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Specific serum immunoglobulin D, detected by antibody capture enzyme-linked immunosorbent assay (ELISA), in cytomegalovirus infection

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SUMMARY

An antibody capture enzyme-linked immunosorbent assay (ELISA) was developed for the detection of immunoglobulin D (IgD) antibodies to cytomegalovirus (CMV) in sera from blood donors and various groups of patients infected with CMV. This method has previously been found especially valuable in detecting specific antibodies of the IgM, IgE, IgA and IgG class in patients with CMV infection. Specific CMV IgD antibodies were found in 37% of CMV seropositive blood donors and in 47 (88%) of the 53 patients investigated, including bone marrow transplant and renal allograft transplant patients, patients with CMV mononucleosis, neonates with CMV infection and AIDS patients with CMV infection. The highest IgD reactivity was found in patients having either a primary post-transplant CMV infection or CMV mononucleosis. The IgD reactivity in patients with AIDS and in neonates was low. It was also found that in the acute phase of CMV infection the development of CMV antibodies of the IgD class was similar to the development of antibodies of the other classes. The maintenance of IgD activity in some patients together with the presence of CMV IgD antibodies in a great proportion of the blood donors indicates that the development of CMV IgD antibodies resembles that of the IgG class. Determination of specific IgD antibodies offered no advantage over determination of specific antibodies of the IgM, IgE and IgA classes in the diagnosis of CMV infection.

Keywords CMV IgD antibody capture ELISA

INTRODUCTION

Immunoglobulin D (IgD) was first described by Rowe and Fahey (1965). IgD is a major receptor antibody on B lymphocytes in human peripheral blood together with immunoglobulin M (IgM) (Van Boxel *et al.*, 1972), and recent studies have indicated a possible role of IgD in the modulation of antibody responses (Xue *et al.*, 1984; Coico *et al.*, 1985; Jacobsen *et al.*, 1985). This immunoglobulin also seems to play a role in humoral immunity (Rowe, Crabbé & Turner, 1968), but investigation has been difficult because of the low levels of IgD usually present in serum. Furthermore, IgD is very susceptible to proteolytic degradation. Nevertheless, elevated IgD levels and specific reacting IgD antibodies have been detected in different patient categories (Buckley & Trayer, 1972; Lertora, Gomez-Perez & Leslie, 1975; Luster *et al.*, 1976; Luster, Leslie & Bardana, 1976). As most patients investigated had chronic antigenemia it consequently was proposed that circulating IgD antibodies are directed against chronically present antigens (Leslie, Correa & Holmes, 1975). Some investigators have also been able to detect specific IgD in sera from patients with acute disorders like acute viral infections (Bahna, Heiner & Horowitz, 1984; Salonen *et al.*, 1985), suggesting an active role of IgD following primary antigenic challenge.

The purpose of this study was to determine cytomegalovirus (CMV) specific IgD in sera from various groups of patients infected with CMV using an antibody capture enzyme-linked immunosorbent assay (ELISA) with enzyme-labelled CMV antigen. Similar antibody-capture ELISAs have been described as reliable and sensitive methods for detecting CMV antibodies of the IgM, immunoglobulin A (IgA), immunoglobulin E (IgE), and immunoglobulin G (IgG) classes (Schmitz, Von Deimling & Flehmig, 1980; van Loon *et al.*, 1985; Nielsen, Sørensen & Andersen 1988). Antibody capture ELISA does not appear to have been applied in the detection of specific IgD. Furthermore, we found it of interest to compare the development of CMV antibodies of the IgD class with the development of CMV antibodies of the other immunoglobulin classes during CMV

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infection in an attempt to find a possible role of IgD in diagnosing CMV infection, and to elucidate a possible role of serum IgD following antigenic challenge with CMV.

