Neutralization Anti-IgG Test for Antisera to Venezuelan Equine Encephalomyelitis

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SUMMARY

A precise, reproducible, and sensitive serum neutralization test was developed to estimate Venezuelan equine encephalomyelitis neutralizing antibody activity within 24 hr. The test depends on the interactions of virus with antiviral globulins and of the resultant complexes with anti-gamma globulin (IgG) antibodies. The 50% serum neutralizing dilutions were calculated from the reduction of fluorescent cells in McCoy cell monolayers resulting from the neutralization of infective virus by antibody. In comparative estimates of the neutralizing activity of human and monkey antisera, the sensitivity of the serum neutralization anti-IgG test was several hundredfold greater than that of the conventional serum neutralization test in mice.

INTRODUCTION

Recently, a quantitative assay of Venezuelan equine encephalomyelitis (VEE) virus was developed that depends on the enumeration of cells containing fluorescent viral antigen (Hahon & Cooke, 1967). This assay system was very suitable for characterization of the in vitro interaction between virus and neutralizing antibody (Hahon, 1969a). The neutralization reaction proceeded initially at a constant rate and then decreased to give a constant level of residual infectivity even in the presence of excess antiviral serum. This was due to the formation of infective virus-antibody complexes (sensitized virus) which could be further neutralized by anti-IgG antibodies not directed against the virus (Hahon, 1969b). An attempt was made, therefore, to increase the sensitivity of the conventional serum neutralization test by the use of anti-IgG serum.

This report describes the development and standardization of a neutralization anti-IgG test for VEE antisera that depends on both the interaction of virus with antiviral globulins and of the resultant complexes with anti-IgG antibodies.

METHODS

Virus and antiviral serum. The source and preparation of the TRINIDAD strain of VEE virus was described previously (Hahon & Cooke, 1967).

VEE antisera were obtained from rhesus monkeys exposed 1 month earlier to an aerosol of about 1000 cell infecting units (CIU) of virus. Human antiviral sera were obtained from immunized personnel and from a convalescent patient. Sera were inactivated at 56°C for 30 min.

Anti-gamma globulin (IgG) serum. Goat anti-monkey IgG serum was obtained from Microbiological Associates, Bethesda, Maryland. Normal goat serum was purchased from Pentex Laboratories, Kankakee, Illinois.
**Cell line.** Cells of the McCoy line were used for assay of residual infectivity. Nutrient medium for the cells consisted of Parker's medium 199 containing 0·5 % (w/v) lactalbumin hydrolysate, 10 % foetal calf serum, and 50 μg. of streptomycin and 75 μg. of kanamycin/ml. Cells were maintained in medium 199 plus 5 % foetal calf serum. They were cultivated on circular coverslips (15 mm. diameter) inserted in flat-bottomed glass vials (19 by 65 mm.). One ml. of cell suspension containing 10^6 to 3 x 10^6 cells was introduced on to coverslips which were then incubated at 35° for 24 hr, or until a complete cell monolayer was formed. Cell monolayers were washed in phosphate-buffered saline (PBS), pH 7·1, free of calcium and magnesium ions, before the addition of inoculum.

**Serum neutralization anti-IgG test.** Appropriate dilutions of antiviral or normal serum were mixed with equal volumes of a constant quantity of virus (7·4 x 10^4 CIU/ml.). Virus and serum dilutions were routinely prepared in PBS. Test mixtures were incubated at 35° for 2 hr. To each test mixture, a 1/2 dilution of goat anti-monkey IgG serum was added in a volume to give a final dilution of 1/6. After incubation at 35° for 10 min., 0·2 ml. of each mixture was introduced on to one of three coverslip cultures of McCoy cells for assay of residual infectivity.

The immunofluorescent cell counting technique (Hahon & Cooke, 1967) was employed for assay of virus. Briefly, the virus inoculum was deposited on cell monolayers by centrifugation at 19 to 30 x 10^3 g for 15 min. Residual fluid was then removed and 1 ml. of maintenance medium was added. After incubation of coverslip monolayers at 35° for 1 hr to ensure virus penetration into host cells, the cell monolayers were held at 35° for 20 hr in the presence of 1 ml. of overlay consisting of 1/20 antiviral serum in medium 199. This overlay blocked a second cycle of infection. If cell monolayers were fixed at 12 hr, the antiviral serum overlay was omitted. Infected cell cultures were fixed with cold (-60°) acetone and either stored at -60° or prepared immediately for immunofluorescent staining and counting of infected cells.

