Enzyme-linked immunosorbent assay for measurement of antibody against cytomegalovirus and rubella virus in a single serum dilution

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SUMMARY Enzyme-linked immunosorbent assays (ELISA) were developed for quantifying cytomegalovirus (CMV) and rubella antibodies using a single serum dilution (1/800) in conjunction with a standard curve. A near linear relation was found between the logarithms of absorbance values of sera at a dilution of 1/800 and the titres as determined by an end point dilution ELISA. The reproducibility of the single dilution ELISA was good; the within-test coefficients of variation averaged 7.5% for CMV antibody and 12.4% for rubella antibody. A close correlation was found between ELISA and complement-fixing (CF) antibody titres to CMV and between ELISA and haemagglutination-inhibition (HI) antibody titres to rubella virus. The titres in ELISA were 200 to 1000 times higher than in CF for CMV and 50 to 100 times higher than in HI for rubella virus.

Many reports have described the application of the enzyme-linked immunosorbent assay (ELISA) for laboratory diagnosis of infectious diseases.^{1 2} With respect to sensitivity, specificity, reproducibility, and ease of performance, ELISA has distinct advantages over existing techniques for determining antibody titres to different aetiological agents including cytomegalovirus (CMV)³⁻⁶ and rubella virus.^{3 7-10} CMV and rubella antibody levels in ELISA have been determined by examining serial serum dilutions^{4 6 7 10} or a single serum dilution.^{3 5 8} It is apparent from these studies that there is a need to standardise methods to distinguish between specific and non-specific reactivity.

We report an ELISA procedure which permits a precise and reproducible quantification of CMV and rubella antibodies by examination of a single serum dilution in conjunction with a standard curve. The sensitivity of the test is compared with that of complement fixation (CF) for CMV and haemagglutination inhibition (HI) for rubella virus.

Material and methods

ANTIGENS

The AD-169 strain of CMV was used. For ELISA

a nuclear antigen was prepared from CMV-infected human diploid fibroblasts essentially according to the procedure described previously.^{11 12} The optimal antigen dilution in ELISA as determined by block titration¹³ was 1/100. Control nuclear antigen for CMV was prepared in a similar way from uninfected cells. The protein content of CMV and control nuclear antigens as determined by the Lowry method was approximately 0.4 mg/ml. CMV antigen for the CF titres was prepared by glycine extraction of CMV-infected human diploid fibroblasts.¹⁴

The Gilchrist strain of rubella virus was used to prepare antigen for ELISA. Virus was grown in Vero cells¹⁵ and purified using a combination of ultrafiltration and discontinuous sucrose gradient centrifugation. The protein content of the antigen was approximately 0.3 mg/ml. The optimal antigen dilution for ELISA was 1/100. Control antigen for rubella virus was prepared from a suspension of uninfected Vero cells in phosphate-buffered saline (PBS) at pH 7.4 by sonication and subsequent clarification at low speed centrifugation.

CF AND HI TESTS

The CF test procedure was the microtitre technique described by Casey.¹⁶ The HI test was performed according to the technique of Stewart *et al.*¹⁷ using pigeon erythrocytes and a commercial rubella virus antigen (Behringwerke AG, Marburg). Sera were

treated with MnCl_2 -heparin¹⁸ to remove non-specific haemagglutination inhibitors.

SERUM SAMPLES

Sera submitted for rubella screening from hospital personnel and pregnant women and sera from patients clinically suspected for rubella or CMV infection were available. The sera were stored at -20°C for several weeks before testing by ELISA.

ELISA PROCEDURE

The test was performed essentially as described previously.^{13 19 20} In the end point dilution ELISA serial twofold dilutions of serum (0.1 ml per well) were incubated at 37°C for 2 hours in antigen-coated plates using PBS-Tween (0.05%) with 2% fetal bovine serum as diluent for tests with CMV and PBS-Tween (0.1%) with 10% calf serum and rubella control antigen at a concentration of 0.1 mg/ml as diluent for tests with rubella virus. In the single dilution ELISA 0.1 ml of 1/800 diluted serum was added to each of two antigen-coated wells. Conjugate (commercial peroxidase-conjugated anti-human immunoglobulin G, γ chain-specific, Institut Pasteur, Paris) diluted 1/1000 in PBS-Tween (0.05%) with 2% fetal bovine serum was used for tests with CMV and conjugate diluted 1/5000 in PBS-Tween (0.1%) with 10% calf serum was used for tests with rubella virus. After addition of substrate solution and 30 minutes' incubation, the colour intensity was determined by spectrophotometry (Vitatron DCP); the results were expressed as the absorbance at 492 nm.

DETERMINATION OF ANTIBODY TITRES IN ELISA

To determine antibody titres by end point dilution the absorbance value of each serum dilution was compared with that of the same dilution of the negative control serum examined in the same plate. The titre of a patient's serum to CMV or rubella was considered to be the dilution with an absorbance value which was 1.7 and 2.5 times, respectively, the absorbance value of the corresponding dilution of the negative control sera for the two viruses. The end point was calculated by interpolation with readings at succeeding serum dilutions. The rationale of this approach will be presented in the first section of results.

Antibody titres in the single dilution ELISA were calculated from the mean value of the duplicate tests with a 1/800 diluted patient's serum in conjunction with a standard curve. Serum pools with varying titres were used to construct standard curves. A standard curve for CMV was obtained by plotting the absorbance values of each serum pool examined at a dilution of 1/800 against its antibody titre as

previously determined by the end point dilution test. A standard curve for rubella virus was constructed by plotting "specific absorbance" values against antibody titres since a better fit was obtained in this way. "Specific absorbance" values were obtained by subtracting the absorbance of the 1/800 diluted negative control serum from the absorbance of each serum pool.

