Comparison of enzyme-linked immunosorbent assay (ELISA) technique and complement-fixation test for estimation of cytomegalovirus IgG antibody

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SUMMARY The ELISA technique has been found to be reliable for the detection and titration of cytomegalovirus-specific IgG antibody in serum. It is about six times more sensitive than the CF test although some discrepancies were found between the antibody titres determined by the two methods.

Cytomegalovirus (CMV) is an important cause of fetal damage and of opportunistic infection in immunosuppressed patients. Serological diagnosis of infection has relied heavily on the complement-fixation (CF) test, although more recently the immunofluorescence technique has been widely used to detect both IgG and IgM antibody (Hanshaw *et al.*, 1968; Thé *et al.*, 1974; Betts *et al.*, 1976). Other techniques, such as passive haemagglutination (Bernstein and Stewart, 1971; Fuccillo *et al.*, 1971), immune adherence (Dienstag *et al.*, 1976), and radioimmunoassay (Forghani *et al.*, 1976; Knez *et al.*, 1976), have also been used and found to be satisfactory but have not been generally adopted.

The latest technique is the enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1972; Voller *et al.*, 1976), which has been shown to be more sensitive than passive haemagglutination for detecting IgG antibody (Castellano *et al.*, 1977) and comparable in sensitivity to immunofluorescence for detecting IgM antibody (Schmitz *et al.*, 1977). The present report compares the ELISA technique with the CF test in examining for CMV-specific IgG antibody in sera from patients.

Material and methods

PREPARATION OF ANTIGENS

Confluent monolayers of MRC-5 diploid human fibroblasts (Jacobs *et al.*, 1970) were grown in Roux bottles. They were infected with CMV strain AD 169, at a multiplicity of 0.1 plaque-forming units per cell, and then incubated in Eagle's minimal

essential medium with 10% fetal calf serum at 37°C. After 7-10 days, when gross cytopathic effects had developed, the cells were scraped off the bottles into a small volume of phosphate-buffered saline (PBS), pH 7.2, washed three times in PBS, and resuspended in a volume of 0.1 M glycine-NaCl-NaOH buffer, pH 10, equivalent to a final concentration of 1 ml per culture bottle of cells. The concentrated cells were immediately disrupted by ultrasonication for 5 s and the cellular debris was removed by centrifugation at 800 *g* for 10 min. The final supernatant was stored, in 0.25 ml quantities, at -70°C. Control antigen was prepared in the same way from uninfected MRC-5 cells. These antigens were used for both the CF and ELISA tests.

COMPLEMENT-FIXATION TEST

This was carried out by the microtitre technique (Sever, 1962) with 2 units of antigen and 3 MHD₅₀ of complement. Antibody titres were read as the reciprocal of the highest dilution of serum giving 50% (2+) fixation.

ELISA PROCEDURE

This was done by the method of Voller *et al.* (1976). Flat-bottomed wells in polystyrene microtitre plates (Cooke Microtitre M29AR, Dynatech Ltd) were coated with viral or control antigen by the addition of 0.3 ml of the antigen, diluted to optimal concentration in 0.1 M carbonate-bicarbonate buffer, pH 9.6, and incubated overnight. The plates were then washed three times in PBS containing 0.05% Tween 20 and 0.02% NaN₃ (PBST) and shaken dry. They were used immediately or after storage at -70°C (stored plates were found to be satisfactory

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even after nine months). Test sera were diluted in PBST, and 0.3 ml of each dilution was added to duplicate wells. The plates were incubated overnight at 4°C, washed three times in PBST, and shaken dry. Conjugate was then added, in 0.3 ml amounts, and the plates were left for 2 h at room temperature. After three further washes in PBST, 0.3 ml of the substrate was added to each well. Exactly 30 min later the reaction was stopped by the addition of 0.05 ml of 3 M NaOH. The intensity of the colour change produced in the substrate was measured at 400 nm in a Gilford 300-T-1 spectrophotometer with a vacuum-aspirated microcuvette accessory. The absorbance (E₄₀₀) for each serum sample was calculated as the mean of the duplicate readings minus the 'background' activity in the test; the latter was measured as the mean of the readings from four duplicate wells incubated with PBST instead of serum.

PHOSPHATASE SUBSTRATE

This was a 0.1% solution (w/v) of *p*-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8, with 0.02% NaN₃ and 0.01% MgCl₂.6H₂O.

CONJUGATE

The conjugate was sheep anti-human-IgG anti-serum labelled with alkaline phosphatase (Type VII, Sigma Chemical Co) by the method of Voller *et al.* (1976). The working dilution was determined by coating the wells of microtitre plates with a 1/100 dilution of viral antigen, challenging with a 1/100 dilution of a pool of CMV CF antibody-positive human sera, and then adding, to duplicate wells, a range of conjugate dilutions from 1 in 100 to 1 in 2000. A control test was set up at the same time with a 1 in 100 dilution of control antigen. The highest dilution of conjugate that gave an E₄₀₀ reading of 1.0 with the viral antigen, compared with 0.05 with the control antigen, was 1 in 400, and this was used as the working dilution in all subsequent tests.

