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Specific and non-specific serological markers in the screening for congenital CMV infection

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SUMMARY

IgM antibodies specific for cytomegalovirus (CMV) were demonstrated in 15 (2·6%) of 575 umbilical cord sera obtained from newborns in Kuwait. Some 93% and 50% of these CMV-IgM positive cord sera displayed markedly raised (more than normal mean + 2 S.D.) content of total IgM and IgA respectively. In contrast, only 0·2 and 1·8% of the CMV-IgM negative cord sera had elevated total IgM and IgA, respectively. Rheumatoid factor (RF) was demonstrable, at concentrations of 30 IU/ml or more, in 67% of the CMV-IgM positive as compared with 3·2% of the CMV-IgM negative sera whereas interferon alpha was found in the serum of only one of these infants. These results indicate that raised total immunoglobulin, in particular IgM, concentrations and the detection of RF in cord blood are useful non-specific markers for the identification of congenital CMV infection.

INTRODUCTION

Congenital cytomegalovirus (CMV) infections are generally diagnosed by virus isolation from urine obtained from babies during the first 2 weeks of life and/or by the demonstration of specific CMV IgM antibodies in umbilical cord sera (Griffiths *et al.* 1982; Griffiths, 1984; Stagno *et al.* 1985). More recently, virus has been isolated in cell culture and early antigens detected by immunofluorescence within 48 h using monoclonal antibody (Griffiths *et al.* 1984; Alpert *et al.* 1985; Paya, Wold & Smith, 1987). CMV infection, as defined by virus excretion, is probably the most common of all congenital virus infection whose incidence ranges from 0·5 to 3·4% in different populations (Stagno *et al.* 1977, 1983). A previous serologic survey showed the frequency in Kuwait to be at least 1·8% (Al-Nakib *et al.* 1985).

With the advent of antiviral chemotherapy and the possibility that congenital CMV infection could be amenable to treatment in the future, it is imperative that simple and reliable markers of congenital CMV infections are identified in order that they can be used to screen a large number of babies and identify those at risk who could then be treated as early as possible after birth. In the present study we have attempted to identify congenital CMV infections by detecting specific (CMV-IgM) and non-specific (total IgM, IgA, rheumatoid factor and interferon) markers

in umbilical cord sera, to obtain information about possible methods that could be used for wide-scale screening and identification of babies who may have had CMV infection *in utero*.

MATERIALS AND METHODS

Serum specimens

Five hundred and seventy-five cord blood together with corresponding maternal and neonatal blood were randomly collected from the Sabah Maternity Hospital. Kuwait. Sera were separated and stored in aliquots at −20 °C.

Determination of total IgM and IgA

Total IgM, and IgA in the cord and maternal blood were determined by laser nephelometry (LN) (Behringweke, Marburg/Lahn, West Germany) and single radial immunodiffusion (SRID) (S-Partigen, Behringweke). The procedures followed in the above assays were those recommended by the manufacturers. Mean values, given by the manufacturer, for total IgM and IgA in cord sera by SRID and LN were 15 mg/dl, 0·3 mg/dl and 25 mg/dl, 0·5 mg/dl, respectively.

Rheumatoid factor (RF) assay

Cord sera were tested for the presence of RF by latex agglutination (Reumagen AR, Biokit, Spain) as described by the manufacturer. The test was calibrated by the manufacturer to give a positive reaction for the presence of RF at concentrations of at least 30 IU/ml. All sera that were positive for CMV-IgM assays were absorbed with heat aggregated IgG as described by Krishna *et al.* (1980) to remove RF, and subsequently retested for CMV-specific IgM.

Enzyme immunoassays for the determination of CMV-specific IgM

Two enzyme-linked immunosorbent assays (ELISA), both based on the principle of reaction of IgM antibodies with solid phase-bound antigen, were used. Kits for one of these assays (Enzygnost) were purchased from Behringweke, West Germany and the other ELISA, hereinafter referred to as ELISA-1, was developed in our laboratory utilizing partially purified CMV prepared according to the method of Schmitz, Von Deimling & Flehmig (1980). Tests using Enzygnost were performed according to instructions by the manufacturer. For screening purposes employing ELISA-1, sera were diluted 1 in 40 and tested using microplates coated with CMV or its control antigen. Test sera were incubated with the antigen for 1 h at 37 °C, washed and reincubated for 1 h at 37 °C with goat anti-human IgM alkaline phosphatase conjugate (Behringwerke). Plates were washed and the substrate, *P*-nitrophenyl phosphate, was added. Following incubation for 45 min at room temperature, the enzymatic reaction was stopped by the addition of 0·1 N NaOH and results were read using a Titertek multiscan (Flow Laboratories. Irvine, Scotland). A specimen was considered positive for CMV-specific IgM if its mean optical density was > 0·2 at 405 nm. This cut off value (identical to that of Enzygnost), was taken as twice the highest O.D. value of the negative serum pool obtained in the presence of the antigen and was predetermined by repeated testing of pools known CMV-IgM positive, negative and single reference sera. Reference

positive and negative sera were included as controls in all assays. Umbilical cord sera that were positive for CMV-IgM in screening assays were further titrated in dilutions of 1 in 40 to 1 in 1280 and the results were evaluated as described above.

