The Neutralization of Rous Sarcoma Virus

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SUMMARY

The neutralization of Bryan standard strain Rous sarcoma virus (Bs-RSV) followed first-order kinetics after 15 min. incubation. No non-neutralizable fraction was observed when about 10^5 focus-forming units of representatives of subgroup A and subgroup B viruses were neutralized by homologous sera.

The antigenicity of a strain of Bs-RSV was changed by growth on relatively resistant cells. The antigenicity of an A subgroup Bs-RSV 'pseudotype' was changed by semi-cloning the virus. Both changes of antigenicity were expressed by a change of slope and a reduction of titre on cross-neutralization by the parent virus antiserum.

Antisera to a number of subgroup A leukosis viruses were examined for neutralization of the homologous Bs-RSV 'pseudotype' and other subgroup A 'pseudotypes'. The slopes of the neutralization curves for the heterologous systems differed from those of the homologous system. Neutralization indices derived from single points on the neutralization curves, because of the slope differences, did not show consistent cross-neutralization relationships of the viruses tested. When the slope differences were taken into account by comparing areas under the curves, the homologous system had an index at least 20% greater than the heterologous systems.

INTRODUCTION

The avian leukosis/sarcoma group of viruses can be divided into two subgroups, A and B, on the basis of one or more of the following properties determined by the viral envelope: host range, interference and antigenicity detected by neutralization tests (Vogt & Ishizaki, 1966a, b). The viral envelope of defective sarcoma viruses is provided by an associated leukosis virus. The antigenicity of these viruses as detected by neutralization is also provided by the associated leukosis virus (Hanafusa, Hanafusa & Rubin, 1963, 1964; Vogt & Ishizaki, 1966b). Cross-neutralization is only seen between viruses belonging to the same subgroup; however, there is clearly antigenic heterogeneity amongst viruses of a single subgroup (Ishizaki & Vogt, 1966; Churchill, 1968).

Early studies at this laboratory had noted that the slope of the neutralization curve differed for the same antiserum against different preparations of the same strain of Rous sarcoma virus. This paper presents the results of a study of the neutralization of Rous sarcoma virus to investigate the cause of this phenomenon.
METHODS

Virus strains. Bryan standard strain Rous sarcoma virus (Rs-RSV) was kindly supplied by Dr R. M. Dougherty as preparation RSV3 (Dougherty & Simons, 1962). Wing-web tumours induced by this preparation in commercial Light Sussex chicks were processed by the Moloney (1956) technique to give the Bs-RSV-R2B/2 preparation. Similarly, the Bs-RSV-R3/2 preparation was prepared from tumours induced by the Bs-RSV-R2B/2 preparation in Houghton Poultry Research Station L151 White Leghorn chicks (HPRS L151 WL) (Biggs & Payne, 1967).

The lymphoid leukosis viruses HPRS-F45 and HPRS-F42 were isolated from field outbreaks of the disease (Biggs & Payne, 1964). HPRS-I lymphoid leukosis virus was isolated from a canine distemper vaccine (Payne et al. 1966) and HPRS-2 lymphoid leukosis virus was isolated from Bs-RSV-R3/2 by end-point dilution by the method of Rubin & Vogt (1962). These viruses have been shown to produce predominantly lymphoid leukosis, but also other mesodermal tumours when inoculated into chickens (Biggs & Payne, 1964; Biggs, 1967, unpublished).

‘Pseudotypes’ of these leukosis viruses were prepared by activating non-producing cells of Bs-RSV-R3/2 by the method of Hanafusa et al. (1964). The term ‘pseudotype’ does not imply any previous cloning of the leukosis viruses used for activation.

All the virus strains were shown to consist predominantly of subgroup A viruses by the criteria of host range, interference inducing properties and neutralization.

Cells. Chick embryo fibroblasts of specific phenotype were derived from embryos of the Reaseheath C, I and W inbred lines of chicken which have been shown to provide C/A, C/O and C/B cells respectively (Payne & Biggs, 1966). The designation C/A cells indicates phenotypic resistance to infection with A subgroup viruses, C/B resistance to B subgroup viruses and C/O susceptibility to both subgroup viruses (Vogt & Ishizaki, 1965).