**Immunofluorescence procedures.** Rhesus monkey VEE antiserum was conjugated with fluorescein isothiocyanate (Riggs et al. 1958); unbound dye was removed from conjugated globulin by passing through a column of Sephadex G-25. To reduce non-specific fluorescence, 5 ml. of conjugated globulin was diluted with an equal volume of PBS and adsorbed twice with 200 mg. of acetone-dried mouse liver powder (Coons & Kaplan, 1950). The direct method was used to demonstrate immunofluorescence of viral antigens in infected cells. Fixed cell cultures were washed twice with PBS and stained with conjugated antiserum for 30 min. at room temperature. Coverslip cell monolayers were then washed twice with PBS and stained with conjugated antiserum for 30 min. at room temperature. Coverslip cell monolayers were then rinsed in two changes of PBS and mounted in a semi-permanent medium (Rodriguez & Deinhardt, 1960).

An American Optical microscope equipped with a Fluorolume illuminator (model 645), exciter filters (Corning no. 5840 and Schott BG-12) and barrier filter (E.K. no. 2A) was used to examine stained monolayers. With this system at ×430 magnification, 1064 microscopic fields were contained in the area of a 15 mm. coverslip. For each coverslip cell monolayer, 50 microscopic fields were examined for fluorescent cells. To calculate the number of CIU of virus/ml., the average number of fluorescent cells/coverslip was multiplied by the number of fields/coverslip, the reciprocal of the dilution of virus inoculum, and by a volume factor for conversion to ml.

**Serum neutralization test in mice.** In comparisons between this method and the serum neutralization anti-IgG test, a similar protocol and conditions of reactants and reaction were used. Normal goat serum, however, instead of goat anti-monkey IgG serum was added.
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to each virus-antiviral serum mixture. For each test mixture, eight Swiss mice of 10 to 14 g.
were each inoculated intraperitoneally with 0.2 ml. Survivors were recorded daily for 10 days.
The 50 % serum neutralizing dilutions were calculated by the method of Reed & Muench
(1938).

RESULTS

Parameters of neutralization

Parallel tests were performed to determine the 50 % neutralizing dilution of VEE monkey
antiviral serum in the presence of either normal goat serum or goat antiserum to monkey
IgG. The percentage reductions of fluorescent cell counts for each antiserum dilution were

\[
\left( \frac{\text{cell infecting units of residual infectivity}}{\text{cell infecting units of initial infectivity}} \right) \times 100
\]

Fig. 1. Determination of neutralizing activity of anti-VEE serum. VEE virus (7.4 × 10^4 CIU/ml.)
and antiserum dilution incubated at 35 ° for 2 hr before further incubation for 10 min. with normal
goat serum (©) or goat anti-monkey IgG serum (●) at 1/6 dilution overall. Assay of residual
infectivity by immunofluorescent cell counting technique. The dilution of antiserum for 50 %
reduction of cell infecting units was obtained by interpolation.

then plotted on probability paper against the logarithm of the corresponding final dilutions
of antiserum. A linear relationship was fitted which gave by interpolation the dilution of
antiserum that neutralized 50 % of virus. The 50 % serum neutralizing dilutions in the pre-
ence of anti-IgG or normal goat sera were 1/105,000 and 1/40,000, respectively (Fig. 1).
The serum neutralizing activity was more than twofold higher when anti-IgG serum was
incorporated into the virus-antiviral serum mixtures.

Experiments using the serum neutralization anti-IgG test were made on the influence of
various conditions of incubation and reagent concentration on the indication of serum
neutralizing activity. The 50 % serum neutralizing activities obtained were comparable
whether virus-antiviral serum mixtures were incubated at 35° for 2 hr or for longer periods. The additional factor of incubation with anti-IgG serum for 10 or 60 min. at 25 or 35° did not affect serum neutralizing activity. For the same incubation time of \( \frac{1}{2} \) hr, serum neutralizing activities were approximately twofold higher at 50° than at 35°. At 50° for \( \frac{1}{2} \) hr, neutralizing activities were comparable to those obtained at 35° for 2 hr. In subsequent tests, virus-antiviral serum mixtures were incubated at 35° for 2 hr and then with anti-IgG serum at 35° for 10 min.

