Detection of IgM Antibodies to Cytomegalovirus (CMV) Using an Enzyme-labelled Antigen (ELA)

By H. SCHMITZ, U. VON DEIMLING AND B. FLEHMIG

Abteilung für Virologie, Bernhard-Nocht-Institut für Schiffs- und Tropenkrankheiten, Bernhard-Nocht-Str. 74, 2000 Hamburg, 4, West Germany and
Abteilung für Medizinische Virologie, Hygiene-Institut der Universität, Tübingen, West Germany

(Accepted 2 April 1980)

SUMMARY

We have applied a peroxidase enzyme-labelled antigen (ELA) for the detection of IgM antibodies to cytomegalovirus: microtitre plates were coated with anti-IgM immunoglobulin. The IgM fraction of human serum was selectively bound to the precoated plates and the virus-specific IgM antibody was then detected by the enzyme-labelled antigen. A very efficient technique for the labelling of virus antigen is described. The IgM antibody was detected simply and specifically, Rheumatoid factor IgM did not interfere with this test.

INTRODUCTION

The detection of virus-specific IgM antibodies is of great value in the diagnosis of acute virus infections. In diseases with an early rise of antibody titre such as infectious mononucleosis (Schmitz et al. 1972) or hepatitis A (Duermeyer & Van der Veen, 1978; Flehmig, 1978; Flehmig et al. 1979) the presence of specific IgM antibodies in a serum specimen may confirm the suspected diagnosis. Similarly, in the absence of typical clinical symptoms such as in cytomegalovirus (CMV) infection (Schmitz et al. 1972) or rubella in pregnancy (Vesikari & Vahteri, 1968) the close correlation between recent infection and IgM response is of great clinical significance.

Virus-specific IgM antibodies can be demonstrated by numerous methods, some of which are time consuming but give unequivocal results, while others are more rapid but false negative or false positive results frequently occur. Among the latter are the indirect techniques using FITC, $^{125}$I or enzyme-labelled anti-IgM globulin. However, with these techniques rheumatoid factors in human sera often give rise to false positive results (Fraser et al. 1971; Reimer et al. 1975). Interactions between rheumatoid factor IgM and human IgG can be efficiently avoided by isolating the IgM fraction from the serum (Reimer et al. 1975; Schmitz et al. 1977) and separation techniques such as column chromatography or ultracentrifugation have often been applied, although the handling of large numbers of specimens is laborious. As a simpler alternative, IgM can be separated from other serum constituents by selective binding to plastic material coated with anti-human $\mu$-chain immunoglobulin. The antiviral activity of the immobilized IgM antibody can then be assayed by adding a corresponding virus antigen, which in turn can be detected by $^{125}$I- or peroxidase-labelled antibody as has been described for the detection of IgM antibodies to hepatitis A antigen (Duermeyer & Van der Veen, 1978; Flehmig, 1978). Similarly, rubella IgM antibody has been demonstrated in human sera, the binding of the rubella haemag-
glutinating antigen being detected by the adsorption of appropriate erythrocytes (Krech & Wilhelm, 1979).

In this report we have applied a peroxidase enzyme-labelled antigen (ELA) for the detection of IgM antibodies against CMV. The efficiency of the labelling as well as the sensitivity and specificity of the test were studied in detail.

**METHODS**

*Sera studied.* Paired serum specimens obtained from 20 patients with acute CMV infections were included in this study. The diagnosis was based on virus isolation (18 patients), on significant rise of complement-fixing antibody to CMV (10 patients) and on a significant decrease of the virus-specific IgM antibody level with an initial titre of $> 512$, which was found in all patients by indirect immunofluorescence. Moreover, the IgM fractions of these sera, obtained by sucrose gradient centrifugation (Vesikari & Vaheri, 1968), were also shown to be positive by anti-complement immunofluorescence (Schmitz et al. 1977). The clinical diagnosis of the patients' illness was hepatitis or mononucleosis (ten cases), Guillain Barré syndrome (six cases) and congenital CMV infection (four cases). Negative control sera of 535 patients and 100 blood donors without clinical or serological evidence of CMV infection were also studied. Among the patients' group 68% had complement-fixing antibodies to CMV. As a further control, 42 sera containing both rheumatoid factor and IgG antibody titres to CMV of $> 128$ were also investigated as well as 3 sera containing IgM antibodies to nuclear antigens.