Testing for rubella and toxoplasma specific IgM

In order to further ensure the specificity the CMV-IgM ELISA, all sera that were positive by this assay were also tested by toxoplasma IgM immunofluorescence test and by rubella IgM ELISA. The latter test (Rubella Enzygnost, Behringwerke) utilizes the same principle as the CMV-IgM ELISA employed and was therefore particularly well suited for specificity checking.

Interferon determinations

Umbilical cord sera that were found to contain CMV-specific IgM by ELISA and a number of CMV-IgM negative sera were tested for the presence of interferon-alpha and interferon-gamma using commercially available radioimmunoassay kits (Abbott Laboratories, West Germany and Celltech Ltd, England, respectively). The tests were performed as described by the manufacturers, except that incubation times were extended to 24 h in order to increase the sensitivity of the tests, which then reproducibly detected interferon (IFN) at concentrations of 5–10 IU/ml.

RESULTS

Screening for CMV-IgM antibodies

With ELISA-1, 19 (3·3%) of 575 cord sera and corresponding neonatal sera were found to give a positive reaction at a dilution of 1 in 40 or higher compared with only 8 (1·4%) sera detected as positive by the Enzygnost assay. All these eight specimens were also positive by ELISA-1. Enzygnost was negative in 7 of the ELISA-1 positive umbilical cord sera but further Enzygnost testing of the corresponding neonatal sera gave positive results in 4 of these 7 cases (Table 1).

All 15 CMV-IgM positive sera by ELISA-1 were found to be negative in the toxoplasma and rubella IgM tests.

False positivity due to rheumatoid factor

The latex agglutination test, indicating the presence of rheumatoid factor (RF), was positive in 20 out of 575 cases (3·5%). Fourteen of these cases were observed among the 19 specimens that were positive for CMV-IgM. Following absorption with heat aggregated IgG to remove RF, four were negative for CMV IgM and were therefore considered to be false positives. Thus, as seen in Table 1, out of the original 575 sera 15 (2·6%) contained specific CMV-IgM.

Total immunoglobulin levels and rheumatoid factor (RF) in cord sera

The mean total IgM and IgA levels were clearly raised in the CMV-IgM positive cord sera (mean 69 and 0·56 mg/dl, respectively) when compared with CMV-IgM negative cord sera (mean 17 and 0·26 mg/dl, respectively) (Table 2). The IgA and the IgM levels were in all cases higher in sera from the mothers than in the

Table 1. *True CMV-IgM positive cord sera* following RF removal*

Specimens	ELISA-I positives/total (%)	Enzygnost positives/total (%)
Cord sera	15/575 (2·6)	8/575 (1·4)
Neonatal sera	15/575 (2·6)	12/575 (2·1)

* At dilutions of 1 in 40 or higher.

Table 2. *Levels of total immunoglobulins as determined by SRID in CMV-IgM-positive and negative umblical cord sera*

CMV-IgM determined by ELISA-I	Immuno-globulin	No.	Mean value total immuno-globulins	No. with values exceeding normal	
				mean + 1 S.D.	mean + 2 S.D.
Positive	IgM	15	69 mg/dl	14	14
Negative	IgM	469	17 mg/ld	3	1
Positive	IgA	12	0·56 mg/dl	9	6
Negative	1gA	221	0·26 mg/dl	17	4

corresponding cord sera. When only marked increases in immunoglobulin concentrations, e.g. more than 2 S.D. above total means, were considered (Table 3), it was evident that CMV-IgM positivity was consistently associated with significant increase in IgM (93%) but less well correlated with raised total IgA (50%). Some 67% (10 of 15) of the CMV-IgM positive cord sera as compared with only 1·3% of the CMV-IgM in negative cord sera were positive for RF indicating an excellent correlation between CMV-IgM positivity and the presence of RF (Table 3).

Comparison between SRID and laser nephelometry (LN)

As seen in Table 4 there was a rather poor correlation between results obtained with SRID and LN regarding detection of raised levels of IgM, and similar results were obtained for IgA. This was primarily due to the fact that exceedingly high values were obtained in the LN assay in several cases, apparently due to lysis of red cells. Thus the false positivity rate seemed to be much higher with LN than with SRID. Also falsely negative results appeared to be more often encountered with LN than with SRID. This was supported by the finding that high IgM values (> mean + 2 S.D.) were found in 12/13 (92%) of CMV positive cases by SRID but in only 9/13 (69%) with LN.