MATERIALS AND METHODS

Sera

A total of 116 sera were studied from nine bone marrow transplant (BMT) patients, nine renal allograft transplant (RAT) patients, 10 neonates with CMV infection, 17 patients with CMV mononucleosis and eight patients with acquired immunodeficiency syndrome (AIDS). Eleven of the transplant patients were classified as having a primary CMV infection indicated by seroconversion in CMV complement fixing (CF) antibodies and in CMV specific antibodies of the IgM, IgA, IgE and IgG class (Nielsen *et al.*, 1988). Seven transplant patients with pre-existing CMV CF antibodies and a significant rise (four-fold or greater) during the early post-transplant period were classified as having a recurrent CMV infection (reactivation or reinfection). Two to four sera were studied from each patient. Twenty-two sera were studied from the 10 neonates from whom CMV had been isolated within the first 4 months of life. The patients with CMV mononucleosis were previously healthy persons in whom CMV IgM and CMV CF antibodies had been found. At least two blood specimens taken at an interval of at least a month were available. Clinically, the patients had a self-limiting disease characterized by prolonged fever, malaise, and lymphocytosis. Most had evidence of liver involvement with or without vomiting or diarrhoea. Seven of the patients had a significant rise in CF antibodies, and the rest exhibited a rise to a CF titre of 32 or more. Infectious agents other than CMV were excluded, and all patients reacted negatively in the mononucleosis agglutination test for heterophile agglutinins (Cellognost-Mononucleosis, Behringwerke AG). Two to three sera per patient were studied. The eight patients with AIDS all had signs of CMV infection during their last year of life, as CMV was isolated either from urine, saliva, or sputum, and all sera examined had CF titres between 8 and ≥ 256. Three to six sera covering the last year of life were studied.

In addition, single serum samples from 75 healthy blood donors were examined. Forty-five of the donors were negative for CMV CF and neutralizing (NT) antibodies during a two-year period. Thirty of the donors were CF seropositive with serum CF titres between 4 and 128.

Antigens

Antigens were prepared as described previously (Nielsen *et al.*, 1986). Briefly, CMV antigen was prepared from nuclei of human embryonal lung (HEL) cells infected with the Ad 169 strain of human CMV. When a nearly complete cytopathic effect was observed, cells were trypsin dispersed and suspended in hypotonic buffer at 0°C for 30 min. Nonidet p-40 was added and the cells were disrupted in a tight-fitting Dounce homogenizer. When the nuclei were largely freed from the cytoplasm the suspension was sedimented by centrifugation and the pellet was suspended in cold phosphate-buffered saline (PBS). The nuclei were separated from nuclear debris by centrifugation (1 200 *g* for 15 min at 4°C) through a layer of 17% Ficoll 400 in PBS. The pellet was suspended in 0·01 M sodium carbonate buffer (pH 9·5). The suspension was sonicated and clarified by centrifugation (5 000 *g* for 10 min). The supernatant was used as antigen for enzyme labelling. Control antigen was prepared from nuclei of uninfected HEL cells by the same procedure.

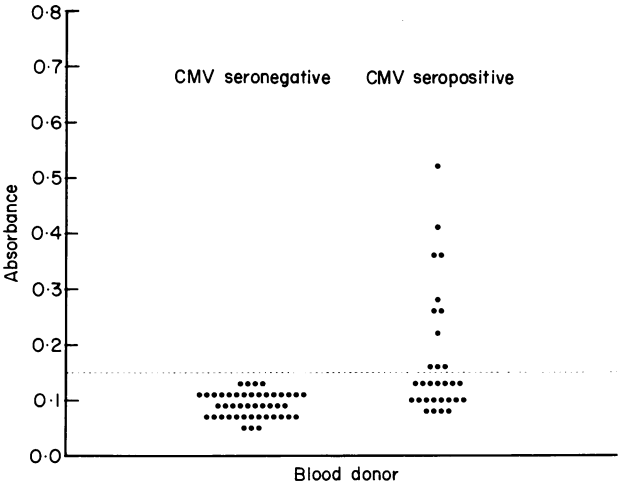

Fig. 1. Detection of CMV specific IgD antibody in sera from CMV CF and NT negative ($n=45$) and CF positive ($n=30$) blood donors. The cut-off level (. . . .) is indicated.

