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Quantitative measurement of cytomegalovirus-specific IgG and IgM antibodies in relation to cytomegalovirus antigenaemia and disease activity in kidney recipients with an active cytomegalovirus infection

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SUMMARY

In a longitudinal investigation 103 kidney recipients were studied with respect to the development of cytomegalovirus (CMV) specific antibodies of the IgG and IgM class, in relation to the detection of CMV antigenaemia (immediate early antigen, IEA), in weekly obtained blood samples during the first 3 months after transplantation. In 15 out of 49 (31%) seronegative patients a primary infection occurred, which was characterized by a quick rise in IgM antibody followed by a slower production of IgG antibody, high maximum numbers of IEA^+ cells, and a CMV syndrome in 11 patients. In 35 out of 54 (65%) seropositive patients a secondary infection occurred. After a post-operative fall in the IgG antibody, which was also found in patients without an active infection and which was accompanied by a similar drop in serum albumin and IgG, a second dip in IgG antibody was found 6 days before the first IEA^+ leucocyte appeared in the blood. This was followed by a significant increase, indicative of an active immune response in consequence of the infection, 18 days later. In 31 of these 35 patients an IgM response was found. This could be ascribed to the presence of rheumatoid factor activity in 20 of them. Eight patients who showed a transient rise in IgG antibody between the two dips could be distinguished from the remaining ones by a lower maximum number of IEA^+ cells and less severe disease symptoms. The described results suggest that (i) an adequate humoral immune system may prevent symptomatic CMV disease in secondary infections; and (ii) CMV-specific antibodies may be removed from the circulation by antigens present in infected tissues before CMV antigenaemia becomes detectable.

Keywords cytomegalovirus antigenaemia test antibody response

INTRODUCTION

Active cytomegalovirus (CMV) infection is a major cause of morbidity and mortality in renal transplant recipients (Glenn, 1981; Rubin & Colvin, 1986). This may result from reactivation of latent virus in the recipient due to the immunosuppressive treatment or from transmission of the virus from the graft if this is obtained from a seropositive donor, in primary as well as in secondary infection (Grundy *et al.*, 1988).

The incidence and severity of CMV disease is higher in seronegative recipients than in patients with a pre-transplant immunity against CMV (Pass, Griffiths & August, 1983; Smiley *et al.*, 1985; Rubin & Colvin, 1986). Therefore, knowledge of the serostatus of the kidney recipient is important.

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Diagnosis of an active CMV infection is hampered by the fact that clinical symptoms are non-specific and may not distinguish it from other infections or from rejection. The diagnosis of a CMV infection therefore is dependent on laboratory parameters, i.e. isolation of virus from clinical samples such as blood, urine and saliva, and the detection of a specific antibody response.

Recently, a rapid and reliable method for the early diagnosis of an active CMV infection has been developed in our laboratory (van der Bij *et al.*, 1988a, b). With the aid of monoclonal antibodies against the major 67–72 immediate early antigen (IEA) protein of CMV-infected cells produced in our laboratory, this antigen could be detected in peripheral blood leucocytes, even before clinical symptoms of the infection became manifest. A relation was found between the level of CMV antigenaemia and clinical disease activity (van der Bij *et al.*, 1989).

This finding and the above-mentioned influence of pre-transplant immunity on the clinical manifestations of the infection prompted us to measure in detail the antibody response against CMV in these patients in relation to CMV antigenaemia and severity of the disease in order to gain insight into the relevance of the humoral immune response for the course of this virus infection. To this end an ELISA was developed for the quantitative assessment of CMV-specific antibodies, which was based on the method described by Middeldorp, Jongsma & The (1984). This method was applied for the sequential quantification of both IgG and IgM antibodies.

MATERIALS AND METHODS

Patients

In the period between April 1986 and December 1987 103 patients receiving a cadaveric renal allograft could be longitudinally studied with respect to their humoral immune response against CMV. Serum was obtained on the day of transplantation and at weekly intervals thereafter for at least 3 months. After this period blood was obtained at irregular intervals.

The initial immunosuppression regimen consisted of cyclosporin A, combined with low-dose prednisolone in case of a second transplant or in the presence of anti-HLA antibodies. In five patients this regimen was preceded by an induction course of monoclonal antibodies (OKT3) with azathioprine, in one patient initial immunosuppression consisted of anti-thymocyte globulin.