In all the experiments with the ELA IgM test a serum with an IgM antibody titre to CMV of 1000 in indirect immunofluorescence was included at a dilution of 1:1000 as positive control. A negative serum with only IgG antibodies to CMV (titre 5000, indirect immunofluorescence) served as a negative control at a dilution of 1:100.

*Indirect immunofluorescence.* The method has been described in detail elsewhere (Schmitz et al. 1972). Briefly, CMV-infected fibroblasts were grown in Petri dishes on a special arrangement of plastic cover slips (Medac, Hamburg, West Germany). Before fixation in ethanol the infected cells were swollen in 0.4% KCl for 5 min. Immediately after fixation the cover slip cultures were stored frozen in 50% glycerol in PBS. FITC anti-γ chain or anti-γ chain conjugate (Dakopatts, Copenhagen, Denmark) was used for the final staining step.

*Purification of virus antigen.* The cells of one Roux bottle of CMV-infected human fibroblasts were harvested by trypsinization and washed once in PBS. The cells were suspended in 1 ml TE buffer (0.01 M-tris-HCl, pH 8.5 plus 1 mM-EDTA) containing 2% NP40 (non-ionogenic detergent; Shell, Hamburg, West Germany). Cells were disrupted by sonication in an ice bath for 10 x 3 s (Microtip, Branson Sonifier, Danbury, Conn., U.S.A.) and cellular debris was removed by centrifugation (3000 g for 6 min). For further purification of the supernatant, a solution of dextran T 10 (Pharmacia, Uppsala, Sweden) was applied (Perdue et al. 1974) which was precentrifuged (3 h at 200000 g) to remove precipitates. The supernatant was layered on to 10 ml of 30% dextran T 10 and ultracentrifuged for 1 h at 180000 g at 4 °C (Beckman SW41 rotor). The sediment was resuspended in 0.5 ml of carbonate buffer (0.05 M, pH 9) by brief sonication as described above. The protein concentration was determined according to the method of Lowry et al. (1951).

*Activation of peroxidase.* The activation was carried out as described by Wilson & Nakane (1978). Four mg horse-radish peroxidase (type IV, Sigma, St. Louis, Mo., U.S.A.) were dissolved in 1 ml distilled water and 0.2 ml 1 M-NaIO₄ was added. The mixture was incubated at 20 °C for 20 min. The activated peroxidase was then dialysed against 1 mM-sodium acetate buffer (pH 4.4) overnight at 4 °C.
Enzyme-labelled antigen

Fig. 1. The individual steps of the ELA IgM test. (a) The plate is coated with anti-IgM immuno-globulin. (b) Serum is added and IgM is selectively bound to the plate. (c) The specificity of the IgM antibody is tested by adding peroxidase-labelled antigen (AG, antigen; p, peroxidase).

Peroxidase labelling of virus antigen. To 0.5 ml of purified CMV antigen (about 1 mg protein) in carbonate buffer, 0.05 M, pH 9, 0.25 ml (0.8 mg) of activated peroxidase was added and the mixture incubated for 2 h at 4 °C. The conjugate was then stored at −70 °C or was lyophilized in the presence of 5% calf serum.

Anti-IgM coating of the plates. Flexible PVC flat-bottom microtitre plates (Cooke Engineering, Alexandria, U.S.A.) were coated with 100 µl per well of anti-IgM immunoglobulin (protein concentration 10 mg/ml; rabbit origin; Dakopatts) at a dilution of 1:500 in PBS, pH 7.2, plus 1 mg/ml NaN3. After 24 h incubation at room temperature in a moist chamber, 10% calf serum was added, and they were again incubated at room temperature for 24 h. The plates were sealed with tape and could be stored at 4 °C for at least 6 months without loss of antibody activity.

Fractionation of the labelled material. In order to characterize the labelled antigen further, it was centrifuged in a linear density gradient prepared with Urografin (Schering, Berlin, Germany) with a density range of 1.1 to 1.4 g/ml at 20 °C for 1 h at 180,000 g in a Beckman SW41 rotor. The gradient was fractionated from the bottom of the tube in 1 ml volumes. Additionally, 1 ml of the labelled antigen was chromatographed through a Sephacryl S-300 (Pharmacia) column (2 × 80 cm; elution buffer: PBS; flow rate: 6 ml/m/cm²/h; fraction size: 3 ml). The fractions obtained by column chromatography or by density gradient centrifugation were tested in the ELA IgM assay against negative and positive control sera. Moreover, the activity of overall peroxidase was tested by adding 1 µl of each sample to 100 µl o-phenylenediamine substrate (Merck, Darmstadt, West Germany) as described below.

