A Practical and Quantitative Microtest for Determination of Neutralizing Antibodies against Epstein-Barr Virus

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Much information regarding the association of the Epstein-Barr virus (EBV) with infectious mononucleosis, Burkitt’s lymphoma and nasopharyngeal carcinoma has been derived from serological test procedures, involving a number of distinct antigen–antibody systems related to EBV. These relationships have been recently reviewed (Henle & Henle, 1972).

Tests for neutralizing antibodies have been hampered by the lack of cells fully permissive for EBV. In addition, infectious virus can be obtained at present only from a few carrier lines and it undergoes essentially one abortive cycle of replication in appropriate lymphoblasts with the synthesis of EBV-induced early antigen (EA) and EBV-determined cell membrane antigens (MA), but little if any EB-virus capsid antigens (VCA) (Henle et al. 1970; Gergely, Klein & Ernberg, 1971; Durr et al. 1970). Nevertheless, three procedures for determination of neutralizing antibodies have been described. Pearson et al. (1970) based their test on the reduction of EA-synthesizing cells following exposure of lymphoblasts to virus-serum mixtures as compared to virus controls. For this test, relatively large numbers of cells must be exposed to sufficient virus to yield 10 to 30 % immunofluorescent cells in 48 h in the controls so that several cell smears can be prepared for assay and calculation of a reproducible neutralization index. Durr et al. (1970) have shown that severe, cytopathological changes induced by EBV can be prevented by sera which show antibody coating of enveloped virus particles, but not by negative sera. Finally, Miller, Niederman & Stitt (1972) have demonstrated that in the course of infectious mononucleosis antibodies appear which neutralize the capacity of EBV to transform peripheral lymphoid cells, thus preventing the establishment of continuous lymphoblast lines. These techniques are not readily adaptable to accurate quantitation of neutralizing antibodies, they are limited in sensitivity, are not suitable for large-scale applications and require, in part, long-term observations. The present report presents a microneutralization test based upon the observation that colony formation by lymphoblasts is inhibited by EBV infections (Rocchi et al. 1973).

The Raji cell line (Pulvertaft, 1964) was used because: (a) a high viability is constantly maintained in stock cultures; (b) it was found to be highly susceptible to abortive EB virus infection measured by immunofluorescence and reduction in colony formation; (c) it has a high cloning efficiency in semi-solid agar (Rocchi et al. 1973), and (d) when the cells are seeded in fluid medium in micro-wells they grow in clumps, analogous to colonies in semi-solid agar; these clumps will therefore be referred to as colonies. RPMI 1640 medium (Grand Island Biological Company) with 20 % foetal calf serum (Microbiological Associates, Inc.) was used for growing the cells and as a diluent in all test procedures.

All EBV preparations were produced from HR-1 cells as described (Henle et al. 1970) and stored in liquid nitrogen before use. Each lot of virus was titrated in Raji cells both for its ability to inhibit formation of cell colonies and to induce synthesis of the D component of the EA complex. Cytopathogenicity titrations were carried out in sterile flat bottom tissue culture plates (Falcon Microtest II). The virus was diluted in 0.5 log₁₀ steps and 25 µl volumes were added to the wells containing 25 µl of medium. Next, 25 µl of a
Raji cell suspension (3.5 x 10^4 cells/ml) were added to each well (875 cells/well). This cell concentration gave between 100 and 200 colonies/well. The plate was incubated for 1 h at 37 °C and shaken at 15 min intervals. After the 'virus exposure period' 0.1 ml of medium was added and the plate was incubated for 4 to 6 days at 37 °C in a humidified atmosphere of 5% CO_2 in air. Results were read under an inverted microscope by counting the colonies present in the wells. The highest dilution of virus, that reduced colony formation by more than 95%, as compared to uninfected controls, was chosen as the dose employed for neutralization tests. Each colony that grew in the controls was considered to arise from one infectable unit. The concentration of virus causing 50% inhibition of colony formation was calculated by the method of Reed & Muench (1938) and used for comparison of the effects of different cell-virus ratios on the results of virus titrations.

Titrations of virus by immunofluorescence assays followed the procedure described by Henle, Henle & Klein (1971). The virus samples used showed considerable variations in titres as measured by both assay methods, due to as yet uncontrollable fluctuations in virus production by the HR-1 line.