Interferon in cord sera

All sera that were positive for CMV-IgM by ELISA were tested for the presence of IFN-alpha and IFN-gamma. As seen in Table 3, one of the 15 CMV-IgM positive sera contained demonstrable IFN-alpha, whereas none of 66 tested CMV-IgM negative sera were positive in this assay. In the IFN-alpha positive case, the IFN level was 60 IU/ml in the cord serum and 140 IU/ml in the maternal blood sample. None of the sera tested (15 CMV-positive and 20 CMV-negative cord sera)

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Table 3. *Frequency of elevated concentrations of total immunoglobulins* and rheumatoid factor in CMV-IgM ELISA-positive and negative cord sera*

	Number/total CMV-IgM positive (%)	Number/total CMV-IgM negative (%)
Raised† IgM (> 41 mg/dl)	14/15 (93)	1/469 (0·2)
Raised† IgA (> 0·58 mg/dl)	6/12 (50)	4/221 (1·8)
RF positives	10/15 (67)	10/560 (1·8)
IFN-alpha positives	1/15 (6·7)	0/66 (0)
IFN-gamma positives	0/15 (0)	0/20 (0)

* As determined by SRID.
† Raised is defined as a value greater than the mean + 2 standard deviations.

Table 4. *Correlation between results obtained with SRID and laser nephelometry (LN) for detection of total IgM*

	SRID	
	Positive*	Negative
LN positive*	9 (9)	15 (0)
LN negative	7 (3)	447 (1)

* Concentration higher than mean + 2 S.D. Figures in parenthesis refer to the number of CMV-IgM positive sera. Two CMV-IgM positive sera that were tested by SRID only are not included in the figure. Both of these sera contained 81 mg/dl of IgM.

contained demonstrable IFN-gamma. In the IFN-alpha positive case, the IFN level was 60 IU/ml in the cord serum and 140 IU/ml in the maternal blood sample.

DISCUSSION

In a previous study we were able to demonstrate a significant association between the detection of a congenital CMV infection and the elevation of total IgM (> 16·4 mg/100 ml) in cord blood (Al-Nakib *et al.* 1985). In that study, we used a simple, rapid (5 min) latex agglutination test calibrated to give a positive test of the concentration of total IgM in the cord blood was > 16·4 mg/100 ml.

A prerequisite for any method to be employed for general screening would be that it should be not only sensitive and specific, but also simple, rapid and inexpensive. In this study, we compared two methods for immunoglobulin determination namely single radial immunodiffusion (SRID) and laser nephelometry (LN). The latter method although amenable to automation and hence suitable for large-scale screening, unfortunately gave a higher proportion of false positives and higher mean levels of immunoglobulins than SRID, probably because of partial haemolysis in some cord sera. This source of error made LN less well suited for the screening of cord sera. Thus in this study using a single radial immunodiffusion procedure, we were able to show that 93 and 50% of the ELISA CMV-IgM positive cord sera had elevated total IgM and IgA compared with only 0·2 and 1·8% of the ELISA CMV-IgM negative cord sera, respectively.

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Furthermore, 67% of the ELISA CMV-IgM positive cord sera, compared with 1·3% of the ELISA CMV-IgM negative sera had RF at concentrations of at least 30 IU/ml.

Since virus isolation was not attempted, a proportion of congenitally infected babies might have been missed. However, since infected babies who have no demonstrable CMV-IgM antibodies generally have a better prognosis, the identification of these cases may not be absolutely necessary (Stagno *et al.* 1977; Griffiths *et al.* 1982).

Lebon *et al.* (1985) found the presence of serum interferon to be a good indicator of a congenital rubella infection. Only one (6·7%) of 15 CMV-IgM positive cord sera in this study had this marker. Therefore, the presence of interferon in the cord sera does not appear to be a useful marker for congenital CMV infection.

The specifity of our CMV ELISA (ELISA-1) was confirmed by the fact that the majority of the sera (80%) were confirmed positive using a commercially available ELISA (Enzygnost), that following RF absorption with heat aggregated IgG, the ELISA was still positive and that none of the CMV-IgM positive sera were positive when tested in the rubella indirect ELISA IgM test, which had the same principal as our test, or the toxoplasma IF IgM test. Therefore, the data presented in this and in our previous study (Al-Nakib *et al.* 1985) show that elevation of total IgM, IgA and/or the presence of RF in cord sera are useful non-specific markers for identifying large numbers of infants who may have had a CMV infection *in utero*. Sera or urine samples from these infants may then be submitted to specialized laboratories for confirmation of congenital CMV infection by demonstrating the presence of virus in urine during the first few weeks of life. Indeed, simple, rapid and inexpensive screening of large number of infants who may be at risk of congenital CMV infection may prove to be essential in the future should antiviral chemotherapy for CMV becomes available. Recent data suggest that ganciclovir or DHPG (Collaborative DHPG Treatment Study Group, 1986; Erice *et al.* 1987) is effective against CMV infections in man, although clearly large scale double blind placebo controlled trials are needed to demonstrate its safety and efficacy unequivocally. Early treatment of infants identified as having CMV infection *in utero* most probably will result in better prognosis.

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