Detection of IgM antibodies to CMV using ELA (ELA IgM test). The individual steps of the ELA IgM test are shown in Fig. 1. The sealing tape was removed and the plates washed three times for 1 min with PBS containing 0.05% Tween 20 (Serva, Heidelberg, West Germany). The serum specimens were diluted 10⁻², 10⁻³ and 10⁻⁴ in PBS and added to appropriate wells in 50 µl amounts. After incubation for 1.5 h at room temperature, the serum was washed away as described above and the ELA was added at optimal dilution (usually 1:100) in PBS containing 0.5% Tween and 5% calf serum. After incubation at
room temperature for 1.5 h the plates were washed again and to each well 50 μl of 0-
phenylenediamine (10 mg/ml in 0.1 M-phosphate buffer, pH 6, containing 0.01% H2O2) were added. After 5 min 100 μl of 2 m-H2SO4 were added to stop the enzyme activity. Test plates were read at 492 nm in an 8 channel automatic photometer (Flow Laboratories, Bonn, West Germany), absorbance values exceeding that of the arithmetic mean (x = 0.140) of the negative serum pool (n = 636) by three standard deviations (3 s = 0.1, x + 3 s = 0.24) were considered to be positive (confidence limits 99.8%; 0.1% false positives).

RESULTS

Optimal test conditions

The individual steps of the ELA IgM are shown in Fig. 1. Several parameters of the test procedure were varied to obtain a specific and sensitive test. As shown in Fig. 2(a), the optimal incubation time of the serum specimens was investigated. The absorbance at 492 nm of the positive control divided by the absorbance at 492 nm of the negative control serum (see Methods) reached a maximum (A492 positive/A492 negative = 22) using an incubation period of about 1.5 h. In a similar experiment (Fig. 2b) an incubation time of 2 h for the ELA, diluted 1:100, seemed to be sufficient.

Composition of the labelled antigen

On fractionation of the ELA by Sephacryl S-300 chromatography two peaks were obtained, one eluting with the void volume which contained the ELA activity and a second one, which probably represented unbound peroxidase (Fig. 3a). For further characterization of the ELA, the material was subjected to density gradient centrifugation (Fig. 3b). Only a very low specific activity was associated with the visible nucleocapsid band in the middle of the gradient (arrow), most of the specific activity being found near the top.

Improved conditions for the preparation and composition of the ELA were examined. As shown in Table 1, several parameters such as concentration of the detergent, the pH at which the cells were lysed and the amount of calf serum and Tween in the final product were tested. Optimum results (A492 positive control serum/A492 negative control serum = 21.5) were obtained with antigens prepared from cells sonicated at pH 8 in the presence of 2%
Enzyme-labelled antigen

Fig. 3. Fractionation of the ELA by (a) column chromatography and (b) density gradient centrifugation. The antigen-bound peroxidase activity in the ELA IgM test (A) and the total peroxidase activity (B) were determined in each fraction. $A_{492} < 0.1 = -; 0.1 \text{ to } 0.4 = (+); 0.4 \text{ to } 0.9 = +; 0.9 \text{ to } 1.5 = ++; > 1.5 = +++$.
Table 1. Testing of several parameters for an optimal composition of the ELA giving maximum values for the fraction $A_{492}$ positive/$A_{492}$ negative

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP40 concentration of cell lysate</td>
<td>1%</td>
</tr>
<tr>
<td>pH of cell lysate</td>
<td>7</td>
</tr>
<tr>
<td>Calf serum in ELA</td>
<td>1%</td>
</tr>
<tr>
<td>Tween 20 in ELA</td>
<td>0.05%</td>
</tr>
<tr>
<td>$A_{492}$ pos./$A_{492}$ neg.</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Fig. 4. Mixtures of 1% serum (containing an IgG antibody titre to CMV of 10000 by indirect immunofluorescence) and different percentages of rheumatoid factor (RF) serum were tested in the ELA assay. $A_{492}$ is plotted against RF concentration.

NP40. The addition of 5% calf serum and 0.5% Tween to the ELA reduced background reactions with the negative serum, thus increasing the fraction $A_{492}$ positive/$A_{492}$ negative serum. It should be mentioned that the effectiveness of the calf serum in reducing the background varied from batch to batch.