Allograft rejection was defined as an increase in serum creatinine of $\geqslant 20\%$, combined with one or more of the following: a swollen, painful kidney; fever; decreased urinary output; and salt retention or findings compatible with rejection in a renal biopsy. Anti-rejection therapy in patients on cyclosporin A-based regimens consisted of a bolus of 1000 mg methylprednisolone given intravenously on 3 consecutive days; in patients on an azathioprine-based immunosuppressive regimen rejection was treated with 200 mg oral prednisolone, in 3 weeks slowly tapered to the original prednisolone dose. Treatment with anti-thymocyte globulin was instituted after failure of first-line anti-rejection treatment. In case of documented vascular rejection six courses of plasmapheresis were given.

CMV syndrome was defined as the occurrence of unexplained fever for at least 3 days in combination with a laboratory diagnosis of active CMV infection, i.e. seroconversion or a significant increase in IgG antibody and/or detection of CMV antigenaemia or viraemia, and one of the following: arthralgia; leucopenia (WBC $< 3{\cdot}10$ exp 9/l), thrombocytopenia (platelets $< 100{\cdot}10$ exp 9/l), liver enzyme rise (ALAT > 50 U/l) or pneumonitis without other cause (Cheeseman *et al.*, 1979).

Detection of CMV antigenaemia (IEA test)

This test was performed exactly as described by van der Bij *et al.* (1988a,b). Briefly, leucocytes were isolated from EDTA-blood, obtained at weekly intervals after transplantation for 3 months, by dextran sedimentation and cytocentrifuged (Shandon) on microscopic slides. The presence of CMV-IEA in the granulocytes was detected by immunoperoxidase staining, using a mixture of two monoclonal antibodies directed against the major 67–72 kD IEA antigen, horseradish peroxidase labelled rabbit anti-mouse immunoglobulin antibodies (Dakopatts) and as substrate H_2O_2+AEC (amino-ethyl-carbazol). Results are expressed as the number of positive cells, i.e. cells showing nuclear staining, per 50 000 cells screened.

Preparation of CMV antigens for serology

Batches of CMV-LA (late antigens) were prepared exactly as described by Middeldorp *et al.* (1984) from fetal fibroblasts which were infected with AD169 (1 PFU/cell), harvested after 6–7 days, and extracted with an alkaline glycine buffer. A control antigen preparation was obtained from mock-infected fetal fibroblasts from the same batch.

Determination of CMV antibodies (CMV-ELISA)

Wells of microtitre plates (Greiner) were coated with 100 μl of a solution of CMV-LA antigens in 0·1 M carbonate buffer, pH 9·6. Optimal coating concentration of different batches was determined by block titrations, and always resulted in a coating concentration of 4–5 μg protein/well.

In each plate, wells coated with the control antigen preparation, used at the same protein concentration, were included to detect non-specific (IgM) binding in patients' sera.

Plates were left at 4°C for at least 48 h. After washing the plates (Titertek microplate washer) with wash buffer (0·3 M NaCl, 0·01 M Tris, pH 8·0, 0·05% Tween 20), the wells were filled with 100 μl of two-fold dilutions of the serum samples diluted in wash buffer + 1% BSA, starting with 1/100, and the plates were incubated at 37°C for 1 h. In each plate two rows were filled with dilutions of the standard serum. After washing the plates, the wells were filled with 100 μl of the appropriate dilution of horseradish peroxidase conjugated goat anti-human IgG or IgM (Kallestad) and incubated at 37°C for 45 min. After washing the plates the wells were filled with 100 μl substrate solution (20 mg OPD, orthophenylene diamine, in 100 ml phosphate buffer, pH 5·6, and 0·0045% H_2O_2 added just before use). After 15–20 min incubation at room temperature the reaction was stopped with 2 N H_2SO_4 and OD at 492 nm was read with a Titertek Multiscan. Results of patients' serum samples were calculated in relation to the standard serum using an on-line computer and a program developed in our department, based on log-logit transformation of the measurements (Rodbard & McClean, 1977). The results of positive IgM tests, which might be due to the presence of IgM-rheumatoid factors (RF), were always checked after absorption of the serum with a sufficient amount of pansorbin (Calbiochem) onto which heat-aggregated normal human IgG was coated. The pansorbin binding capacity was not saturated so that partial absorption of patients' IgG was still possible (resulting in about 50% reduction of the IgG antibody content), in view of the preferential binding of some RFs to patients' own IgG (Champsaur, Fattal-German & Arranhado, 1988; and unpublished observations). The quantity of pansorbin routinely used for absorption was calculated to be sufficient to remove all of the RF activity generally present. This was checked with a sensitive RF-ELISA and sera with known RF concentrations (kindly done by Miss Rina de Jong, Department of Immunochemistry).

