Single-radial-haemolysis test for diagnosing flavivirus infections, particularly Japanese encephalitis*

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Use of the single-radial-haemolysis (SRH) technique for the diagnosis of flavivirus infections is described. A large number of paired and single convalescent serum samples collected from cases of encephalitis during two major outbreaks in Kolar district of Karnataka State in India during 1977 and 1979 were tested by this technique. The results were compared with those obtained in the haemagglutination inhibition (HI) test in all cases, and the complement fixation (CF) and neutralization tests in some cases. Japanese encephalitis virus was shown by the SRH test to be the major etiologic agent responsible for both epidemics. This was corroborated by the HI, CF and neutralization test results. The single-radial-haemolysis test was found to be simpler and more specific and sensitive than the haemagglutination inhibition test.

The single-radial-haemolysis (SRH) test was originally developed for the detection and assay of antibody to influenza virus haemagglutinins (1, 2) but has been successfully adapted for detection of several other haemagglutinating viruses, e.g., rubella virus (3, 4), mumps virus (5, 6), coronavirus (7, 8), parainfluenza viruses (9), and measles virus (10). It has been applied to togaviruses also (11-14). In this paper we describe our attempts at the application of the SRH test for the diagnosis of flavivirus infections, with particular reference to Japanese encephalitis, which is one of the major public health problems in India. The results of the SRH test were compared with those obtained in the haemagglutination inhibition (HI) test, and in some cases with those of the neutralization and complement fixation (CF) tests.

MATERIALS AND METHODS

Viruses and immune sera. The Indian reference strains of Japanese encephalitis virus (JE, P20778 strain), West Nile virus (WN, G22886 strain) and dengue type 2 virus (DEN-2, P23085 strain) and their respective mouse immune sera were used in this study.

Human sera. The following human sera were employed in the study. (1) Paired sera from 4 cases and single convalescent sera from 22 cases collected during the 1977 epidemic of encephalitis in Kolar district of Karnataka State, India. (2) Paired sera from 171 cases and single convalescent sera from 30 cases obtained during the 1979 epidemic of encephalitis in Kolar district.

Antigens. Initially, sucrose-acetone extracted (SA) antigens were used. Later, a crude 20% suspension of infected mouse brains in borate saline, pH 9.0, was used; this antigen was termed “SRH antigen”. Both the SA and SRH antigens were treated with protamine sulfate. Both were stored in the frozen state (–50 °C) and their HA titres were determined before use.

Preparation of SRH immunoplates. Erythrocytes from 2-4-day-old chicks were washed three times in phosphate-buffer saline (PBS), pH 7.2, and finally suspended in 0.4% bovine albumin borate saline (BABS) at the optimal pH for virus haemagglutination to make a 15% suspension. To 1 ml of the erythrocyte suspension was added 1 to 2 ml of SA or SRH antigen having an HA titre of 1280 to 2560. The mixture was kept at 4 °C for 30 minutes with occasional shaking. The antigen-sensitized erythrocytes were washed three times in 0.4% BABS and finally suspended in the same diluent to make a 15% suspension.

Erythrocytes treated with an equal volume of normal mouse brain “antigen” were used for the preparation of control plates. Normal erythrocytes were also used for the preparation of additional control plates.

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To prepare one SRH plate, 0.3 ml of the 15% suspension of sensitized erythrocytes was added along with 0.1 ml of undiluted guinea pig complement to 2.6 ml of 1.5% agarose (Indubiose A-37) in PBS, pH 7.2, held at around 40 °C in a water-bath and poured into an immunoplate kept on a levelling table. The gels were allowed to harden for about 30 minutes after which wells of 2.0 mm diameter were punched in the agarose, and 5 μl of inactivated serum was added to each well. The plates were covered with their lids, transferred to a humid box, and incubated at 37 °C for 18-20 hours. The diameters of the zones of haemolysis were measured with the aid of a micrometer eyepiece calibrated in 0.1 mm divisions.

**HI, CF and neutralization tests.** These were performed according to standard procedures employed in our Institute (15-17).

**Criteria for positive sera in the SRH test**

A haemolytic zone of ≥2.5 mm diameter was accepted as a positive reaction (13).

Paired sera giving the following reactions were considered to be diagnostically positive in SRH:

(a) Seroconversions, where the acute serum sample gave no haemolytic zone but the convalescent serum gave a haemolytic zone of ≥3.0 mm diameter.

(b) Increase or decrease of ≥2.0 mm between the diameters of the zones produced by the acute and convalescent sera (5, 6).

