DESIGN OF A STANDARD CURVE FOR THE MEASUREMENT OF IgG ANTIBODIES AGAINST HUMAN CYTOMEGALOVIRUS IN A SINGLE SERUM DILUTION BY ULTRA MICRO-ELISA (UMELISA).

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SUMMARY

The use of a standard curve in an ultra-micro-ELISA (umELISA) for detection of IgG antibodies against Human Cytomegalovirus (HCMV) is reported. Based on the end-point titration by linear regression, 33 sera showing $r^2 = 0.98$ were chosen and 4 standard curves were constructed which related the natural logarithm of the obtained umELISA fluorescence for the 4 dilutions and the natural logarithm of the previously determined end-point titer. The curve corresponding to the 1:40 dilution was selected ($r^2 = 0.93$) and used to evaluate 50 sera with a known approximate titer by the traditional four dilutions method. A coincidence of 85 % was found with respect to the latter. The 15 % divergence is attributed to the lack of sensitivity of the graphic method in those points close to the cut-off line.

RESUMEN

Se reporta la utilización de una curva patrón en Ultra-Micro ELISA (umELISA) para la detección de Inmunoglobulina G humana contra Cytomegalovirus. Basándonos en el título a punto final calculado por regresión lineal, se escogieron 33 sueros con r^2 0.98 y se construyeron cuatro curvas patrones que relacionaban el logaritmo natural de la fluorescencia (umELISA) para cuatro diluciones y el logaritmo natural del título a punto final determinado previamente. Se escogió la curva correspondiente a la dilución 1:40 ($r^2 = 0.93$) y con ellas se evaluaron 50 muestras de suero de las cuales se conocía el título aproximado por el método gráfico tradicional de las cuatro diluciones. Se encontró una coincidencia del 85% con respecto a este último. La divergencia del 15% es atribuída a la falta de sensibilidad del método gráfico en los puntos cercanos a la línea de corte.

INTRODUCTION

Human Cytomegalovirus (HCMV), belonging to the *Herpesviridae* family, is widely distributed among humans and other mammals. The virus can reside in a latent state in host cells and tissues causing asymptomatic infections or without anspecific symptomatology (Griffiths, 1987). The detection of specific antibodies in serum allows the recognition of previous or recent exposure to the virus, the latter case if the test is designed to detect IgM class antibodies (Filice *et al.*, 1980). According to its features of sensitivity, specificity, reproducibility and execution, the ELISA assay for detection of anti HCMV antibodies has proven advantageous with respect to other serological methods (Sarov *et al.*, 1980). The Ultra Micro-Analytic System (SUMAtm), Immunoassay Center CIE, Havana, Cuba; has introduced the Ultra Micro-ELISA fluorescent Assay (10 μl) (umELISA), fully automated (Otero *et al.*, 1984). The standardization of an umELISA for detection of antibodies against HCMV (Laferté *et al.*, 1992) allowed the massive screening of IgG levels using a graphic method with four sera dilutions

The use of intra-assay standard curves in a 100 vl ELISAs has permitted to overcome the need of evaluation of all serial dilutions, simplifying the test performance and reducing its cost since it allows the study of a greater number of sera in each plate. These results have been expressed as antibody titers (Van Loon and Van der Veen, 1980; Van Loon *et al.*, 1981) or international units (Coulson *et al.*, 1989).

This study presents the optimization of a standard curve confectioned from sera samples with a broad spectrum of antibody titers against HCMV. The curve was used to calculate the antibody titers in sera samples screened in a single dilution in order to facilitate a better automation in the system and a substantial increase of the number tested sera in each plate.

MATERIALS AND METHODS

Antigens

The HCMV antigen was prepared from diploid human fibroblastic cells infected with the AD 169 HCMV strain. A control antigen was prepared also from the same type of cells but uninfected, according to previously described methods and adjusted to a coating concentration of 40 μg/ml in an UMELISA (Laferté *et al.*, 1992).

