An Improved Procedure for Measuring Neuraminidase Antibodies by Hemagglutination-Inhibition

ALAN P. KENDAL, ELVA MINUSE, and FRED M. DAVENPORT

Department of Epidemiology, School of Public Health, The University of Michigan, 109 Observatory Street, Ann Arbor, Michigan 48104

This work is dedicated to our friend and colleague, Professor Werner Schäfer, on the occasion of his 60th birthday

(Z. Naturforsch. 27 b, 241—245 [1972] ; received December 31, 1971)

Neuraminidase antibodies are known to inhibit hemagglutination by X-15 and X-15 (HK) recombinant viruses. However, the level of inhibition observed varies when different batches of chicken erythrocytes are employed, and the test generally detects neuraminidase antibodies with less sensitivity than an enzyme inhibition test. By titrating neuraminidase antibodies in the presence of anti-IgG globulins, with appropriate specificity, the hemagglutination-inhibition activity of neuraminidase antibodies is enhanced and the effect of cell variation is minimized. Consequently results obtained with this modified method for titrating neuraminidase antibodies become comparable to those obtained by measuring enzyme-inhibition. The improved hemagglutination-inhibition procedure possesses the important advantages of greater convenience and economy. Similar enhancing effects may also be obtained with egg white and guinea pig serum.

Neuraminidase (E.C. 3.2.1.18) is an enzymic component of influenza viruses, and shows antigenic specificity and variation independent of viral hemagglutinin\textsuperscript{1–4}. Methods for detection of antibodies to neuraminidase have until recently been based on chemical determination of free sialic acid\textsuperscript{5,6}. An alternative method for titrating antibodies to the neuraminidases of Asian and Hong Kong influenza viruses is by measuring hemagglutination inhibition with a suitable recombinant influenza virus\textsuperscript{7–9}. This report describes improvements in the latter method, by use of which results become comparable to those obtained with the chemical test and are more readily achieved. In consequence, studies on the possible epidemiological significance of neuraminidase antibodies are facilitated.

Materials and Methods

**Human sera**

For use in most experiments, pools were prepared employing equal aliquots from 24 or more serum pairs. These serum specimens were obtained from army recruits bled before and after vaccination in 1968 with 300 CCA units of A\textsubscript{2}/Aichi/2/68 virus. In one experiment paired specimens from this vaccine group and from another group receiving 250 CCA units of A\textsubscript{2}/Japan/305/57 vaccine in 1957 were employed. The bleeding interval was two weeks in each case. Sera were used untreated in the neuraminidase enzyme inhibition (NI) test, and were treated with trypsin-periodate\textsuperscript{10} for the hemagglutination inhibition (HI) test. All specimens were devoid of antibodies to the hemagglutinin of A/Equine/1/Prague/56 virus.

**Viruses**

Seeds of recombinant viruses X-15 and X-15(HK) were kindly supplied by Dr. E. D. Kilbourne. Both viruses contain hemagglutinin antigen derived from A/Equine/1/Prague/56 virus. However X-15 contains neuraminidase antigen derived from A\textsubscript{2}/RI/5/57 virus\textsuperscript{11}, whereas X-15(HK) contains neuraminidase.
antigen derived from A2/Hong Kong/16/68 virus (J. L. Schulman, personal communication). Viruses were grown in chicken eggs and partially purified from allantoic fluid by one cycle of red-cell adsorption-elution.

**Biological materials used to enhance hemagglutination-inhibition**

Chicken egg white was freshly prepared from 1 day eggs. Guinea pig serum, frozen at −65°C in small lots, was thawed when required. For some tests complement was inactivated by heating the serum to 56°C for 30 minutes. Fluorescein-conjugated anti-human IgG globulin (rabbit) was obtained from Sycco; (Lot 081169-C); Fluorescein-conjugated anti-human IgG globulin (goat) from Microbiological Associates (Lot 6122). All materials were diluted in phosphate buffered saline, pH 7.2.

**Hemagglutination-inhibition (HI) tests**

These tests were performed in perspex trays. To determine the concentration of biological materials which could rightfully be employed in the HI test, each substance was first titrated to find the lowest dilution that did not show HI activity in a standard HI test using four virus agglutinating doses. Since such concentrations of biological materials might nevertheless bind some virus even though complete HI was not observed, a further step was necessary. In this serial 0.2 ml dilutions of virus were prepared containing 4, 2, 1 and 0 agglutinating doses, which were added either 0.2 ml of saline or dilutions of biological materials. These mixtures were then reacted with 0.4 ml of 0.5 percent chicken erythrocytes. In this way it was possible to establish the lowest dilution of each material that did not inhibit even one virus agglutinating dose. To measure HI by neuraminidase antibodies in the presence of biological enhancing materials the following procedure was adopted. 0.2 ml of virus antigen (4 agglutinating doses) was added to serial 2-fold dilutions of human serum and reacted at room temperature for 30 minutes. Then 0.2 ml of a solution of biological enhancing material was added, and reacted for 30 min. before adding 0.2 ml of a 1% chicken erythrocyte suspension. Hemagglutination patterns were read 1 hr. later, and the reciprocal of the highest dilution of human serum causing HI was taken as the serum titer. Enhancer dilutions referred to in results are initial dilutions, and were the highest at which maximum enhancing activity was obtained, consistent with a lack of non-specific HI.