All neutralizations were tested on C/O Houghton Poultry Research Station Brown Leghorn (HPRS BrL) fibroblasts (Biggs & Payne, 1967).

Virus assay. Sarcoma viruses were assayed by the focus technique of Temin & Rubin (1958) on chick embryo fibroblasts. Foci counts of whole plates were made macroscopically 10 to 14 days after the cells had been challenged with virus, as described by Dougherty & Simons (1962).

Neutralization. An appropriate dilution of the test virus was made with Puck's D1 phosphate buffered saline containing 2% calf serum, and 200 units penicillin and 200 μg. streptomycin/ml. Equal volumes of diluted virus and serial dilutions of antiserum were mixed and incubated at 30° for 2 hr (Dougherty, Stewart & Morgan, 1966). As controls, equal volumes of diluted virus and diluent were mixed and incubated for the same period. The final virus concentration was estimated to be approximately 500 focus-forming units (f.f.u.)/ml. The mixtures were then chilled and 0.2 ml. was inoculated on to each culture plate. At least two and usually three plates were used per mixture. The mixture was allowed to adsorb for 1 hr at 37° before removing excess fluid and overlaying with nutrient agar.

Kinetic and multiplicity curves. The rate of neutralization was measured by a method similar to the normal neutralization procedure except that the volumes of the neutralizing mixtures were sufficient for the withdrawal of several samples for
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assay of surviving virus 15, 30, 60 and 120 min. after the start of incubation. Control virus was assayed immediately and at the stated time intervals.

The multiplicity of neutralization was also measured in a similar manner to the normal neutralization procedure, except that higher concentrations of virus (10⁴ to 10⁶ f.f.u./ml.) were used.

Antiserum. Antisera to sarcoma viruses were produced by inoculating 5 to 20 f.f.u. of virus into the wing webs of 6 week-old HPRS L 151 WL chickens. After tumour regression the birds were rechallenged with 10⁴ f.f.u. of virus and the serum collected one week later. Antisera to leukosis viruses were induced by inoculation of 10⁵ tissue culture infectious doses intravenously into adult HPRS L 151 WL cockerels. At least one further dose at the same virus concentration was given intramuscularly 1 week later. Sera were collected 4 weeks after the last virus inoculation. All sera were inactivated at 56° for 30 min. before use. No preinoculation sera neutralized the viruses used in these experiments.

RESULTS

Neutralization

Thermal inactivation over the 2 hr period at 30° was found to be negligible. The results of two experiments were combined to construct kinetic curves of neutralization of Bs-RSV-R 3/2 (Dulbecco, Vogt & Strickland, 1956) by several dilutions of an homologous serum (Fig. 1). Similar curves with an homologous serum were obtained for Bs-RSV (HPRS-2). The curves show the normal pattern of an exponential rate of neutralization which was greatest with the highest serum concentration. As the first observation was at 15 min. (excluding adsorption time on the chick embryo fibroblast monolayers) no observations were made as to the existence of a lag phase (Lafferty, 1963).

Table 1. Rate constant values (k) at 30 min. incubation for Bs-RSV-R 3/2 and an homologous serum (see Fig. 1)

<table>
<thead>
<tr>
<th>Log. serum dilution</th>
<th>3.0</th>
<th>3.3</th>
<th>3.5</th>
<th>3.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>123.6</td>
<td>—</td>
<td>107.3</td>
<td>—</td>
</tr>
<tr>
<td>Expt 2</td>
<td>113.5</td>
<td>116.5</td>
<td>—</td>
<td>113.2</td>
</tr>
</tbody>
</table>

\[ k = \frac{\log (D \cdot D \log (C_0/C_I))}{t} \]

where \( D \) = serum dilution, \( C_0 \) = initial virus concentration, \( C_I \) = virus survival at incubation time \( t \).

The rate constant values (k) of McBride (1959) calculated for different serum dilutions at a fixed time of neutralization (30 min.) were similar within and between experiments for each of the two virus preparations tested. The k values for the kinetic curves of neutralization of Bs-RSV-R 3/2 are shown in Table 1.