Results

DETERMINATION OF ANTIBODY TITRES BY END POINT DILUTION ELISA

Fig. 1 compares the dose response relation of sera with varying antibody levels to CMV (a) and rubella virus (b) with that of negative sera. Intercepts with the dashed line representing the mean negative value of each dilution plus three standard deviations of the mean indicate the titre. In subsequent tests a negative control serum consisting of a pool of negative sera was used for determining positive/negative cut-off values. An absorbance value 1.7 and 2.5 times the absorbance of the negative control serum corresponded with the mean value of negative sera plus three standard deviations of the mean for CMV and rubella virus, respectively. Since the absorbances of a large number of negative sera were found to have a nearly normal distribution in both tests (data not shown) it may be calculated that the probability of obtaining a false positive result is less than 0.5% in tests with both viruses.

REPRODUCIBILITY

Four replicates of a positive serum and the negative control serum for CMV and rubella virus were examined at a dilution of 1/100 in consecutive tests (Table).

DETERMINATION OF ANTIBODY TITRE BY SINGLE DILUTION ELISA

The titre of an individual serum was calculated in conjunction with a standard curve constructed for each plate similar to the experiment depicted in the inserts of Fig. 1. Linear regression coefficients of standard curves exceeded 0.98. Comparison of the absorbance values of a single dilution (1/800) and end point dilution titres of 77 rubella sera provided evidence of the validity of using a single dilution (Fig. 2).

COMPARISON WITH CF FOR CMV ANTIBODY AND HI FOR RUBELLA ANTIBODY

Fig. 3 shows that the single dilution ELISA was more sensitive than the CF test for detecting CMV antibody. Antibody titres in ELISA increased proportionally with increasing CF titres; Spearman's

Fig. 1 End point dilution ELISA for antibody to CMV (top, a) and rubella virus (below, b) using five positive and 27 negative human sera in each assay. The lower line represents the mean absorbance values of the negative sera. The broken line denotes the values three standard deviations above these means. The inserts show the relation between antibody titres of the positive sera and their absorbance at a 1/800 dilution.

Reproducibility of enzyme-linked immunosorbent assays (ELISA) for antibody to cytomegalovirus (CMV) and rubella virus

Antibody	Within-test*		Between-test†	
	+ve serum	-ve serum	+ve serum	-ve serum
Cytomegalovirus	5.3% (1.5-9.8)	7.5% (4.0-13.2)	1.18 (1.01-1.40)	0.13 (0.09-0.17)
Rubella virus	6.4% (4.0-7.0)	12.4% (9.0-20.0)	1.30 (0.98-1.85)	0.08 (0.05-0.12)

*Mean variation coefficient and range. Four replicates of each serum were examined in seven different tests for CMV and five different tests for rubella virus by single dilution ELISA.

†Mean absorbance and range in these tests.

Fig. 2 Relation between antibody titres of 77 sera to rubella virus as determined by end point dilution ELISA and their absorbance at a 1/800 dilution.

Fig. 3 Comparison of CMV antibody titres of 113 sera determined by single dilution ELISA and the CF test.

rank correlation coefficient (r_s) was 0.88 ($p < 0.001$). Titres in ELISA were approximately 200 to 1000 times higher than in CF titres.

As seen in Fig. 4, similar results were obtained when sera were examined by ELISA and the HI test for rubella antibody ($r_s = 0.94$, $p < 0.001$). Antibody titres in ELISA were approximately 50 to 100 times higher than in HI titres.

Fig. 4 Comparison of rubella antibody titres of 231 sera determined by single dilution ELISA and the HI test.

Discussion

One of the main problems in developing ELISA is to distinguish between low levels of specific reactivity and non-specific reactivity. The use of a predetermined fixed cut-off level as described by other investigators^{3 5 10 21} does not take into account day-to-day variation and decrease of non-specific reactivity when sera are further diluted. This will hamper standardisation of the test and will cause a relative underestimation of high antibody titres. A better procedure is to determine the cut-off level from the frequency distribution of the absorbance values of negative sera.^{19 22-24} Since in accordance with previous reports^{22 23} the absorbance values of negative sera were normally distributed in both tests, we assumed that sera with absorbance values three standard deviations above the mean value of negative sera contained specific antibody. If absorbance values of negative sera are not normally distributed other statistical methods (transformation, non-parametric methods) should be used to establish a cut-off level.²⁴

In ELISA for CMV antibody the use of control antigen^{4 6 25} was found to be unnecessary since none of the 113 sera tested showed a significantly increased reactivity in wells coated with control antigen. In contrast, the use of control antigen was required in ELISA for rubella antibody since some sera showed an increased nonspecific reactivity in wells coated with control antigen. ELISA for rubella antibody was more practical, economical, and precise when sera were diluted in buffer solution containing control antigen compared with the use of control antigen-coated wells as described by other investi-

gators.^{8 10 21} Purification of the rubella virus antigen further reduced non-specific reactivity.^{7 9 10}

There was a linear relation between logarithms of antibody levels and absorbance values over a wide range of serum dilutions as has been reported previously for other antigen antibody systems.^{19 24 26 27} Furthermore, the slopes of the linear portions of dose response curves of six CMV and eight rubella acute-phase sera (data not shown) appeared to be approximately the same as those of the late-convalescent sera depicted in Fig. 1. These data indicate that antibody titres could be reliably determined by examining only a single (1/800) serum dilution in conjunction with a standard curve. An advantage of using a high dilution is that complications due to saturation phenomena or to the presence of other immunoglobulin classes are avoided.²⁴ Furthermore, it allows for detection of antibody in very small sample volumes. When the problems related to the presentation of ELISA results²⁴ are sufficiently solved, we expect that ELISA will rapidly become a generally accepted procedure in routine sero-diagnosis.

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