(c) Both the acute and convalescent serum samples gave similar haemolytic zones of ≥6.0 mm diameter.

Single convalescent serum samples giving haemolytic zones of ≥6.0 mm diameter were considered positive.

Sera reacting with more than one antigen were considered positive for that antigen with which the haemolytic zonal diameter was at least 2.0 mm, greater than that produced by other reacting antigens. Sera giving haemolytic zones on control plates were excluded.

**Criteria for positive sera in the HI test**

Those serum pairs which showed conversions and those which showed a fourfold or more increase or decrease in titres were considered to be diagnostically positive. Also those pairs in which both the acute and convalescent sera had similar high titres of ≥1:160 were considered to be diagnostically positive. Single convalescent serum samples with titres of ≥1:160 were also considered positive.

**RESULTS**

The homologous and heterologous reactions of JE, WN and DEN-2 viruses and their immune sera in the SRH and HI tests are given in Table 1. Haemolytic zones were consistently larger in the homologous reactions (10-12.5 mm), compared with the heterologous reactions (0-7.5 mm); with the DEN-2 antigen, haemolysis was observed only with the homologous antisera. In the HI test employing the same sera, all the antigens reacted not only with the homologous immune sera, but also with the other two at lower titres.

The 1977 encephalitis epidemic

Four paired serum samples were tested in the SRH, HI, CF and neutralization tests. None of the sera produced nonspecific zones of haemolysis on the control plates containing unsensitized erythrocytes or erythrocytes treated with normal mouse brain antigen. Three pairs gave a monotypic response for JE virus in the SRH test, with a decrease in zone diameter of 2-4 mm between the first and second specimens. These three pairs reacted monospecifically for JE virus in the CF test, and they were also positive in the neutralization test with JE virus. However, in the HI test, all the three pairs gave positive J + WN + DEN-2 reactions; the increase in the zone diameters in these three pairs was accompanied by a decrease in the HI titres also. The fourth pair showed conversion in both SRH and HI tests for WN virus only, and the neutralization test with JE virus was negative; low levels of CF antibodies (1:4) for WN virus could be detected in the second serum sample.

The reactions of the 22 single convalescent sera in the SRH test were: 10, monotypic JE; 2, monotypic WN; 9, J + WN; and 1, negative. None of the sera produced nonspecific zones of haemolysis on the control plates. In all the 10 monotypic JE cases, the CF reaction was also monotypic for JE: 8 of these 10 tested in the neutralization test with JE virus were also positive. The reactions of these 10 sera in HI were: 6, monotypic JE; 3, J + WN; and 1, J + WN + DEN-2. The two sera which gave a monotypic WN reaction in SRH failed to react in both CF and HI. Both were negative in the neutralization test with JE virus. Eight of the 9 sera which gave a J + WN reaction in SRH gave a monotypic JE reaction when tested in CF. All the 9 sera with a J + WN reaction in SRH were positive in the neutralization test with JE virus. The reaction in HI of these 9 sera were: 3, monotypic JE; 3, J + WN; and 3, J + WN + DEN-2.

In all, 19 out of the 22 single convalescent sera reacted in SRH with JE antigen (mean zone diameter,
Table 1. Homologous and heterologous reactions of JE, WN and DEN-2 viruses with mouse immune sera in SRH and HI tests

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Immune sera against</th>
<th>Normal mouse serum (control)</th>
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<tbody>
<tr>
<td></td>
<td>JE P20778</td>
<td>WN G22886</td>
</tr>
<tr>
<td>JE P20778</td>
<td>10 0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.75 (320)</td>
</tr>
<tr>
<td></td>
<td>(640)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>WN G22886</td>
<td>7.5 (320)</td>
<td>12.5 (1280)</td>
</tr>
<tr>
<td>DEN-2 P23085</td>
<td>0 (40)</td>
<td>0 (40)</td>
</tr>
<tr>
<td>Normal mouse</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>brain (control)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> SRH zone diameters expressed in mm.
<sup>b</sup> Figures in brackets indicate HI titres expressed as reciprocals of serum dilutions.

8.78 mm) and 11 sera reacted with WN antigen (mean zone diameter, 5.45 mm). In HI, 19 sera were positive for JE virus with a geometric mean (GM) titre of 165.95; 10 were positive for WN and 4 were positive for DEN-2 with GM titres of 80.0 and 33.64, respectively.