Non-specific reactions

To evaluate the possible role of rheumatoid factor IgM in producing false positive results (Fraser et al. 1971; Reimer et al. 1975; Schmitz et al. 1977), 42 sera containing rheumatoid factor as shown by strong agglutination in the latex agglutination test (Behring, Marburg, West Germany) were tested for IgM antibodies to CMV. All 42 sera had IgG antibody titres to CMV of >128 as shown by indirect immunofluorescence. Therefore, all sera reacted positively at a dilution of 1:32, if CMV IgM antibodies were detected by indirect immunofluorescence. In contrast, all sera remained completely negative ($A_{492}$ < 0.05) when assayed in the ELA IgM test. Moreover, false positive reactions could not be found in the ELA IgM test (Fig. 4) when an artificial mixture of a serum with only IgG antibodies to CMV (titre $10^4$ by indirect immunofluorescence), with a serum containing large quantities of rheumatoid factor IgM, was applied.

As has already been discussed, sera with antinuclear antibodies of the IgM type produced false positive results in an indirect enzyme-immunoassay for CMV IgM antibodies (Schmitz et al. 1977). Three sera with IgM antibodies to nuclear material were therefore tested in the ELA IgM assay at a dilution of 1:100. Surprisingly all sera remained completely
Enzyme-labelled antigen

Table 2. Correlation between IgM antibody titres to CMV determined by indirect immunofluorescence and by the ELA IgM test*

<table>
<thead>
<tr>
<th>Titre in ELA IgM test</th>
<th>Titre obtained by indirect immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000000</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>100000</td>
<td>&gt; 64</td>
</tr>
<tr>
<td>1000</td>
<td>64</td>
</tr>
<tr>
<td>100</td>
<td>&gt; 32</td>
</tr>
<tr>
<td>50</td>
<td>&gt; 32</td>
</tr>
<tr>
<td>&lt; 50</td>
<td>&lt; 32</td>
</tr>
</tbody>
</table>

* \( r_s = +0.951; P < 0.0005. \)

Fig. 5. The regression line (\( \log y = -0.66 + 1.72 \log x \)) was calculated from the data of Table 2 (\( y < x, \text{ if } x < 8.13 \)).

negative \( (A_{492} < 0.1) \), indicating that the ELA had been freed of nuclear material during the purification process.

Early sera of patients with infectious mononucleosis occasionally produced low positive reactions in the ELA IgM antibody assay. Similar immunofluorescence observations on the cross-reactivity of the IgM antibody to Epstein-Barr virus have been known for many years (Schmitz et al. 1972).

Sensitivity of the method

The correlation between the titres obtained by the ELA IgM assay and by indirect immunofluorescence are shown in Table 2. 636 sera were negative with both methods \( (A_{492} < 0.24; \text{ see Methods}) \). Fifty-six sera of 20 patients with acute CMV infections showed positive reactions in the ELA test, while two of these sera, which were derived from neonates, remained negative using the indirect immunofluorescence method. Both sera had IgG antibody titres to CMV of 1.024 by indirect immunofluorescence. After separation of the IgM by sucrose gradient ultracentrifugation, the IgM fractions were positive using indirect immunofluorescence testing. It is likely, therefore, that this phenomenon is due to blocking of the IgM antibody binding by high concentrations of specific IgG antibody.

The sensitivity of antibody detection by the ELA IgM test does not seem to be much better than indirect immunofluorescence. With high concentrations of specific IgM antibody the ELA IgM test produces titres exceeding those of indirect immunofluorescence by 10- or even 100-fold. However, such titre increase cannot be obtained with low specific IgM
antibody concentrations. From the mathematical calculation of the regression line (Fig. 5) obtained from the data of Table 2 it can be concluded that the immunofluorescence test would be even more sensitive than the ELA IgM test, if serum dilutions of less than 1:8 could be investigated (titre in indirect immunofluorescence: $x = 8$, equals ELA IgM titre $y = 7.8$).

**DISCUSSION**

During our investigations the virus subunits or precursors, which are present in infected cells in large quantities (Schmitz *et al.* 1980) were most efficiently labelled by peroxidase. The virus particles seem to be labelled less efficiently, which might be explained by a reduced number of accessible amino groups on the virions compared to the virus subunits. Our experiments have further shown that the elimination of unbound peroxidase from the ELA does not significantly reduce the background reactions in our ELA IgM test. Obviously, most of the unreacted peroxidase is eliminated during the final washing procedure. Similar observations have been reported for immunohistological staining experiments (Avrameas & Ternynck, 1971).