Standard sera

Standards for CMV antibodies of the IgG and IgM class respectively were prepared from mixtures of different serum samples and put at 100 U.

For the IgG standard two volumes of serum from healthy controls with a high normal level of IgG antibody were mixed with one volume of a mixture of several patients' sera with high IgG antibody levels due to a secondary CMV infection. This mixture did not contain detectable IgM antibody.

For the IgM standard, two volumes of serum from a normal seronegative individual who showed no aspecific IgM binding to either the CMV-LA or the control FF antigen preparation were mixed with one volume of a mixture of several serum samples from patients with a primary CMV infection which were obtained before the IgG antibody reached undesirably high levels. Thus prepared the IgM standard contained 4 U IgG antibody. Both standards were kept frozen at −80°C in small aliquots.

Normal sera

Normal sera were obtained from healthy blood donors ($n=84$) and laboratory personnel and students ($n=27$) to determine the local incidence of CMV infection and the normal mean level and range of IgG and IgM antibody concentrations in seropositive individuals when using our quantitative ELISA assay.

Statistical analysis

Differences between groups of patients and normals were statistically analysed using Student's t-test, Wilcoxon's rank sum test, and χ^2 test. Correlation analysis was performed with the Spearman rank correlation test.

RESULTS

CMV-ELISA

Reproducibility of the ELISA results was investigated by duplicate and triplicate measurements of 92 randomly chosen serum samples containing IgG and/or IgM antibody. Intra-assay variability appeared to be negligible. Inter-assay variability was shown to be less than 15%. In view of this finding and the clinical data on active CMV infection of the patients an increase in IgG antibody concentration of 50% in relation to the previous value was considered to be conclusive for a secondary infection.

CMV antibodies in healthy controls

Results obtained with serum samples from healthy individuals, among them known seronegative and seropositive persons previously determined by immunofluorescence (The, Klein & Langenhuysen, 1974), showed that seronegatives always had less than 2 U IgG antibody in the quantitative ELISA. Therefore individuals with an IgG binding >2 U were considered seropositive. With respect to the IgM-ELISA it was found that although most normals, seropositive as well as seronegative, had IgM levels ≤ 1 U compared with the standard, some seronegative individuals showed weak aspecific IgM binding, which never exceeded 4 U, so an IgM antibody level of 5 U or more in a previously seronegative individual was considered to be decisive for a seroconversion.

The prevalence of CMV IgG antibody in our series was found to be 53% (59/111). The mean level of IgG antibody concentrations, which were log-normally distributed, in these 59 seropositive individuals was 17 U with a range of 4–72 U.

Table 1. Incidence of cytomegalovirus infection in kidney transplant recipients

Patient	n	Infection	%
Seronegative	49	15*	31
Seropositive	54	35†	65

* Immediate early antigen (IEA) test, $n=15$, IgM antibody test, $n=14$.

† IEA test, $n=31$, significant rise in IgG antibodies, $n=35$.

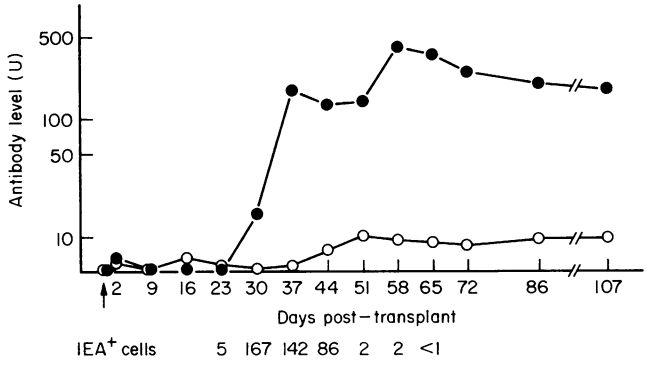

Fig. 1. Course of cytomegalovirus (CMV) specific antibodies in a seronegative patient with a primary CMV infection, in relation to CMV antigenaemia. ●, IgM; ○, IgG. IEA, immediate early antigen.

CMV antibodies in kidney transplant patients

CMV status before transplantation. Of the 103 patients studied, 49 were seronegative and 54 were seropositive. The incidence of previous CMV infections in this group therefore was comparable with that of the group of healthy controls.