The 1979 encephalitis epidemic

**Paired sera.** The results of the SRH and HI tests on the 171 paired sera obtained during the 1979 outbreak of encephalitis in Kolar district are as follows. The SRH test results in the case of 138 patients (80.7%) were consistent with a recent flavivirus infection. In 13 cases antibodies to one or more of the antigens were detected but were not at the diagnostic level; the remaining 20 cases were negative in the SRH test. Fifty-seven of the 72 cross-reactive cases were diagnosed as JE on account of the zone diameters to JE virus being larger by $\geq 2.0$ mm than the diameters of the zones for the other reacting antigens; the remaining 15 cross reactions were unresolvable.

In the HI test, 115 pairs (67.3%) fulfilled the diagnostic criteria for one or more of the antigens employed in the test. In 20 pairs, antibodies to one or more of the antigens were present, but only at the non-diagnostic level; 36 pairs were negative. Out of the 56 cross-reactive cases in HI, 43 were compatible with a diagnosis of JE on account of the HI titre being at least fourfold higher for JE virus. The remaining 13 cross reactions were unresolvable.

**Single convalescent sera.** Of the 30 single convalescent sera tested in SRH, one gave a nonspecific reaction on the control plates and was excluded. The reactions of the remaining 29 sera in the SRH test were: 13, monotypic JE; 14, JE + WN; 1, JE + WN + DEN-2; and 1, negative. In all the 15 cross-reactive cases, the zone diameter for JE virus was $\geq 2.0$ mm bigger than that of the WN and DEN-2 zones. However, only 19 of the 29 single convalescent sera fulfilled the diagnostic criteria for JE virus, i.e., with zones of $\geq 6.0$ mm diameter.

In the HI test, out of the 29 single convalescent sera, 9 gave a monotypic JE response; 15 gave a JE + WN response; 3 gave a JE + WN + DEN-2 response; and 2 were negative. On the whole, only 13 sera fulfilled the diagnostic criteria for JE, i.e., an HI titre of $\geq 160$ and fourfold higher titre for JE in cases of cross-reactive sera.

** Confirmation of the specificity of the SRH reaction**

There were a few sera which produced haemolytic zones of varying diameters, but were negative in the HI test. Attempts to determine whether such SRH reactions in the absence of HI reactions were specific or not were undertaken by means of the neutralization tests. Seventy-one sera giving haemolysis with JE virus, but negative in HI, were tested for the presence of JE virus neutralizing antibodies; 48 (67.6%) of these were found to be positive, which proved that more than two-thirds of the SRH-positive but HI-negative sera contained JE virus neutralizing antibodies. The remaining one-third of the sera might have had too low a level of antibodies to become positive in the neutralization test. All these sera except one produced small zones of $\leq 5.0$ mm (mean zone diameter, 3.21 mm), indicating low levels of antibody. Also, the neutralization test done by the intracerebral route may not have been as sensitive as the intraperitoneal neutralization test in infant mice.

Another 45 sera selected at random were also tested for the presence of JE virus neutralizing antibodies and 38 were positive. When the results of the neutralization tests were correlated with those of the SRH and HI tests, it was found that 25 sera were positive in all three tests and five sera were negative in
all of them. Thus, full agreement between the results of the SRH, HI and neutralization tests was found in 30 cases (66.6%). Twelve sera were positive in the SRH and neutralization tests but negative in HI, whereas one serum was positive in SRH and HI but negative in the neutralization test. One serum was positive in SRH only and another in the neutralization test only. Perfect agreement between the results of any two or all the three tests was found in 43 cases (95.5%).

Correlation of SRH and HI test results

For this, all the 392 serum samples collected from the 171 cases of encephalitis during the 1979 epidemic in Kolar district were investigated. When the SRH and HI test results of these sera were correlated, it was found that relatively significantly more positive results were obtained by the SRH test than the HI test for both JE virus (χ² = 62.11, P < 0.05) and WN virus (χ² = 17.88, P < 0.05) (Tables 2 and 3). The individual haemolytic zone diameters of all the sera positive in SRH were correlated with their individual HI titres in the case of JE and WN antigens (Fig. 1 and 2). There was good correlation between the two methods of antibody measurement, although there was some scatter of zone diameters for each HI titre.

For JE and WN viruses, the mean zone diameters of the patients’ sera were regressed on the log, HI titres and a linear relationship was found between the two. The regression coefficients were: for JE virus, r = 0.945 (P < 0.01) and for WN virus, r = 0.909 (P < 0.05). The observed mean zone diameters at each HI titre along with the expected values arrived at statistically for JE and WN antigens (260 and 111 positive sera, respectively) also showed good agreement.