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Sera sample

A first group was constituted by 100 samples previously titrated in an umELISA for detection of anti-HCMV antibodies, using the graphic four dilutions method and determining the end-point titer to each one of these in an individual linear regression. Thirty three of these samples were chosen to construct four standard curves (see forward).A second group consisted in 50 randomly selected sera with a previously determined anti-HCMV titer by ther dilutions method and served to compare their titers with those found by the standard curve method using a single dilution.

Indirect ultra-micro-ELISA

The procedure was based on an Indirect ultramicro Assay (Laferté *et al.*, 1992) and consisted in the incubation of previously diluted samples (1:40, 1:160, 1:640, 1:1280) in a 15 mM/l Tris buffer containing Tween 20 at a 0.05 % (TRIS-T) and 5 % calf serum on PVC plates (SUMA, CIE), sensitized with both control and HCMV antigens at a concentration of 40 ug/ml. Plates were incubated during 30 minutes at 37 °C. An anti-human IgG conjugate (Alkaline Phosphatase) was used (CIE, Cuba) diluted 1:1000 in the same sera diluent. Fluorogenic substrate (4-methyl-umbelliferil phosphate) was incubated at room temperature during 30 minutes. Washing procedures were carried dispensing 25 ul per well of TRIS-T buffer. Reading of plates was carried out by an automatic spectrofluorimeter (SUMA-121(tm)) and results expressed in fluorescence percentages (F%).

Determination of the sera approximated titer in UMELISA by the four dilutions method

To the percentual value of fluorescence (F%) corresponding to the antigen in each tested dilution, the F% value for the control antigen was subtracted (delta F). The difference was divided by the delta F value obtained with the positive control of the test in order to obtain an adjusted delta F. The antibody titer against HCMV for each serum was estimated selecting the dilution whose adjusted delta F was the lowest one over a cut-off value (0.2). This value was previously determined in the same assay from a frequency hystogram obtained after a preliminary study of blood donor samples (Laferté *et al.*, 1992).

End-point titer of sera by linear regression using an UMELISA

The end-point titer was determined plotting the cut-off value in the linear regression curve for the natural logarithms of adjusted delta F values against the natural logarithms of the reciprocal for each dilution for each serum sample. Titers obtained covered a range of values from less than 1:40 up to more than 1:1280 in five groups. This allowed us to select 33 sera of them whose r^2, was 0.98 or better. Four curves were constructed with these sera which related the natural logarithm of the adjusted delta F with the natural logarithm of the end-point titer for each one of these 33 sera, and one corresponding to each group of adjusted delta F values for the 1:40; 1:160; 1:640; 1:1280 dilutions respectively (Van Loon *et al.*, 1981).

Statistical analysis

Linear regression of the data with a logarithmical transformation and the Spearman's Ranks correlation test were used.

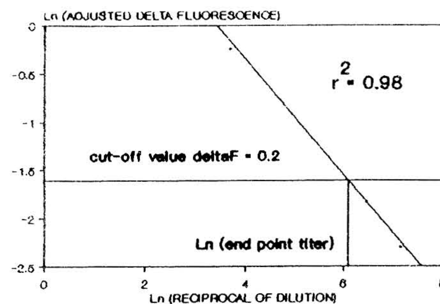

Fig.1 Calculation of the vs. HCMV end-point titers using a linear regression between the natural logarithm of the adjusted flourescence delta and natural logarithm of the reciprocal of the dilution. Serum randomly selected among a group of 33 with a $r^2 > 0.98$.

RESULTS

End-point antibody titers against HCMV by UMELISA

Figure 1 shows the linear regression between the natural logarithm of the adjusted delta F and the natural logarithm of the reciprocal of the dilution for one of the selected sera, used for the construction of the standard curve. It should be noticed that the plotting of the natural logarithm of the cut-off value (0.2) in the regression line directly reflects the value for the natural logarithm of the end-point titer which was analytically calculated by the regression equation. This titer coincides with the rank expression provided by the graphic four dilution method in all of the 33 selected sera with an $r^2 = 0.98$.