Because previous studies showed that the union of sensitized virus (infective virus-antibody complexes) with anti-IgG antibodies occurred within 30 sec. (Hahon, 1969b), tests were made with an initial mixture of virus, antiserum, and anti-IgG serum incubated at 35° for 2 hr in order to determine whether virus neutralization would occur. It might thus be possible to eliminate the second incubation period used for anti-IgG interaction with sensitized virus. Control mixtures with normal goat serum were also tested. Neutralization tests were also made in which virus-antiviral serum mixtures were preincubated at 35° for 2 hr. before either anti-IgG or normal goat serum was added and the mixtures incubated at 35° for 10 min. The 50 % serum neutralizing dilutions were determined for each condition.

Table 1. Effect of VEE virus concentration on indication of antibody activity

<table>
<thead>
<tr>
<th>Virus concentration in CIU/0.2 ml. test mixture</th>
<th>Antiviral serum dilutions</th>
<th>Antibody activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/60,000</td>
<td></td>
</tr>
<tr>
<td>3.3 x 10⁴</td>
<td>75</td>
<td>98,000</td>
</tr>
<tr>
<td>1.6 x 10⁴</td>
<td>70</td>
<td>100,000</td>
</tr>
<tr>
<td>6.5 x 10⁵</td>
<td>84</td>
<td>100,000</td>
</tr>
<tr>
<td>3.3 x 10⁶</td>
<td>70</td>
<td>108,000</td>
</tr>
</tbody>
</table>

* Reciprocal of 50 % serum neutralizing dilution.

When virus, antiviral serum, and anti-IgG serum were initially mixed and incubated, the serum neutralizing activity (700) was about 50-fold lower than that in mixtures containing normal goat serum (40,000). The serum neutralizing activity was unchanged when normal goat serum was added to mixtures initially or after the primary incubation period. When anti-IgG serum was added to virus-antiviral serum mixtures after the primary incubation period, the serum neutralizing activity was 150-fold higher (105,000) than that obtained by mixing these reactants together before incubation. These data emphasize the importance of incubating virus-antiviral mixtures for a suitable time before adding anti-IgG serum.

To determine the effect of virus concentration on the serum neutralizing activities, neutralization tests were made which differed only in the quantity of virus that reacted with appropriate dilutions of antiviral and anti-IgG sera. Tenfold differences in virus concentration did not significantly change the 50 % serum neutralizing activity (Table 1). This is consistent with the ‘percentage law’ of Andrewes & Elford (1933).

To determine the influence of different dilutions of anti-IgG serum on the serum neutralizing activity, a constant quantity of virus was mixed with appropriate dilutions of antiviral serum and incubated at 35° for 2 hr. Samples of each virus-antiserum mixture were then taken and to each was added a different dilution of anti-IgG serum. The neutralizing activity was unchanged when 1/6 or 1/30 anti-IgG serum was reacted with virus-antiviral serum mixtures (Table 2). Sufficient anti-IgG antibodies were apparently present at these dilutions to saturate sensitized virus. With dilutions of anti-IgG serum beyond 1/30, the
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...indicated neutralizing activity decreased. To ensure that anti-IgG antibodies were available in excess in serum neutralization tests, a final dilution of 1/6 of anti-IgG serum was routinely employed.

Table 2. Effect of different anti-IgG serum dilutions on indication of VEE antibody activity

<table>
<thead>
<tr>
<th>Final dilution of goat anti-monkey IgG serum added to previously incubated virus-antiviral serum mixtures</th>
<th>Antibody activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/6</td>
<td>100,000</td>
</tr>
<tr>
<td>1/30</td>
<td>100,000</td>
</tr>
<tr>
<td>1/60</td>
<td>75,000</td>
</tr>
<tr>
<td>1/120</td>
<td>47,000</td>
</tr>
<tr>
<td>Normal goat serum</td>
<td>44,000</td>
</tr>
</tbody>
</table>

* Reciprocal of 50% serum neutralizing dilution.

Table 3. Reproducibility of estimation of VEE antibody activity

<table>
<thead>
<tr>
<th>Test date</th>
<th>Control infected cell count</th>
<th>Final dilution of antiviral serum</th>
<th>Antibody activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1/600,000 1/90,000 1/120,000</td>
<td></td>
</tr>
<tr>
<td>20 Nov.</td>
<td>158</td>
<td>70 60 43</td>
<td>108,000</td>
</tr>
<tr>
<td>25 Nov.</td>
<td>306</td>
<td>84 60 34</td>
<td>100,000</td>
</tr>
<tr>
<td>2 Dec.</td>
<td>110</td>
<td>80 65 40</td>
<td>110,000</td>
</tr>
<tr>
<td>9 Dec.</td>
<td>60</td>
<td>82 63 38</td>
<td>105,000</td>
</tr>
</tbody>
</table>

* Reciprocal of 50% serum neutralizing dilution.