DISCUSSION

Since flaviviruses such as Japanese encephalitis, West Nile and dengue viruses are of considerable public health importance in India, the development of a simple, sensitive and type-specific test for the

Table 2. Correlation of SRH and HI test results with JE virus

<table>
<thead>
<tr>
<th>SRH test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HI test:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>175 (44.6)</td>
<td>8 (2.0)</td>
<td>183 (46.7)</td>
</tr>
<tr>
<td>Negative</td>
<td>85 (21.7)</td>
<td>124 (31.6)</td>
<td>209 (53.3)</td>
</tr>
<tr>
<td>Total</td>
<td>260 (66.3)</td>
<td>132 (33.7)</td>
<td>392</td>
</tr>
</tbody>
</table>

* Figures in parentheses indicate percentages.

rapid diagnosis of these diseases is extremely important. Conventional serological tests like HI, CF and the neutralization test have well-known limitations. The single-radial-haemolysis test is simple, sensitive, type-specific, and rapid and therefore its usefulness in the diagnosis of flavivirus infections was determined.

In the present study, the SRH test was applied successfully for the determination of the etiology of two outbreaks of encephalitis which occurred in India. During the 1979 outbreak, 138 out of the 171 cases (80.7%) where paired sera were available could be diagnosed by the SRH test as due to flavivirus infection, compared with 115 (67.3%) diagnosed by the HI test. Of the 138 positive cases in SRH, 120 could clearly be identified as JE virus infections and 3 as WN virus infections. By the HI test, only 100 could be clearly diagnosed as JE virus infections and 2 as WN virus infections.

The number of sera reacting with DEN-2 antigen in both SRH and HI tests was small and in no case did DEN-2 appear to be etiologically involved. Generally, primary JE cases are marked by the absence of a DEN-2 response. However, cross reaction with the closely related WN virus is known to occur. The reactions in both SRH and HI tests were mostly with the JE and WN antigens. The antibody level and the number of positives to WN virus were lower than those to JE virus. For example, the geometric mean HI titre for WN virus was only half (29.4) of that for JE virus (58.3). The mean zone diameter was also smaller for WN virus (5.4 mm), compared with that for JE virus (6.05 mm).

Among the encephalitis cases in 1977, JE virus could be incriminated by SRH as the causative agent in 3 out of the 4 cases where paired sera were available and in 19 out of 22 cases where only single convalescent sera were available; in one case, WN virus was the probable etiologic agent although neutralization tests could not be carried out with WN virus owing to insufficient quantity of the serum. Compared with this, only 2 paired sera and 13 single sera fulfilled the diagnostic criteria for JE virus by HI.
The comparatively high mean zone diameter of 8.78 mm for JE virus as against 5.45 mm for WN virus, and also the high geometric mean HI titre of 165.95 for JE virus as against 80.0 for WN virus, confirm that the JE virus was the major etiologic agent in the 1977 outbreak. Our SRH and HI results also show that the 1979 epidemic of encephalitis was caused by JE virus, the WN virus being responsible for only two cases.

The serological diagnosis of Japanese encephalitis and other flavivirus infections is often difficult owing to cross reactions in all the three conventional tests (HI, CF and neutralization), particularly in areas where more than one member of the group is present. In such situations, the SRH test would be advantageous as it was found to be less cross-reactive in the present study.

Several workers have reported type specificity as one of the main advantages of the SRH test with arboviruses. Odelola (13) found no cross reaction between WN and yellow fever viruses in SRH, whereas they cross-reacted in HI. Gaidamovich & Melnikova (11) found the SRH test useful in the diagnosis of laboratory infection with Venezuelan equine encephalomyelitis virus as well as for sero-
surveys. Gaidamovich et al. (12) employed this technique for the diagnosis of dengue fever in Bangladesh. They found no cross reaction between DEN-2 and JE viruses in SRH whereas it was present in HI. Duca et al. (14) found the SRH test simple and more specific than the classical haemagglutination inhibition test in the quantitative assay of specific antibody to WN and Sindbis viruses. They reported that dengue immune human and rat sera, which reacted in HI with the WN virus, were consistently negative in the SRH test with WN virus.