The sera for titration of neutralizing antibodies were selected on the basis of their antibody titres to VCA (Henle & Henle, 1966; Henle et al. 1969) to cover a range from very high to negative values (see Table 2). They had been stored at -20 °C and were inactivated at 56 °C for 30 min before use.

Microtitre equipment (Cook Engineering Co., Alexandria, Virginia) was used for the neutralization test. The sera were diluted in twofold steps in microplates, according to the technique described by Sever (1962), starting with 1:5, and 25 μl of appropriately diluted virus was added to each well containing 25 μl serum dilution. To duplicates of the first three serum dilutions, medium was added instead of virus as a control of serum toxicity. A virus control (no serum) as well as virus titration of several further series 0.5 log_10 dilutions accompanied each test. The serum-virus mixtures were incubated for 1 h at 37 °C and the plates shaken every 15 min. Additional overnight incubation at 4 °C was carried out in one test. At the end of the incubation period Raji cells and subsequently medium were added as described for virus titrations. After 4 to 6 days of incubation at 37 °C in an atmosphere of 5% CO_2 in air, the wells were scanned under an inverted microscope and scored for the approximate numbers of colonies. A well showing colonies amounting to > 30% of the number found in uninfected controls was considered to show evidence of neutralization. After microscopic examination the plates were returned to the CO_2 incubator for an additional 4 to 5 days and then incubated at the same temperature in normal atmosphere overnight. At this point virus neutralization was evident also from an acid pH (metabolic activity) as shown by the yellow colour of the indicator (phenol red).

In early experiments cells were exposed to serum–virus mixtures and then seeded in soft agar to observe the effect on colony formation (Rocchi et al. 1973). It was noted subsequently that agar is not essential as a supporting medium and all neutralization tests were therefore performed in fluid medium. Fig. 1 shows the sequential growth of uninfected Raji cells over a 4-day period, and the complete abrogation of colony formation after infection with EBV.

In order to determine the minimal dose of virus required for the test, experiments were performed to evaluate the influence of cell–virus ratios on colony formation. Volumes of 1 ml of serially diluted virus were therefore incubated with 1 ml volumes of a cell suspension containing 4 x 10^4, 40 x 10^4 and 400 x 10^4 cells/ml, respectively, and incubated at 37 °C for 1 h. The cell concentrations in the last two sets of preparations were then adjusted to that of the first set (2 x 10^4/ml), by dilution with medium and 40 μl of each preparation was
Fig. 1. Colonies of normal Raji cells in fluid medium. Frames A–D show sequential development of colonies from day 2 to day 5 after seeding a cell suspension in micro-wells. Arrows point to one developing colony. Frames E and F (day 2 and 5, respectively) illustrate that EBV infected cells failed to grow.

The colonies were counted 5 days later as described. Table 1 shows that with a decrease in the numbers of cells used for infection, the virus titre increases roughly corresponding to the increase in the cell–virus ratio. It was evident that the smallest number of cells needed for formation of sufficient numbers of colonies in micro-wells (about 900 cells/well) would be suitable for the neutralization tests.

Table 2 presents the results of neutralization tests carried out with 14 selected sera on five separate occasions and with different virus preparations. There was close agreement among the five separate tests. The two sera that were negative for anti-VCA in indirect immunofluorescence tests also failed to show neutralizing antibodies. The 12 anti-VCA positive sera all revealed neutralizing activity. The serum of Burkitt the tumour patient, F.M., and the negative serum from J.H. have been used as standard positive and negative controls in further tests and were examined thus more than 30 times, always giving the same results.
Table 1. *EBV* titrations with different cell concentrations: average number of colonies observed per well after 5 days of incubation

<table>
<thead>
<tr>
<th>Virus preparation</th>
<th>Cell concentration (× 10⁴/ml) at exposure</th>
<th>Cell concentration (× 10⁴/ml) at plating</th>
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<td>40</td>
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<tr>
<td><strong>Virus dilution</strong></td>
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<tr>
<td>10⁻⁶</td>
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<td>0</td>
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<tr>
<td>10⁻³</td>
<td>0</td>
<td>13·0 (1·80)</td>
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<tr>
<td>10⁻²</td>
<td>2·5³ (0·67)</td>
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<td>10⁻¹</td>
<td>33·5 (3·95)</td>
<td>94·8 (2·30)</td>
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<td>10⁰</td>
<td>84·3 (8·00)</td>
<td>96·0 (12·00)</td>
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<tr>
<td>10¹</td>
<td>84·0 (1·97)</td>
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<td><strong>Cell control</strong></td>
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<td><strong>TCD₅₀</strong></td>
<td>10⁻³⁻⁰⁸</td>
<td>10⁻²⁻⁰¹</td>
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* Average number of colonies developing in four wells of a microplate; standard error is given in parentheses.