DETERMINATION OF OPTIMAL CONCENTRATION OF ANTIGEN

This was determined by a 'chess-board' titration against the CF antibody-positive human serum pool. Duplicate wells were coated with doubling dilutions of viral antigen or control antigen and challenged with doubling dilutions of antiserum. The E₄₀₀ readings for each dilution of the antigens were plotted against the serum dilutions (Fig. 1). The highest reading, indicating saturation of binding sites on the surface of the microtitre wells, was achieved with a 1 in 400 dilution of the viral antigen.

Fig. 1 E₄₀₀ values of serial dilutions of CMV antigen in the presence of increasing dilutions of specific antiserum. Dilutions of antigen: ○—○ = 1 in 100; ●—● = 1 in 200; △—△ = 1 in 400; ▲—▲ = 1 in 800; □—□ = 1 in 1600; ■—■ = 1 in 3200. ○---○ = control, uninfected host-cell antigen.

Results

RELATIONSHIP OF E₄₀₀ VALUES TO CONCENTRATION OF CMV ANTIBODY IN TEST SERA

Preliminary studies were carried out with 33 antisera, four devoid of CF antibody (titre <8) and 29 with CF antibody titres ranging from 32 to 5792. Doubling dilutions of each serum were examined by the ELISA technique, and their E₄₀₀ values were plotted on a graph against serum dilution. Figure 2 shows the results from eight representative sera, two CF antibody-negative and six CF antibody-positive. Sera without antibody (CF antibody titre <8) commonly gave, at low dilutions, some minimal reactivity (always <0.25). This was probably due to nonspecific, residual attachment of serum or conjugate since similar low readings were obtained from wells that had been coated with control antigen or pretreated only with PBST. The curves for the antibody-positive sera, particularly those with higher titres, were obviously sigmoid in form. However, whether the antibody titre was high or low (CF antibody titres 32-5792), the body of the curve, over a wide range of serum dilutions, was rectilinear. Moreover, the curves over their rectilinear sections, for all 29 positive sera examined, were found to be closely parallel, indicating that a standard curve could be used to determine the ELISA antibody titre of each serum from the E₄₀₀ value

Fig. 2 Relationship between antibody concentration and E₄₀₀ values in six known CMV antibody-positive (CF antibody titres 32-5792) and two antibody-negative (CF antibody titre < 8) sera.

obtained from any single suitable dilution of that serum.

The ELISA antibody titre of each of the 29 sera was measured, from their curves described above, as the reciprocal of the dilution that had an E₄₀₀ value of 0.5, and this was plotted on a graph against the E₄₀₀ value obtained for a 1 in 300 dilution of the same serum (Fig. 3); 1 in 300 was chosen as a suitable dilution from Fig. 2, which showed that it lay on the linear part of curves representing both high-titred and low-titred antisera. A straight-line, directly proportional relationship was obtained (correlation coefficient $r = 0.93$, $P < 0.001$), confirming that the antibody titre of a serum could be reliably calculated from the E₄₀₀ value of a single 1 in 300 dilution. The lowest E₄₀₀ reading among the 29 positive sera examined at this dilution was 0.22; the readings for the four negative sera were all < 0.09; 1 in 300 was, therefore, used as the standard test dilution for sera in subsequent experiments.

COMPARISON OF ELISA AND CF TESTS FOR DETECTION OF ANTIBODY

A single batch of antigen was used to examine the 29 CMV-antibody-positive antisera described above by both the ELISA and the CF techniques. The results obtained by the two techniques, that is, the E₄₀₀ values at a 1 in 300 dilution and the CF antibody titres, showed good correlation ($r = 0.73$, $P < 0.001$) (Fig. 4), although a few sera with almost identical E₄₀₀ values had CF antibody titres

Fig. 3 Comparison of the ELISA antibody titres of 33 sera, calculated from the highest dilution of serum giving an E₄₀₀ value of 0.5, with the E₄₀₀ values of the same sera examined at a 1 in 300 dilution.

Fig. 4 Comparison of the CF antibody titres of 29 CMV antibody-positive sera against the E₄₀₀ values of the same sera examined at a 1 in 300 dilution.

that differed as much as 64-fold. Extrapolation of the regression curves to the abscissae on Figs 3 and 4 showed that the ELISA technique was about six times more sensitive than CF for detection of CMV antibody.