Incidence of CMV infection after transplantation (Table 1). Of the 49 seronegative kidney recipients, 15 (31%) contracted a primary CMV-infection which was diagnosed by a positive IEA test at day 34 ± 14 post-transplant in all 15 and a subsequent humoral immune response, characterized by a quick rise of IgM antibody followed by a gradual increase of IgG antibody in 14 patients. Maximal values reached in the CMV-IEA test ranged from 5 to 356, with a median of 63. Eleven patients had a CMV syndrome. IgM antibody reached maximum values of 40– ≥ 700 U within 2–3 weeks and gradually declined thereafter. IgG antibodies were first detected 0–14 days after the IgM response and continued to rise during the period studied (a typical example is shown in Fig. 1).

In the group of 54 seropositive transplant patients 35 (65%) had a secondary CMV infection. Of these, 31 were diagnosed by both a positive IEA test (maximal values 1–334, median 7) and a significant increase in IgG antibody, while the remaining four repeatedly had negative IEA tests and only showed a significant rise in IgG antibody. Two patients had a very late infection, i.e. > 100 days after transplantation, and the exact date of their first positive IEA test could not be determined. The mean period between transplant and the first positive IEA test for the remaining 29 patients was 33 ± 13 days. A significant increase in IgG antibody followed at day 45 ± 10 after transplant.

Table 2. Concentration (%) of pre-transplant levels in 26 kidney recipients with and without cytomegalovirus (CMV) infection

		Time		
	n	8 days	4 weeks	8 weeks
Total protein	26	76±4	81±6	91±9
Albumin	26	72±5	80±9	88±12
IgG				
Non-infected	10	70±9	80±14	87±17
CMV infected	16	70±9	68±13	87±31
CMV antibodies (IgG)				
Non-infected	10	71±13	73±13	110±19
CMV infected	16	71±13	63±18	310±5000*

* Median 700.

Course of IgG antibody in seropositive patients after transplantation. The pre-transplant values of the 54 seropositive patients varied from 8 to 225 U with a mean level of 50±47. When the six patients with values >100 U were excluded, the mean level was 34±19 U, which was still significantly higher than that of the normals (Students' *t*-test: $P<0{\cdot}001$). After transplant the weekly obtained serum samples of each of the patients showed rather strong variations in antibody content. Further study showed that these were connected to particular events. Firstly, a post-operative dip of CMV antibody occurred at day 8±5 after transplant, both in the 19 individuals who did not get a secondary CMV infection (group 1) and in the 35 patients who did (group 2). When the post-transplant antibody values are expressed as a percentage of the pre-transplant concentration for each individual the minimal antibody concentration varied from 31 to 89% with a mean of 65±14 in group 1 and a mean of 66±15 in group 2. When serum albumin and total IgG levels were measured in 26 randomly chosen patients a similar dip was found (see Table 2). Secondly, after the post-operative dip antibody concentrations in the two groups of patients showed a different course. In group 1 the antibodies rose slowly until at 7 weeks the original level was reached.

In group 2, however, antibody levels showed further variations. In some patients a continuing decrease was found, while in others the antibodies either stabilized at this level or quickly increased to pre-transplant values followed by a second fall in antibody concentration (examples are shown in Fig. 2). The nadir of the second dip was found at day 27±7 and reached values that were significantly lower than those of patients of group 1 at this timepoint, i.e. 61% *versus* 74%, $P<0{\cdot}02$). This dip appeared to be connected with the first positive IEA test, clinical signs of a CMV infection, and a subsequent rise in IgG antibody in this order (see Table 3).

This connection was found to hold for individual patients: the period between the second ab dip and the first positive IEA-test ranged from −12 to +29 days with a mean of 8±10 (only three of 29 patients showed a positive IEA test before the antibody dip), and the period between the first positive IEA test and the significant increase in IgG antibody ranged from 0 to 19 days with a mean of 11±5.