Table 4. Comparative determinations of VEE antibody activity by the serum neutralization anti-IgG test and by a direct serum neutralization test in mice

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>Antibody activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-IgG test</td>
</tr>
<tr>
<td>Human serum</td>
<td></td>
</tr>
<tr>
<td>Convalescent patient</td>
<td>42,000</td>
</tr>
<tr>
<td>Immunization no. 28</td>
<td>10,000</td>
</tr>
<tr>
<td>Immunization no. 23</td>
<td>35,000</td>
</tr>
<tr>
<td>Immunization no. 2</td>
<td>6,000</td>
</tr>
<tr>
<td>Immunization no. 4</td>
<td>10,000</td>
</tr>
<tr>
<td>Rhesus monkey serum†</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100,000</td>
</tr>
<tr>
<td>2</td>
<td>150,000</td>
</tr>
<tr>
<td>3</td>
<td>60,000</td>
</tr>
<tr>
<td>5</td>
<td>45,000</td>
</tr>
</tbody>
</table>

* Reciprocal of 50% serum neutralizing dilution.
† Animals exposed to aerosol of VEE virus; serum obtained 30 days later.

Quantitative evaluation of antibody activity

The precision of the serum neutralization anti-IgG test was estimated in ten determinations made under identical conditions. The standard deviation of antibody activity was 4.7%.
of the mean activity (107,900). In the immunofluorescent serum neutralization test for psittacosis (Hahon & Cooke, 1965), the standard deviation of antibody activity was 3.4%.

To determine the reproducibility of estimates of neutralizing activity, four identical tests were made at intervals over a period of 3 weeks. The reproducibility attained (Table 3) was such that twofold or greater differences in serum neutralizing activity were significant.

The sensitivity of the serum neutralization anti-IgG test was compared with that of the conventional serum neutralization test in mice for estimation of VEE antibodies in human rhesus monkey sera. Goat anti-monkey IgG serum was employed in the former test because it is equally effective in neutralizing virus sensitized by either human or monkey antiviral serum (Hahon, 1969b). Significantly higher antibody activities were indicated for all sera when the serum neutralization anti-IgG test was used (Table 4). The average differences in antibody activity obtained between the two sets were about 400- and 300-fold for human and monkey antisera, respectively.

DISCUSSION

The serum neutralization anti-IgG test described in this report for the estimation of VEE neutralizing antibody activity depends on both the interaction of virus with antiviral antibodies and of the resultant complexes with anti-IgG antibodies. The optimal conditions required for neutralization of virus infectivity in these reactions has been defined. Previously, it was shown (Hahon, 1969a) that residual infectivity was due to the presence of infective virus-antibody complexes (sensitized virus) that could be neutralized by anti-IgG antibody inactive against the virus particle (Hahon, 1969b). It is the latter reaction with anti-IgG antibody that significantly enhances the sensitivity of antibody detection (Notkins et al. 1968). It has been postulated that anti-IgG antibodies form bridges that cover critical infective sites on the virus particle or stabilize the attachment of antiviral antibody to virus (Notkins et al. 1966; Ashe & Notkins, 1966). The mechanism by which infective virus-antibody complexes may be neutralized by anti-IgG serum has not been resolved completely.

The high precision and reproducibility of estimates of 50% serum neutralizing activity by the serum neutralization anti-IgG test are a consequence of the assay procedure employed to measure residual infectivity (Hahon & Cooke, 1967). Twofold differences in antibody activity were significant. In comparative estimates of VEE neutralizing antibodies in human and monkey sera, the sensitivity of the serum neutralization anti-IgG test was from 300- to 400-fold greater than that of the direct serum neutralization test in mice. In addition, the ability of the former test to determine antibody activity within 24 hr is an important advantage for diagnostic purposes. The principle of the serum neutralization anti-IgG test in conjunction with the immunofluorescent cell-counting assay of residual infectivity may be applicable to the studies on other virus systems.

The excellent technical assistance of W. Douglas Zimmerman is gratefully appreciated.

REFERENCES


Neutralization anti-IgG test for VEE antibody


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