To examine further the relationship between the ELISA and CF tests, a larger batch of 90 sera,

randomly collected from patients in hospital, was tested. Twenty-one had CF antibody titres of <8 and E400 values, at a 1 in 300 dilution, of 0.04-0.13 (mean 0.08, standard deviation 0.03). Sixty-nine sera had CF antibody titres of 16-8192 and E400 values of 0.24-2.32. Again there was good general agreement between the two tests ($r = 0.62$, $P < 0.001$), but some scatter of results (Fig. 5). Sera with CF antibody titres of 64 or less had E400 values of <1.0 , and those with titres of >1024 had E400 values of >1.0 , but among the sera with intermediate CF antibody titres of 128-1024 there was a scatter of E400 values, ranging from 0.24 to 2.0. However, examination by ELISA at a 1 in 300 dilution effectively discriminates between antibody-negative sera (CF antibody titre <8 , E400 <0.2) and antibody-positive sera (CF >16 , E400 >0.2). None of the 90 sera showed any significant reactivity in either the CF or ELISA tests against control, uninfected antigen; they were negative for rheumatoid factor when tested at a 1 in 10 dilution by latex agglutination and were not anticomplementary in the CF test.

The reproducibility of the CF and ELISA results was determined by examining the 29 original positive sera on three separate occasions. Their CF antibody titres varied by no more than twofold and their E400 values by only 19%-32% (mean 25%). A further test for reproducibility was carried out by fractionating six of the sera on 15%-35% (w/w) sucrose gradients, for 18 h at 35 000 rpm, in the Beckman SW 40 rotor, and examining the various fractions for antibody. Clearly related curves were

Fig. 5 Comparison of the CMV CF antibody titres of 90 randomly collected sera with the E400 values of the same sera examined at a 1 in 300 dilution.

Fig. 6 Comparison of the ELISA and CF tests for detecting the presence of CMV antibody in sucrose gradient fractions of two sera. ●—● = E400 values at a 1 in 300 dilution of each gradient fraction; ○—○ = CF antibody titre.

obtained for the two techniques with all six sera; results for two of them are shown (Fig. 6).

Discussion

Previous studies have shown that the ELISA technique can be readily applied to the detection of CMV antibody, both IgG (Voller and Bidwell, 1976) and IgM (Schmitz *et al.*, 1977), and that it is comparable in sensitivity to the indirect immunofluorescence test (Schmitz *et al.*, 1977) and 2- to 10-fold more sensitive than the indirect haemagglutination test (Castellano *et al.*, 1977). The present investigation has shown that it is about six times more sensitive than the CF test. Moreover, the antibody titre, determined by ELISA, was found to be proportional to the absorbance (E400) over a wide range of serum dilutions and can therefore be reliably calculated from the E400 reading obtained for a single dilution of the serum; 1 in 300 was found to be a suitable dilution for this purpose. There was good correlation between the antibody titres obtained by the ELISA and CF techniques and excellent agreement in determining the presence (CF antibody titres 16 and more) or absence (CF antibody titre <8) of antibody; at the lower levels of CF antibody the E400 readings were twice those obtained for CF antibody-negative sera. However, none of the sera investigated had really low CF antibody titres of <16 , perhaps because the antigen was a potent, glycine-extracted preparation (Krech *et al.*, 1971; Kettering *et al.*, 1977). Whether exam-

ining sera at a 1 in 300 dilution in the ELISA test will effectively discriminate at these low levels of antibody requires further investigation.

Nevertheless, although the same antigen was used for both the ELISA and CF tests, there were frequent discrepancies between the E400 values and the CF antibody titres, particularly within the middle range of values (Fig. 5). That is, sera with similar E400 values often showed a wide range of CF antibody titres. This could not be satisfactorily explained by poor reproducibility of either test or by the variable presence of nonspecific inhibitors, such as anticomplementary factors or rheumatoid factor, the presence of which has been shown to depress CF antibody titres in several other virus-antibody systems (Stanford, 1972; Haukenes, 1974). Another possibility was the presence in the antigen preparations of the nonspecific, intracytoplasmic F_c receptor that develops in CMV-infected cells (Sakuma *et al.*, 1977). Extracted receptor protein could perhaps attach to the microtitre wells and give spuriously enhanced E400 readings due to reaction with antibody other than CMV antibody; F_c receptor-antibody complexes do not fix complement and would not interfere with the CF test. This was excluded, however, by the lack of significant reaction in the ELISA test with CMV antibody-negative sera. The simplest explanation would be that human sera contain variable mixtures of CMV IgG antibodies (Cremer *et al.*, 1975), only some of which readily fix complement, whereas the ELISA technique, based on a globulin-antiglobulin reaction mechanism, has a much wider recognition range for antibody. Unfortunately, IgG antibodies that do not fix complement have not been described for CMV, but since similar discrepancies have been recognised between the CF test and both the indirect and anti-complement immunofluorescence techniques (Griffiths *et al.*, 1978), they deserve further investigation.

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