Single dilution antibody titer determination by UMELISA

The individual sera titers were calculated using the standard curve of 33 sera shown in figure 2 by linear plotting in a straight line which equation resulted in the expression:

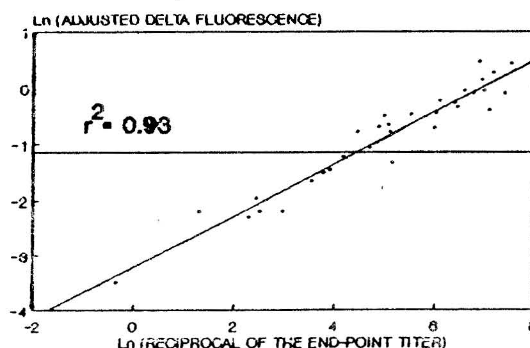

Fig.2 Relation between the logarithms of the antibody titers against CMV in 33 sera (end-point titers) and the logarithms of the adjusted flourescence deltas at a single dilution of 1:40.

$$y = -3.2269 X + 0.4662$$

with a $r = 0.9656$ and a $r^2 = 0.93$ for which the adjusted delta F corresponding to the 1:40 dilution were used.

Comparison between standard curve-obtained antibody titers and those obtained by the four-dilution method

Figure 3 shows the comparison among the titer obtained in both methods. The Spearman's Ranks Correlation Coefficient was $r_s = 0.92$ with an error probability of $p<0.001$.

DISCUSSION

A linear regression was found between the natural logarithm of the adjusted delta F and the end-point titer logarithms as was previously demonstrated with absorbance values and titers in other MicroELISA systems using different antigens (Gerber *et al.*,1980; Parker *et al.*, 1979). In other studies, the 1:800 dilution has been chosen as the single serum dilution, in order to avoid diverse drawbacks like saturation phenomena and presence of other immunoglobulins (de Savigni and Voller, 1980). The selection of the 1:40 dilution in our assay was based on the fact that it was the best among the others (1/320, 1/640 and 1/1280) and the extra advantages provided by the automatic pipette of the SUMA ᵗᵐ System, reducing the possibilities of error caused by hand-pipetting. When comparing both methods (figure 3), a higher sensitivity of the standard curve to detect anti-HCMV antibodies is noticed, since these vary in a continuous magnitude with respect to the graphic method in which titers are expressed in discrete amounts.

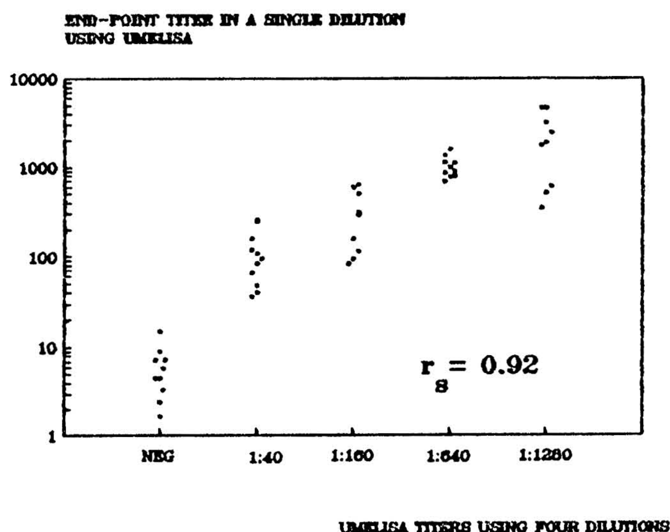

Fig.3 Comparison between the antibodies titers determined with standards curve and those determined by the graphic four dilutions methods.

One of the most important problems to solve in the development of the ELISA system is related to discrimination between the specific reactions occurring at a low level and those non-specific. The use of predetermined cut-off values, do not take in consideration the inter-assay error, even when the cut-off value is expressed in percentual exclusion terms from a frequency distribution of negative sera absorbances.

The single-dilution UMELISA proposed in this paper pretends to eliminate the induced error caused by a graphic estimation of the sera titer in the traditional four-dilution method. Although the use of curves included in each assay plate will increase the accuracy inter assays, the latter results very inaccurate in the estimation of sera titers which fluorescence value is close to the cut-off value. The non coincidence of the two methods is precisely found in those values (the error is considered by the regression). The use of a standard curve in each umELISA plate demands the selection and preparation of standard control sera withan appropriate stability in order to ensure inter-plate and inter-laboratories quality control.

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