The neutralization curve obtained by plotting log. virus survival against log. serum dilutions had the same slope at 60 min. as at 120 min. incubation; however, the former had a distinct flattening above 50% virus survival which was not apparent at 120 min.

The multiplicity curve (Dulbecco et al. 1956) for two homologous sera of Bs-RSV (HPRS-2) (Fig. 2) did not show any non-neutralizable virus. All the virus in the most
concentrated preparations available (about $10^5$ f.f.u./0.2 ml.) was neutralized; the serum concentration range being at least two dilution steps beyond where detectable virus was found. Any non-neutralizable fraction must be below 0.001% virus survival. A similar multiplicity curve was obtained with a subgroup B virus (Schmidt-Ruppin RSV) and its homologous serum.

Factors affecting the slope of the neutralization curve

*The heterogeneity of the virus*

Preliminary experiments indicated that sera gave different neutralization curves with two different preparations of Bs-RSV (R2B/2 and R3/2). Sera prepared against R2B/2 in HPRS L151 WL birds neutralized the R3/2 preparation better than the homologous preparation (Fig. 3). It was considered that this may have been caused by the change of host to HPRS L151 WL for the preparation of R3/2 and for the induction of antisera to R2B/2. This principle could be tested by culturing the virus on chick fibroblasts relatively resistant to the subgroup of sarcoma virus forming the main component of the R3/2 preparation of Bs-RSV.

Reaseheath C line (C/A) chick embryo fibroblasts were challenged with 50 f.f.u. of Bs-RSV-R3/2 and grown in liquid medium for 10 days. The supernatant fluid was
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Table 2. Log. titres/ml. of BS-RSV on genetically different cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Virus</th>
<th>C (C/A)</th>
<th>I (C/O)</th>
<th>W (C/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BS-RSV-R 2B2</td>
<td>2.32</td>
<td>6.6</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>BS-RSV-R 3/2</td>
<td>3.55</td>
<td>8.25</td>
<td>8.27</td>
</tr>
<tr>
<td></td>
<td>BS-RSV-R 3/2/1</td>
<td>6.2</td>
<td>6.2</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>BS-RSV-(HPRS F42)/I</td>
<td>&lt; 2.0</td>
<td>5.9</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>BS-RSV-(HPRS F42)/10</td>
<td>&lt; 2.0</td>
<td>7.1</td>
<td>6.9</td>
</tr>
</tbody>
</table>

The phenotyped cells were from the highly inbred Reaseheath C, I and W lines of chickens.

![Fig. 3](image)

Fig. 3. Comparison of neutralization of BS-RSV-R 2B/2 (■—■), and BS-RSV-R 3/2 (○—○) virus preparations by BS-RSV-R 2B/2 homologous antiserum 1126/4. \( V_o \), initial focus count; \( V \), surviving focus count.

![Fig. 4](image)

Fig. 4. Comparison of neutralization of BS-RSV preparation R 3/2 (○—○), and the preparation R 3/2/1 (■—■) derived from the former virus preparation by growth on resistant cells, using the same antiserum as in Fig. 3.

harvested and used to produce confluent lesions on the chorioallantoic membrane of fully susceptible 11-day-old C/O HPRS BrL embryos. The chorioallantoic membranes were harvested after 8 days and used to prepare a Moloney T2 preparation (Moloney, 1956). This virus BS-RSV-R 3/2/1 was compared with its parent virus by neutralization with the same serum used to show the difference between the R 2B/2 and R 3/2 preparations of BS-RSV (Fig. 4). As can be seen a markedly similar effect to the former neutralization was observed. The R 3/2/1 preparation was poorly neutralized and the neutralization curve had a flat slope. However, the growth characteristics of the R 3/2/1 preparation on genetically phenotyped cells showed that it was different from the other two strains (Table 2) and probably consisted predominantly of a B subgroup virus.