**Neuraminidase-inhibition (NI) tests**

These tests were performed as previously described, using human serum Cohn fraction IV-4 as enzyme substrate. Pre-immunization sera were generally used to give final dilutions of 1/20, 1/40, 1/80, and 1/160, and post-immunization sera 1/20, 1/80, 1/320, and 1/1280. Higher dilutions were used when necessary. Serum dilutions inhibiting 50% of control enzyme activity (measured in the absence of serum) were obtained by graphical interpolation.

**Results**


Since human sera do not contain antibodies to A/Equine/1/Prague/56 hemagglutinin, it is assumed that any HI with X-15 or X-15(HK) viruses caused by human sera is due solely to the presence of neuraminidase antibodies. However, when titrating neuraminidase antibodies by the HI test with X-15 and X-15(HK) viruses it was observed that use of erythrocytes from different chickens significantly affected antibody titer of a standard human serum pool. Moreover, the patterns of HI obtained with erythrocytes of most chickens were indistinct. It seemed likely that by use of appropriate materials which might stabilize HI patterns, end points could be sharpened and variability in test results might be reduced.

To investigate this possibility, human serum pools were prepared, and these were shown by the enzyme inhibition (NI) test to contain neuraminidase antibodies. NI titers in pre-immunization pools were 80 and 20 against X-15 and X-15(HK) antigens respectively, rising in post-immunization pools to about 1000 against both viruses.

The post-immunization serum pools were titrated for their HI activity against X-15 and X-15(HK) virus antigens in the presence of saline or various biological materials. Representative results are given in Table 1, which shows that in the standard HI test antibody activity was observed if erythrocytes from chicken No. 512 were used, but not with erythrocytes from chicken No. 546. However, when the test was carried out using various biological materials as stabilizing agents titers were generally enhanced, and similar values were obtained using erythrocytes from either chicken. These results demonstrate the usefulness of biological materials in overcoming variability in the HI test due to differences in sensitivity of erythrocytes to hemagglutination. They also demonstrate the increased sensitivity of the modified test.

In examples shown, the most distinct cell patterns were observed with a preparation of anti-human
IgG globulin as enhancing agent, and several other commercial preparations were therefore tested for enhancing activity. Considerable differences in activity were found, presumably because of the known variation in content of commercially available immunoglobulin preparations. The most active preparation was a fluorescein conjugated anti-human IgG globulin (goat), and this material was used to examine reproducibility of the HI test. Pre- and post-immunization 1968 serum pools were titrated for HI against X-15 and X-15(HK) virus antigens on several days using erythrocytes from different chickens, and pools of erythrocytes. Table 2 shows the high reproducibility of HI enhancement observed in the presence of anti-human IgG globulin.

To ascertain comparability of results obtained by the HI and NI tests, individual serum pairs were examined. Anti-human IgG globulin (goat) was again used to enhance HI. Ten serum pairs obtained in 1957 from recruits immunized with 250 CCA units of A2/Japan/305/57 did not exhibit antibody titers in the HI or NI tests, against either X-15 or X-15(HK) virus antigens (Table 3). This finding is further indication that use of an enhancer in the HI test does not give false positive results when neuraminidase antibodies are absent. Serum pairs obtained in 1968 from recruits immunized with 300 CCA units of A2/Aichi/2/68 were also tested, and typical results are presented in Table 3 for ten serum pairs. In every case a rise in titer of neuraminidase antibodies occurred, and this was seen in HI and NI tests, although the ratio HI titer : NI titer was not constant throughout. When measuring low antibody levels in pre-immunization sera, one instance was found (serum No. 56 vs. X-15 virus) where neuraminidase antibodies were demonstrated by NI but not by HI. In another instance (pre-immunization serum No. 59 vs. X-15(HK) virus) a low HI titer of 8 was obtained in the apparent absence of neuraminidase antibodies as judged by NI. These minor discrepancies in results obtained by the two different tests may reflect heterogeneity in individual sera of antibody classes and avidity, as well as intrinsic experimental error in the tests.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Chicken Erythrocytes</th>
<th>Virus Antigen</th>
<th>Pre and Post Immunization HI Titer with antihuman IgG**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No. 515*</td>
<td>X-15</td>
<td>&lt;8/32</td>
</tr>
<tr>
<td>2</td>
<td>No. 515 + No. 104</td>
<td>X-15</td>
<td>&lt;8/32</td>
</tr>
<tr>
<td>3</td>
<td>No. 101 + No. 103</td>
<td>X-15</td>
<td>&lt;8/16</td>
</tr>
<tr>
<td>4</td>
<td>No. 102</td>
<td>X-15</td>
<td>&lt;8/32</td>
</tr>
<tr>
<td>5</td>
<td>No. 565</td>
<td>X-15</td>
<td>&lt;8/32</td>
</tr>
<tr>
<td>1</td>
<td>No. 515</td>
<td>X-15(HK)</td>
<td>&lt;8/32</td>
</tr>
<tr>
<td>2</td>
<td>No. 515 + No. 104</td>
<td>X-15(HK)</td>
<td>&lt;8/32</td>
</tr>
<tr>
<td>3</td>
<td>No. 101 + No. 103</td>
<td>X-15(HK)</td>
<td>&lt;8/16</td>
</tr>
<tr>
<td>4</td>
<td>No. 102</td>
<td>X-15(HK)</td>
<td>&lt;8/32</td>
</tr>
<tr>
<td>5</td>
<td>No. 565</td>
<td>X-15(HK)</td>
<td>&lt;8/16</td>
</tr>
</tbody>
</table>