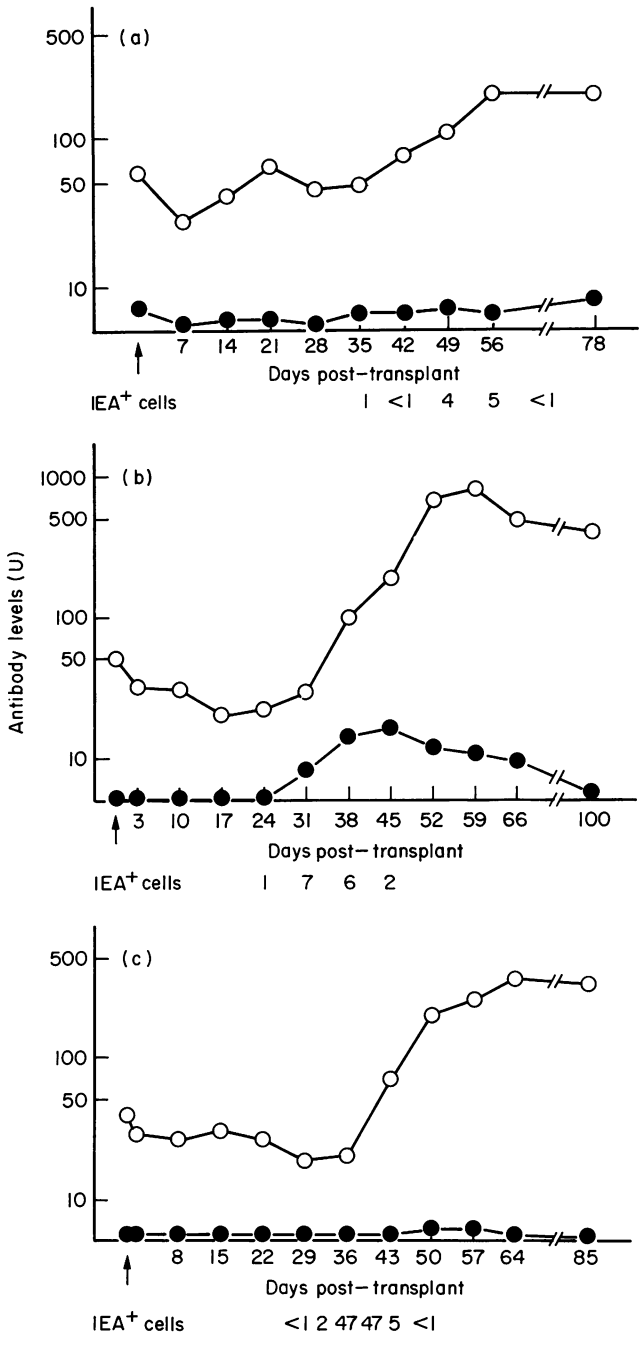

Fig. 2. Examples of different courses of CMV-specific antibodies in three (a, b, c) seropositive patients with active infection. ●, IgM; ○, IgG. IEA, immediate early antigen.

The possible connection of this decrease with the active CMV infection manifesting itself shortly thereafter was further investigated by determining the concentrations of total serum IgG and albumin in the same serum sample, and comparing them with the levels of albumin, IgG and CMV antibodies in patients without an active CMV infection (Table 2).

After the post-operative dip mentioned above, total proteins, albumin, IgG, and CMV antibody gradually increased to pre-transplant levels in patients without an active infection. On the contrary, in patients with an infection a decrease in total IgG

Table 3. Sequence of events related to active secondary CMV infection

	Timepoint (days)
Post-operative dip in CMV antibody level (IgG)	8±5
Second dip	27±7
First IEA-positive test	33±13
Significant rise in CMV antibody (IgG)	45±10

CMV, cytomegalovirus; IEA, immediate early antigen.

was found at the same time that the CMV antibody fall occurred. The mean serum IgG level at 4 weeks was significantly lower than that of patients without an infection ($P<0{\cdot}05$). However, there was no correlation between total serum IgG and CMV antibody concentrations in individual patients ($R_s=0{\cdot}18$). As mentioned above several patients ($n=8$) showed a quick rise in IgG antibody after the first, post-operative dip, which reached the pre-transplant level or a slightly higher one (see for example Fig. 2a). This group of patients showed lower maximum numbers of IEA-positive cells, i.e. 0–34 with a median of 1, than the remaining patients who showed maximum numbers of 0–386 with a median of 11 (Wilcoxon's rank sum test, $P<0{\cdot}05$). They also showed less disease activity, i.e. none of these eight patients had a CMV syndrome, whereas 10 of the remaining 25 had (two of whom died), but this difference did not reach statistical significance ($\chi^2=2{\cdot}85$).

IgM antibody response in secondary CMV infection. In all but four patients, IgM binding became positive at the same time or just before the IgG. Positivity ranged from 5 to 79 U. However, in most cases this could be ascribed to the presence of RF, i.e. absorption with heat-aggregated IgG removed all binding activity. In 11 patients a positive IgM binding was left, with values ranging from 5 to 25 U. No relation could be shown to the maximum of the IgG antibody response nor to the maximal value reached in the IEA test. However, a high IgM response was found in a patient whose IgG antibody response was considerably delayed and whose IEA-test reached very high values (334), comparable to those found in primary infections.