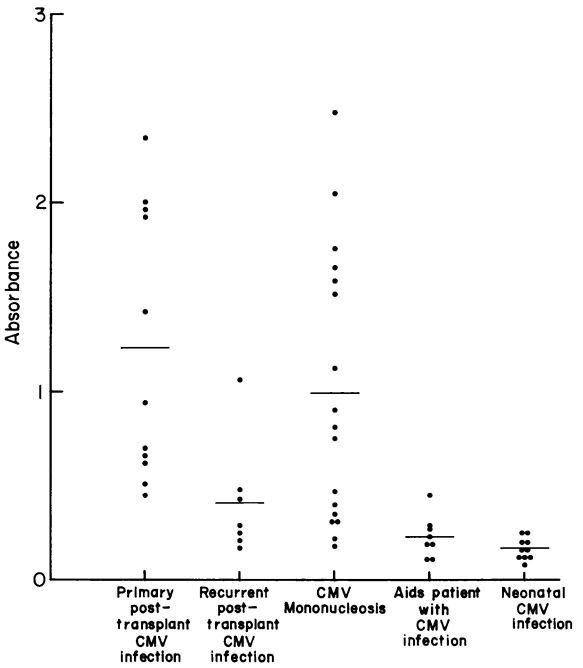

Fig. 2. The highest reactivity of CMV specific IgD antibody in sera from 11 patients with primary post-transplant CMV infection, nine patients with recurrent post-transplant CMV infection, 17 patients with CMV mononucleosis, and 10 neonates and eight AIDS patients with CMV infection. The cutoff level (. . . .) is indicated.

Antigen labelling procedure

The CMV and control antigens were labelled with horseradish peroxidase by the periodate method, essentially as described earlier (Nielsen *et al.*, 1986) and used as conjugates.

IgD antibody capture ELISA

Detection of CMV IgD antibodies by the antibody-capture ELISA was carried out essentially as described previously for detection of CMV IgG antibodies (Nielsen *et al.*, 1986). Briefly, individual wells of ELISA plates (Immunoplate I, NUNC, Copenhagen, Denmark) received 100 μl of anti-IgD (rabbit immunoglobulins to human monoclonal IgD (δ-chain); Dakopatts, Copenhagen, Denmark) diluted 1:400 in carbonate buffer (pH 9·6). The plates were sealed and incubated for 1 h at 37°C. The plates were then washed four times in PBS containing 0·05% Tween 20 at pH 7·4 (PBS-T), and 100 μl of serum diluted 1:10 in PBS-T with 2% fetal calf serum (FCS) was added to each of four wells. The plates were sealed and incubated for 2 h at 37°C. After the plates were washed four times in PBS-T, two wells received 50 μl of CMV conjugate diluted 1:500 in PBS-T with 2% FCS and 13 μg/ml of unlabelled control antigen. The other two wells received control conjugate prepared in the same way. The plates were incubated overnight at 4°C. After the plates were washed in PBS-T, 50 μl of peroxidase substrate was added to each well. The substrate solution was prepared immediately before use by dissolving 2 mg of orthophenylenediamine/ml of 0·1 M citrate phosphate buffer (pH 5·0), followed by the addition of 0·75 μl of 35% H_2O_2/ml substrate. After 8 min of incubation in the dark at room temperature, the reaction was stopped by adding 100 μl of 1 M H_2SO_4 to each well. The A_{490} was determined by spectrophotometry (Immunoreader NJ-2000, NUNC), with A_{620} as the reference. Wells containing 100 μl of H_2SO_4 were used as blanks. The CMV specific IgD reactivity of each sample was calculated as the mean absorbance of the duplicate test with CMV conjugate. By checker-board titration with positive and negative sera the optimal dilutions of anti-IgD, sera and conjugates were determined. The cut-off level (A = 0·15) was determined by testing CF and NT antibody negative sera from blood donors (mean ± 3 s.d., see results). CMV antibodies of the IgM, IgA, IgE and IgG classes were detected by similar antibody capture ELISAs as previously described (Nielsen *et al.*, 1988), and the cut-offs for these were 0·2, 0·07, 0·10 and 0·06, respectively. The ELISA results are shown either as specific reactivity (absorbance) or as positive/negative (P/N) values. P/N-values were calculated as the specific reactivity divided by the cut-off value for the immunoglobulin class studied.

RESULTS

Specific IgD in sera from blood donors

To discriminate between IgD CMV positive and negative sera, the minimum positive absorbance value or cut-off value was determined as the mean absorbance plus three standard deviations (s.d.) for the 45 sera from the CMV seronegative blood donors when tested with the CMV conjugate. Reactions above this cut-off (absorbance 0·15) level were considered positive, indicating the presence of CMV IgD antibodies. The results of testing sera from these 45 donors and from the 30 seropositive blood donors are shown in Fig. 1. Specific IgD antibodies were found in 11 (37%) of the 30 CF-positive donor samples. There was no significant difference concerning age and sex between the donors with detectable CMV IgD in their sera and the donors without detectable CMV IgD in their sera. No correlation was found between the presence of CMV IgD antibodies and the level of CF antibodies against CMV in the seropositive blood donors, as the mean CF antibody titre in the two groups of IgD positive and IgD negative blood donors was 27 and 23 respectively.