As was to be expected, interaction between rheumatoid factors and virus-specific IgG did not occur with the ELA IgM assay, since IgG was effectively eliminated before the antigen was added. Moreover, preformed IgG–IgM complexes, which might be present in serum specimens of rheumatoid factor patients did not give rise to false positive results in the ELA IgM test, although 42 serum specimens containing both rheumatoid factors and IgG antibodies to CMV were tested. On the other hand, rheumatoid factor IgM is efficiently bound to the plastic plates coated with anti-μ chain globulin. During our experiments a strong reactivity of these IgM antibodies to peroxidase-labelled human or animal IgG was observed. The high affinity of rheumatoid factor IgM for aggregated IgG, which is formed during peroxidase labelling (Avrameas & Ternynck, 1971; Wilson & Nakane, 1978) might account for this reactivity. The alternative method using unlabelled antigen and peroxidase-labelled antibody (Duermeyer & Van der Veen, 1978; Gerlich & Lüer, 1979) might, therefore, produce false positive results even with sera containing only low levels of rheumatoid factor IgM. In our experiments, before the application of ELA, this non-specific reaction of rheumatoid factor IgM could be reduced by using peroxidase-labelled F(ab')₂ antibody fragments or by diluting the labelled antibody in aggregated IgG, but the low background values obtained with directly labelled antigen could not be obtained. In contrast to the enzyme labelling such an interfering aggregation of IgG apparently does not occur during ¹²⁵I labelling of antibodies (Flehmig *et al.* 1979). Using indirect immunofluorescence or enzyme-immunoassay techniques, unspecific binding of immunoglobulins to virus antigens can be observed especially with serum specimens at low dilutions (Schmitz *et al.* 1977; Schmitz, 1978). This phenomenon partially depends on the purity of the antigen, but a certain amount of human IgG might adsorb even to highly purified antigens fixed to solid phase plates (Forghani & Schmidt, 1979). In contrast to indirect techniques, the ELA IgM test, using a reverse sequence of staining steps, will not detect traces of immunoglobulin, as ELA can be applied in the presence of human or animal serum in order to prevent or block any further adsorption. Thus a very low background reactivity can be found with negative sera. In addition, blocking of IgM antibodies by IgG, which seems to occur preferentially in newborn sera, will no longer play a role in the ELA IgM test. A further advantage is that ELA seems to be free from immunologically active nuclear material. This is possibly due to the purification process, which is of great importance even with antigens used in indirect enzyme-immunoassays (Schmitz *et al.* 1977; Forghani & Schmidt, 1979). The very high yield of ELA (about 100 ml
final dilution per Roux bottle) now makes the application of optimally purified antigens more economic.

From the antibody levels obtained with the ELA IgM test a high sensitivity might be falsely deduced. However, on critical mathematical analysis, the ELA test does not detect more sera with a low specific IgM antibody level. This phenomenon can be explained by the fact that in a serum specimen containing only a low proportion of specific to total IgM, relatively few specific antibodies are bound to the anti-IgM-coated plates, while most of them are washed away. In contrast, using indirect techniques, more specific IgM antibodies will bind to the antigen-coated plates. Nevertheless, from our mathematical deduction it can be concluded that the sensitivity of the immunofluorescence test would exceed that of the ELA IgM test only with dilutions of less than 1:8. Such dilutions cannot be applied with both tests without considerable loss of specificity. These theoretical considerations suggest that the ELA IgM test is especially suitable for the detection of specific IgM (or IgA) antibodies where the proportion of specific total immunoglobulin is high, while a similar assay for specific IgG antibodies, using anti-\(\gamma\) chain-coated plates, would be highly insensitive.

Compared to the indirect enzyme or indirect immunofluorescence methods, the ELA IgM antibody assay shows increased specificity and comparable sensitivity. It is very easy to perform and due to the efficient labelling it uses only very low concentrations of antigen. The high reproducibility of the method was confirmed in seven different European countries in March 1979 using a set of eight reference sera with and without IgM antibodies to CMV.

Therefore enzyme labelling of further virus antigens might help to improve the detection of various IgM antibodies.

We would like to thank the members of the European Group for Rapid Laboratory Viral Diagnostics for cooperation especially Dr U. Krech for supplying the reference sera and R. Geister for statistical analysis.

REFERENCES


(Received 31 January 1980)