DISCUSSION

In this study an ELISA technique for the quantitative measurement of IgG and IgM anti-CMV antibody is described, which uses a CMV antigen mixture from late stage CMV-infected fetal fibroblasts harvested without the use of trypsin to prevent proteolytic degradation of membrane antigens (Middeldorp *et al.*, 1984). When sera of known seropositive individuals were tested in two-fold dilutions all samples produced the same slope when OD readings were plotted against the serum dilutions, suggesting that they had the same binding efficiency. Furthermore, quantitative calculations were found to be reproducible due to low interassay variations. Therefore we concluded that this ELISA could be used to compare quantitatively consecutive serum samples obtained from different individuals and employed it to investigate the course of CMV antibodies in 103 kidney recipients with and without an active infection.

The mean pre-transplant level of the seropositive patients ($n=54$) was significantly higher than the mean of healthy controls, which might indicate that these patients persistently express higher levels of CMV induced cellular antigens than normals do.

Fifty of the transplant patients had an active CMV infection; 15 of 49 seronegatives (31%) had a primary; and 35 of 54 (65%) seropositives a secondary infection. In all the patients with a primary infection except one (this patient did not produce any antibodies at all and had to be treated with Ganciclovir to cope with his CMV infection) the antibody developed according to the classical pattern, showing a sharp increase in IgM antibody followed by a gradual increase in IgG antibodies.

In the 35 patients with a secondary CMV infection, the IgG antibody levels showed some interesting fluctuations. Firstly, the dip at about 4 weeks, which was found to be related to an active CMV infection as it occurred just before CMV antigens were detected in the blood. At the same time a decrease in the total serum IgG was found in this group of patients. However, no correlation between these two parameters was found within individuals. Therefore, we conclude that the two decreases are independent events, which might have the same cause, i.e. both an active CMV infection and a decrease in serum IgG could be related to a stronger immunosuppressive regimen on account of more rejection episodes in the patients concerned, which indeed was the case in our patients' population. The dip in CMV antibody concentrations might be explained by a specific removal from the circulation through binding to CMV antigens already present in low concentrations in the circulation or in infected tissues. Binding to soluble antigens would result in the formation of immune complexes (IC). These might be detectable as soluble IC in the circulation (CIC). In preliminary experiments serum samples from 15 patients were tested for the presence of CIC with the use of three different techniques, among which the indirect granulocyte phagocytosis test, a technique which is particularly sensitive for CIC in antibody excess (van der Giessen & The, 1986). CIC could not be detected in any of the sera. Secondly, the quick rise of IgG antibody concentrations to pre-transplant levels after the first, post-operative dip in eight patients who could be distinguished from the others by a lower maximum number of IEA^+ cells in the circulation and less severe CMV disease. This suggests that an early (humoral) immune response can limit the extent of CMV antigenaemia, and consequently CMV disease, as these appear to be related (van der Bij *et al.*, 1989).

The antibody response consisted primarily of IgG, but a positive IgM binding was also found in most cases. However, this appeared to be caused by the presence of RF activity. Synthesis of RF in patients with infections is a common finding (Williams, 1977). Even in the seropositive patients in this study who did not get an active CMV infection, a transient increase in IgM binding due to the presence of RF in consecutive serum samples was frequently found. A true IgM antibody response was found only in 11 patients. This finding is in agreement with the results of others on the detection of IgM antibody in secondary infections (van Loon *et al.*, 1981; Rasmussen *et al.*, 1982; Pass *et al.*, 1983; Wreghitt, Gray & Chandler, 1986; Chou *et al.*, 1987; Nielsen, Sørensen & Andersen, 1988). Unfortunately, the CMV status of the organ donor was known only in 17 of the 35 patients: eleven patients received a kidney from a seropositive donor, and six from a seronegative donor. No

significant differences could be detected with respect to maximal numbers of IEA$^+$ cells, number of rejection episodes, or presence of RF activity. However, a tendency existed to a higher incidence of IgM antibody (4/11 *versus* 0/6) and symptomatic disease (5/11, two of whom died, *versus* 1/6) in the group of patients who received an allograft from a seropositive donor. This is in agreement with the findings of Betts & Schmidt (1981) and Grundy *et al.* (1988). We conclude that quantitative determinations of CMV antibodies together with a follow-up of IEA$^+$ blood cells can give good indications about a patient's capability to surmount a CMV infection.

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