Specificity of IgD antibody capture ELISA

To evaluate the specificity of the CMV IgD antibody capture ELISA, all sera were tested against both CMV and control conjugate. Only one of the 191 sera from blood donors and patients had an IgD absorbance above 0·15 (cut-off) when tested

Table 1. CMV class-specific antibody activity in serial samples from patients with CMV mononucleosis

Months after onset of symptoms	Number of sera	Mean CF titre	Mean P/N ratio (s.d.)				
			IgM	IgE	IgA	IgG	IgD
<2	17	43	6·7 (2·6)	13·6 (6·0)	6·5 (4·5)	6·8 (4·9)	6·6 (4·7)
1–2	17	67	3·0 (2·3)	7·5 (5·3)	2·3 (1·6)	4·6 (2·9)	3·1 (1·6)
>2	9	43	1·1 (1·8)	1·3 (2·1)	1·7 (3·0)	3·7 (2·6)	1·5 (0·7)

Table 2. CMV class-specific antibody activity in serial samples from patients with primary post-transplant CMV infection

Months after transplantation	Number of sera	Mean CF titre	Mean P/N ratio (s.d.)				
			IgM	IgE	IgA	IgG	IgD
<1	9	<4	0·4 (0·2)	0·2 (0·1)	0·3 (0·2)	0·5 (0·3)	0·4 (0·1)
2–4*	9	97	7·9 (2·3)	15·0 (6·9)	6·8 (6·3)	15·0 (8·1)	6·3 (4·8)
4,5–10	8	184	3·7 (2·0)	5·8 (4·8)	1·9 (1·2)	14·4 (7·7)	4·1 (3·9)
12–32	7	173	2·0 (0·9)	5·1 (4·6)	1·7 (1·2)	14·5 (8·2)	4·9 (3·6)

* Corresponding with the time just after seroconversion in CF antibodies.

with the control conjugate. This sera with an absorbance of 0·27 was from a patient with a primary post-transplant CMV infection and it also reacted highly positive with CMV conjugate (absorbance 2·34).

The reproducibility of the test was examined by comparing the values of a CMV antibody positive serum obtained in seven different assays. The coefficient of variation was 6·9%, with a mean absorbance of 0·72. In addition, a within-run test of one CMV antibody positive serum was performed in 20 replicates on one plate. The coefficient of variation was 4·8%, with a mean absorbance of 0·97.

Specific IgD in various groups of CMV infected patients

Figure 2 shows the absorbance values for the serum sample with the highest specific IgD reactivity from each patient. All patients with a post-transplant CMV infection or with CMV mononucleosis had detectable CMV antibodies of the IgD class. The group with primary post-transplant infection showed a higher reactivity (mean 1·23, s.d. 0·71) than the group with recurrent infection (mean 0·41, s.d. 0·31). By way of comparison, the reactivity in sera from AIDS patients and neonates was low.

Kinetics of specific IgD during primary CMV infection

The development of specific antibodies of the five immunoglobulin classes during CMV infection, expressed as mean P/N ratios of serial serum samples from 17 patients with CMV mononucleosis and nine patients with primary post-transplant CMV infection, is shown in Tables 1 and 2. Concerning the patients with CMV mononucleosis, a high response was seen in all classes in the first sera taken within the first month after the onset of symptoms. Thereafter, reactivity declined considerably within 4 months. In the acute phase of CMV infection the development of specific IgD antibodies in the transplanted patients followed the development of specific antibodies of the other classes. However, in contrast to the steadily decreasing activity in the IgM, IgE, and IgA class, a maintained activity was seen in the IgD class, resembling the pattern of activity seen in the IgG class.