Table 2. Reproducibility of enhancement by anti-human IgG globulin in the hemagglutination-inhibition test for neuraminidase antibodies. * Chicken laboratory No, ** Goat globulin 1/60.
Table 3. Comparison of results obtained by hemagglutination-inhibition and neuraminidase-inhibition for detection of neuraminidase antibodies in individual human serum pairs. * Obtained using a 1/60 dilution of anti-human IgG globulin (goat) as enhancer, ** Pre-immunization/Post-immunization. < = <8 HI, <20 NI.

Overall, the results in Table 3 show that neuraminidase antibodies in human sera were consistently detected by HI using anti-human IgG globulin as enhancer, and the levels measured were in general of similar magnitude to those observed by NI.

Discussion

The X-15 hemagglutination-inhibition test for titrating neuraminidase antibodies has been regarded as insensitive. Present results show that insensitivity may be overcome either by careful selection of erythrocytes, or by the use of biological materials as enhancing agents; preferably both measures should be taken. We have found that use of anti-human IgG globulins as enhancing agent provides the most distinct cell patterns in the hemagglutination-inhibition test when titrating neuraminidase antibodies in human serum. Data obtained with individual sera indicates that the hemagglutination-inhibition test carried out in this way is a sensitive and reliable method suitable for conveniently measuring A2/1957 and Hong Kong influenza neuraminidase antibody levels in large population groups. To obtain such data with the neuraminidase-inhibition test is more time consuming and expensive. It seems probable that by the use of appropriate virus antigens and enhancing agents, the HI test could be successfully applied to investigate levels of antibodies to other influenza virus neuraminidases.

While the mechanism whereby neuraminidase antibodies inhibit viral hemagglutination is not known, it is suggested that the effect is due to steric hindrance of the viral surface by neuraminidase antibodies. The effect of anti-human IgG globulin described here might be due to increased steric hindrance of the viral surface. Alternately it might be due to increased aggregation of virus particles by antibody.

The observation that guinea pig serum or highly diluted egg white has an enhancing effect in the HI test may be analogous to the use of serum cofactors in the HI test as described by Styk et al. These substances probably all act by increasing the total number of macromolecules bound at the viral surface, so that together with antibodies, sufficient hindrance of the viral surface occurs to prevent hemagglutination. Binding of influenza-specific glycoprotein inhibitors to viral hemagglutinin might
also be facilitated in the presence of neuraminidase antibodies, since antibodies could prevent viral neuraminidase from enzymically inactivating such glycoproteins. Heating guinea pig serum sometimes appeared to reduce slightly its activity as an enhancing agent (Table 1), suggesting that the complement system may contribute to the overall activity observed with unheated serum.

Since completion of this study, it has been reported elsewhere that anti-IgG serum increases the HI activity of influenza anti-serum. However, the relative contributions of hemagglutinin and neuraminidase antibodies to the overall HI were not distinguished.

Technical assistance was provided by Louise Allen and Betty Thomas. Neuraminidase substrate was kindly provided by Dr. D. S. Pepper of the American National Red Cross, Washington. This investigation was conducted under the sponsorship of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported by the US Army Medical Research and Development Command, Department of the Army, under Research Contract DADA 17-70-C-0050.

References:

15. N. Hahon, J. A. Booth, and H. L. Eckert, Inf. and Immunity 4, 508 [1971].