A similar antigenic change to that seen with the BS-RSV-R 3/2 preparation grown on C-line cells could be obtained by a cloning method. The 'pseudotype' BS-RSV
(HPRS F42)/1 was semi-cloned by picking an isolated focus from an overlaid plate of fully susceptible cells with ten to twenty foci and plating the selected focus on to susceptible cells to produce sufficient virus to repeat the procedure. After repeating nine consecutive times, the tenth such clone (BS-RSV (HPRS F42)/10) was grown up and used to produce antisera in HPRS L151 WL birds. The neutralization characteristics of this serum are shown in Fig. 5. The neutralization of the homologous BS-RSV (HPRS F42)/10 virus was of high titre and good slope. The neutralization of the parent virus BS-RSV (HPRS F42)/1 was poor with flat slope. Similarly, with the reverse neutralization, the homologous (HPRS F42)/1 virus preparation was neutralized well with steep slope, whereas the heterologous virus preparation (HPRS F42)/10 was neutralized poorly.

Table 3. Neutralization indices calculated in various ways for antiserum HPRS-1 lymphoid leukemia virus 1333/7

<table>
<thead>
<tr>
<th>Virus BS-RSV</th>
<th>(HPRS-1)*</th>
<th>(HPRS-2)</th>
<th>(HPRS F42)/1</th>
<th>(HPRS F45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extrapolation to X axis (log. serum dilution)</td>
<td>6.0</td>
<td>3.9</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Extrapolation to Y axis (log. virus neutralized by undiluted serum)</td>
<td>2.6</td>
<td>2.4</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Serum dilution to give 50 % virus survival</td>
<td>5.4</td>
<td>3.4</td>
<td>3.6</td>
<td>3.7</td>
</tr>
<tr>
<td>% virus survival at 10^{-1} serum dilution</td>
<td>&lt; 1.0</td>
<td>1.6</td>
<td>&lt; 1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Area value (A^2/B)</td>
<td>15.9</td>
<td>9.4</td>
<td>10.8</td>
<td>5.7</td>
</tr>
<tr>
<td>Normalized area %</td>
<td>160</td>
<td>59.1</td>
<td>68.1</td>
<td>35.9</td>
</tr>
<tr>
<td>Slope of curve (B)</td>
<td>-0.43</td>
<td>-0.62</td>
<td>-0.68</td>
<td>-0.23</td>
</tr>
</tbody>
</table>

* Homologous virus.

All values were calculated from the regression lines in Fig. 7.
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hardly neutralized at all (Fig. 6). This change of antigenicity, however, did not affect the host range of the virus, its characteristics still being of the A subgroup (Table 2).

Cross-neutralization

In order to assess whether change of slope is the important factor for differences of antigenicity, the Rous sarcoma virus ‘pseudotypes’ of the leukosis viruses listed in methods were compared by cross-neutralization. Neutralization with any one antiserum against all the ‘pseudotypes’ was carried out at the same time. An example of the neutralization curves for the various ‘pseudotypes’ against a single serum is shown in Fig. 7. Unless the slopes of the neutralization curves are taken into account an assessment of the degree of cross-neutralization is difficult. Some of the values normally given for neutralizing indices derived from the neutralization curves shown in Fig. 7 are shown in Table 3. The use of the area under the curves after extrapolation to the axes takes the slope of the curves into account (Westaway, 1965). These areas can be normalized (McBride, 1959) by calculating the heterologous areas as a percentage of the homologous area (Table 4). Using the normalized area values shows that the homologous systems have area values at least 20 % greater than the heterologous systems. This difference may not be so apparent when neutralization indices are derived from a single point on the dose response curve (Table 3).

