 | C, IFA | 16 | 64 | + | <10 | 80 | + |
| 2 | IFA | 16 | 16 | − | 10 | 10 | − |
| 3 | C, IFA | 16 | 64 | + | <10 | 20 | + |
| 4 | C, IFA | 4 | 256 | + | <10 | 160 | + |
| 5 | C, IFA | <4 | 4 | + | <10 | 20 | + |
| 6 | C, IFA | <4 | <4 | − | <10 | <10 | − |
| 7 | IFA | <4 | 64 | + | <10 | 80 | + |
| 8 | IFA | <4 | <4 | − | <10 | 10 | − |
| 9 | IFA | <4 | <4 | − | 10 | 10 | − |
| 10 | IFA | <4 | <4 | − | <10 | <10 | − |

[a] Virus was detected after isolation by culture in MDCK cells (C) or from the presence of a group-specific antigen in cells from nasal washings (direct immunofluorescent-antibody [IFA] procedure).

[b] Fourfold-or-greater increase in titer after infection. For the calculation of increases, neutralization and HI-TE titers of <4 and <10 were taken as 1 and 5, respectively (i.e., $4 \times 4^{-1}$ and $10 \times 2^{-1}$).

## DISCUSSION

Endpoints for the neutral red release neutralization test can be determined automatically and reliably in a standard enzyme-linked immunosorbent assay reader without the need for expensive reagents to detect unneutralized virus. In vaccination studies, both the HI-TE and neutralization tests were shown to be superior to the HI-W test (Tables 2 and 3). Although results by the neutralization and HI-TE tests showed significantly more agreement than could be attributed to chance alone, the overall level was only moderate, with 8 and 13% of volunteers seroconverting by one test but not the other. If the HI-TE test were regarded as the standard against which the increases in antibody by the neutralization test could be compared, sensitivities of 88 and 89% were obtained for vaccines A and B, respectively. However, specificity was relatively poor, being only 61% for vaccine A and 60% for vaccine B. Similar differences in specificity between influenza B neutralization and HI-TE responses to both natural infection and vaccination by influenza B antigen were noted by Kendal and Cate (6). Predictive values for the combined positive and negative responses to each vaccine were 82 and 90%, respectively. The levels of agreement between the HI-TE and neutralization tests probably reflect responses to different antigens that are measured by each test, which may have included some contribution from nonsurface antigens. However, for the 10 volunteers with proven natural infections, there was complete agreement between the results obtained by each test (Table 4).

Of interest (Table 4) was the observation that a preexisting neutralizing antibody titer of ≤16 failed to prevent the establishment of natural infection. Levels of 16 or greater have been shown to be protective for influenza B virus when determined by a microneutralization test by Frank et al. (3). Assuming that a level of greater than 16 is protective, the data in Table 2 can be used to determine the efficacy of each vaccine against influenza B virus infection according to the following formula: [no. with a postvaccination titer of >16 − no. with a prevaccination titer of >16 (%)]/(total no. vaccinated − no. with a prevaccination titer of >16). For vaccines A and B, these figures are 32 and 51%, respectively.

An antibody rise was detected in only 5 of 10 volunteers from whom virus was detected by culture or antigen detection or both in the cells from nasal washings, and no rise occurred in volunteer 6, from whom virus was detected by both procedures. However, it should be noted that an antibody rise was measured in four of five cases in which the virus was cultured. These figures suggest that the immune response to influenza B virus infection is variable. They also indicate the possibility of obtaining false-positive results by the use of a direct immunofluorescent-antibody procedure alone for the diagnosis of infection by influenza B virus.

## ACKNOWLEDGMENTS

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