DISCUSSION

Specific antibodies of the IgD class have been detected by various immunological methods, including an immunodiffusion technique (Heiner & Rose, 1970; Lertora *et al.*, 1975), an indirect immunofluorescent technique (Luster *et al.*, 1976) and an indirect enzyme immuno assay (EIA) (Salonen *et al.*, 1985). In this study specific IgD antibodies to CMV were detected by the antibody capture ELISA. This method using enzyme-labelled CMV antigen, first described by Schmitz *et al.* (1980), has been found especially valuable in the diagnosis of CMV infection, as it is possible to detect specific antibodies of the IgM, IgE, IgA and IgG classes (van Loon *et al.*, 1985; Nielsen *et al.*, 1988). We found that it was also possible to develop a sensitive IgD antibody capture ELISA and to detect specific IgD antibodies to CMV, in spite of the relatively low level of IgD found in sera from normal individuals. In contrast to the indirect test for detection of antibodies, the antibody capture ELISA using enzyme-labelled antigen gives no problems with false reactions caused by rheumatoid factors or competitive antibodies (Salonen *et al.*, 1980; van Loon *et al.*, 1981), the latter being of great importance in the detection of antibodies of the minor immunoglobulin classes.

To diminish the influence of test variation on the results, only one CMV conjugate, one control conjugate, and one catching antibody were used in the experiments described here, albeit that several CMV conjugates and two different capture antibodies to IgD were tested (data not shown). The different preparations of CMV conjugate varied in sensitivity and specificity. Non-specific reactions, i.e. reactivity in sera against cellular antigens in the CMV conjugate, were previously particularly common in the IgM test (Nielsen *et al.*, 1987a; 1988), but by adding to the conjugates unlabelled control antigen harvested from non-infected HEL cell nuclei, these reactions could be depressed. We found that non-specific reactions in the IgD test were of minor importance, as only one of the 191 sera investigated in this study reacted against control conjugate.

Specific IgD antibodies were detected in 37% of the seropositive blood donors, although on the whole the reactivity was low. These donors did not have CMV antibodies of the IgM, IgE, and IgA class. However, the presence of specific IgD antibodies in a great proportion of the blood donors is interesting. Thus, antibodies of the IgD class may persist longer than antibodies of the IgA, IgM, and IgE classes, as continuous antigen production may take place in some donors (Leslie *et al.*, 1975). In a study on rubella antibodies, Salonen *et al.* (1985) also found rubella IgD antibodies in some specimens from blood donors. Of the 53 patients investigated we found specific CMV IgD antibodies in 47 (88%), with the highest reactivity being seen in the groups of patients having either CMV mononucleosis or a primary post-transplant CMV infection. The kinetics of the specific IgD class were the same as the kinetics of specific antibodies of the other classes, with an initial high response and a later decline. The decline occurred faster in patients with CMV mononucleosis than in transplanted patients, which is probably due to the immunosuppression of the transplanted patients. However, the maintenance of the IgD activity in some patients, together with the presence of specific IgD antibodies in 37% of the blood donors, indicates that the kinetics of CMV IgD antibodies is much like the kinetics of CMV IgG antibodies.

The serologic response of specific IgD in neonates was weak, similarly to what has previously been described for the CMV IgA response (Nielsen *et al.*, 1988). Studies on the ontogeny of human serum IgD have also shown that neonates possess almost no detectable levels of circulating IgD (Leslie *et al.*, 1975), and therefore it is reasonable to suggest that the low IgD response is a result of a general low capability of neonates to synthesize humoral IgD.

AIDS patients were included in this study because CMV infection is an important opportunistic infection in these patients and because increased levels of IgD were observed during infection with human immunodeficiency viruses (Mizuma *et al.*, 1987). We did not find elevated CMV specific IgD antibodies in AIDS patients which may explain this observation.

Determination of CMV antibodies of the IgD class could be used to diagnose CMV infection. However, the presence of CMV IgD antibodies in a proportion of seropositive blood donors makes the diagnosis of recent CMV infection difficult. Furthermore, the IgD test offers no obvious advantage over the IgM, IgE and IgA test as the background was relatively high,

causing low P/N ratios (Tables 1 and 2). In this respect the IgE test is best, and determination of CMV IgE class antibodies is advantageous both in diagnosing primary CMV infection (van Loon *et al.*, 1985) and in diagnosing infection with CMV in early infancy (Nielsen *et al.*, 1987b; Nielsen *et al.*, 1988).

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