DISCUSSION

Several viruses have a non-neutralizable fraction, e.g. Newcastle disease virus (Granoff, 1965), poliomyelitis, (Dulbecco et al. 1956). We were unable to demonstrate a non-neutralizable fraction for representatives of subgroup A and subgroup B
Table 4. Area values for 'pseudotype' cross-neutralizations

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Normalized area (%)</th>
<th>Area</th>
<th>Slope</th>
<th>Normalized area (%)</th>
<th>Area</th>
<th>Slope</th>
<th>Normalized area (%)</th>
<th>Area</th>
<th>Slope</th>
<th>Normalized area (%)</th>
<th>Area</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRS-1</td>
<td>100</td>
<td>15·8</td>
<td>-0·43</td>
<td>59·1</td>
<td>9·4</td>
<td>-0·62</td>
<td>68·1</td>
<td>10·8</td>
<td>-0·68</td>
<td>35·9</td>
<td>5·7</td>
<td>-0·23</td>
</tr>
<tr>
<td>HPRS-2</td>
<td>33·2</td>
<td>7·9</td>
<td>-0·55</td>
<td>100</td>
<td>23·9</td>
<td>-1·45</td>
<td>37·5</td>
<td>8·9</td>
<td>-0·31</td>
<td>6·8</td>
<td>1·65</td>
<td>-0·31</td>
</tr>
<tr>
<td>HPRS F42/I</td>
<td>48·0</td>
<td>3·43</td>
<td>-0·83</td>
<td>76</td>
<td>5·5</td>
<td>-1·0</td>
<td>100</td>
<td>7·1</td>
<td>-0·95</td>
<td>50·0</td>
<td>3·5</td>
<td>-0·51</td>
</tr>
<tr>
<td>HPRS F45</td>
<td>No neutralization</td>
<td>43·3</td>
<td>3·5</td>
<td>74·2</td>
<td>6·0</td>
<td>-0·84</td>
<td>100</td>
<td>8·1</td>
<td>-0·68</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Areas were calculated from the regression line equation \( Y = A + BX \) as \( (A^2/B)/2 \). The factor 1/2 is common to all values and has been omitted from the calculations (see Fig. 7). Normalized area % is the heterologous area calculated as a percentage of the homologous area for each antiserum.
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Rous sarcoma virus, and have shown that if present it forms less than 0.001 % of the infectious virus.

The cross-neutralization results between bs-RSV and ‘pseudotypes’ formed from subgroup A field isolates of leukosis viruses show, in agreement with Ishizaki & Vogt (1966) and Churchill (1968), that antigenic heterogeneity exists within this subgroup. However, this antigenic heterogeneity expresses itself in cross-neutralization tests not only by differences in titre, but also by differences in slope of the neutralization curves. In this respect our results closely resemble those of Westaway (1965) with arboviruses.

We have shown that the antigenicity of two strains of sarcoma virus (bs-RSV and bs-RSV (HPRS F42)/1) can be changed by subjecting them to selection pressures, i.e. changing host susceptibility and cloning respectively. The change in antigenicity was shown in cross-neutralization tests in which the slope of the neutralization curves and titre of each serum for the homologous virus was different from those for the heterologous virus. This suggests that these strains are mixtures of viruses which are not antigenically identical. These findings suggest an explanation for the different slopes of neutralization curves noted in the homologous systems, and the occasional occurrence of steeper slopes in heterologous tests compared with the homologous test. We suggest that virus mixtures are responsible for these results. The antigenicity of a strain of virus will vary from stock to stock if there are changes in the proportions of the components of the virus mixture or loss or addition of some components, and changes in the degree of phenotypic mixing. Phenotypic mixing is a phenomenon which is likely to occur with virus mixtures (Vogt, 1967). High degrees of phenotypic mixing occur only when relatively high multiplicities of infection are used, whereas changes in the components of the virus mixture are more likely to occur at low multiplicities of infection and where selection pressures, such as changes in host susceptibility, are present. The same factors could explain the difference in neutralizing properties of different sera for the same stock of virus.

It is clear from these studies that because of differences in slope of neutralization curves, estimates of antigenic relationships between strains of virus based on cross-neutralization tests are inadequate where titres are expressed in the conventional way as a single point on the neutralization curve. We suggest that area values are more satisfactory as a method of fully describing the neutralization properties of antisera to viruses of the leukosis/sarcoma group.

We are grateful to Mr B. S. Milne, Miss M. Brand, Mr E. G. Carrington and Mr P. Sewter who gave valuable technical assistance during the course of this work.

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REFERENCES


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