In most cases the agreement between the microscopic and metabolic readings was close. If there was a discrepancy, the titres obtained by metabolic readings were one dilution step lower than those recorded for microscopic readings. This difference is reflected in the geometric mean titres.

In test number 5 the virus-serum mixtures were incubated at 37 °C for 1 h and overnight at 4 °C before adding the cells. With three sera there was a slight indication of an increase in titre, which, however, appeared hardly significant. For each test, different virus preparations were used. While the potency of these lots, as measured by both immunofluorescence and cytopathogenicity assays, varied over a 30-fold range, this fact had no apparent influence on the neutralizing titres of the sera as long as the dilution of the virus used was properly adjusted.

The method described is apparently both sensitive and reliable for detecting neutralizing antibodies to EBV. The performance of the test is simple and the microtitre method for serum dilution is well accepted for determination of titres for a variety of different antibodies. It permits the screening and titration of large numbers of sera for EBV-neutralizing antibodies. Microscopic reading of the plates is rapid when a dose of virus is used which inhibits cell growth and colony formation in controls by 95 to 100 % rather than a dose causing only 50 % inhibition. The clearly negative or clearly positive wells under these conditions need only be scanned, while an actual count of colonies is restricted to those wells near the serum end-point. Reading on the basis of pH changes also depends upon nearly complete inhibition of cell growth so that clearly negative and positive results are evident from the indicator colour. The two methods of evaluation provide an ‘internal’ check of the results. The selected sera revealed a wide spectrum of neutralizing titres ranging from 1:5 up to 1:2560. The reproducibility of these results was good even though in each test different virus preparations of varying potency were used after appropriate dilutions.
Table 2. EBV neutralization by selected sera. Titres determined by microscopic (mic) and metabolic (met) readings

<table>
<thead>
<tr>
<th>Test</th>
<th>Ant-VCA titres</th>
<th>Diagnosis</th>
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Comparative potency of virus preparations

<table>
<thead>
<tr>
<th>Dilution used in neutralization test</th>
<th>Immunofluorescent stained cells at 10° dilution</th>
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<td>No. 5*</td>
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* In this experiment the serum virus mixture was incubated at 37°C for 1 h and at 4°C overnight before adding the cells.
† The antibody titres are expressed as a reciprocal of the serum dilution.
‡ Data not used to calculate the geometric mean titre.
NT, not tested.
Short communications

The neutralization test for EBV differs to a certain extent from neutralization tests used for titration of antibodies against cytopathogenic viruses in permissive cell cultures. Conventionally, the 50% infective dose of a virus suspension is determined and a multiple of this dose, usually 100 TCD 50 is used in the neutralization test. Non-neutralized virus, however little, causes a spreading infection with destructive cytopathic effects. In contrast, the infection of Raji cells by EBV is abortive. There are no detectable second cycles of infection and therefore the number of effectively adsorbed infectious virus particles must be at least as great as the number of exposed cells, in order to detect a high degree of colony inhibition. This consideration implies that in this test system, allowing for non-adsorbed virus, probably about 1800 infectious virus particles must be neutralized by the diluted serum. A small non-neutralized fraction would fail to register, since it could reduce colony formation by only an insignificant amount. The sensitivity of this test seems comparable to the conventional type of neutralization test. Any further increase in sensitivity is limited by the cell numbers which must be used to obtain sufficient colonies in the uninfected controls. The number of cells exposed in the conventional test is immaterial.

From the limited number of sera included in this report it seems evident that those without antibodies to VCA are also free of neutralizing antibodies, whereas all sera containing anti-VCA show neutralizing activity. This correlation requires confirmation by testing additional sera. The ratios between neutralizing antibody and anti-VCA titres of single sera varied, however, over an eightfold range, indicating that different antigens are involved in the two systems, as was noted also by Pearson et al. (1970). Comparative studies on the antibody levels to various EBV-related antigens and neutralizing titres are in progress which at the same time are intended to determine the distribution of neutralizing antibodies in populations at large as well as in the various EBV-associated diseases.

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REFERENCES


Short communications


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