Some advantages which make the SRH test most suitable both for serodiagnosis and for serosurveillance of Japanese encephalitis and other flavivirus infections are: (i) the test is simple and because it is unaffected by nonspecific inhibitors, acetone extraction of sera is not necessary; (ii) since crude mouse brain antigens suffice, this results in considerable saving; (iii) sera can be tested undiluted and only microlitre volumes are required; (iv) no costly equipment is required. The simplicity, specificity, sensitivity and economy of the SRH test render it ideal for the detection of arboviral antibodies, particularly in field areas in India and other developing countries where JE, WN and dengue viruses are endemic.
RÉSUMÉ

L’ÉPREUVE D’HÉMOLYSE RADIALE SIMPLE DANS LE DIAGNOSTIC DES INFECTIONS À FLAVIVIRUS, EN PARTICULIER L’ENCÉPHALITE JAPONAISE

En Inde, les flavivirus tels que ceux de l’encéphalite japonaise, West Nile et de la dengue jouent un rôle considérable en santé publique. Les épreuves classiques destinées à diagnostiquer ces maladies, par exemple l’inhibition de l’hémagglutination (IH), la fixation du complément (FC) et la neutralisation sont laborieuses, longues à exécuter et coûteuses. En outre, il n’est pas toujours possible de faire un diagnostic spécifique de type en raison des réactions croisées observées dans ces épreuves. C’est pourquoi on a essayé d’appliquer l’épreuve d’hémolyse radiale simple au diagnostic de ces infections flavivirales.

Pour cette épreuve, on a sensibilisé les érythrocytes de poulet avec des virus de l’encéphalite japonaise (EJ), West Nile (WN) et de la dengue (DEN-2) et on les a incorporés dans de l’agarose (à 1,5% dans du soluté salin tamponné au phosphate) en même temps que du complément. Des quantités de 5 μl de sérum inactué de malades étaient introduites dans des cupules d’un diamètre de 2 mm pratiquées dans le gel. Les plaques étaient incubées à 37 °C jusqu’au lendemain. Des zones d’hémolyse se sont produites autour des cupules renfermant des sérums qui contenaient des anticorps. Les diamètres de ces zones ont été mesurés au moyen d’un oculaire de micromètre étalonné en 0,1 mm.

Les échantillons éprouvés comprenaient 4 paires de sérum et 22 sérums uniques de convalescents recueillis pendant l’épidémie d’encéphalite qui a sévi en 1977 dans le district de Kolar, État de Karnataka, en Inde, ainsi que 171 paires de sérum et 30 sérums uniques de convalescents recueillis pendant l’épidémie d’encéphalite de 1979 dans le même district.

Sur les 171 cas pour lesquels on disposait de paires de sérum en 1979, 138 (80,7%) ont pu être attribués à une infection à flavivirus au moyen de l’épreuve d’hémolyse radiale simple, contre 115 cas (67,3%) diagnostiqués par l’épreuve d’inhibition de l’hémagglutination. Sur les 138 cas ci-dessus, 120 ont été clairement identifiés comme des infections à virus EJ, 3 comme des infections à virus WN et les 15 restants comme dus à des “flavivirus indifférenciés”. Sur les 120 cas diagnostiqués comme EJ par l’hémolyse radiale simple, 63 ont donné une réaction monotypique alors que 57 présentaient des réactions croisées surtout avec WN. Sur les 115 cas diagnostiqués au moyen de l’inhibition de l’hémagglutination, 100 étaient des EJ, 2 étaient dus à WN et 13 étaient des infections “à flavivirus indifférenciés”. Sur les 100 cas diagnostiqués comme EJ par cette dernière méthode, 75 ont donné une réaction monotypique et 31 donnaient des réactions croisées surtout avec WN. Au total, il a été possible d’identifier nettement l’agent étiologique dans 123 cas (72%) par l’épreuve d’hémolyse radiale simple contre 102 cas (60%) par l’épreuve d’inhibition de l’hémagglutination.

En ce qui concerne l’épidémie d’encéphalite de 1977, le virus EJ a pu être incriminé comme agent étiologique par l’épreuve d’hémolyse radiale simple dans 3 des 4 cas pour lesquels on disposait d’une paire de sérums et dans 19 des 22 cas pour lesquels on possédait seulement un sérum unique de convalescent. Dans l’épreuve d’inhibition de l’hémagglutination, seuls 2 paires de sérums et 13 sérums uniques satisfaisaient aux critères diagnostiques de EJ. Dans un cas, les deux épreuves ont désigné le virus West Nile comme agent étiologique. On a constaté que l’épreuve d’hémolyse radiale simple offrait de nombreux avantages car elle est simple, sensible et donne lieu à moins de réactions croisées que la réaction d’inhibition de l’hémagglutination.

REFERENCES