Antibodies to cytomegalovirus in renal allograft recipients: correlation with isolation of virus

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SUMMARY   A cohort of 47 renal transplant recipients was studied prospectively for up to one year after transplantation. Cytomegalovirus (CMV) was isolated from 21 of the patients. The first time the virus was isolated seven patients were IgM positive, nine showed a significant rise in IgG titres, and 12 had a four-fold or greater rise in complement fixation titre. There was no significant difference in the time at which virus was first detected following transplantation between patients with primary CMV infection and those with reinfection or recurrent infection. In general, patients with primary infection shed virus consistently over long periods. Those with reinfection or recurrent infection shed virus intermittently or not at all. There were considerable differences between individual patients in the timing and pattern of the immune response. Taken overall, a four-fold rise detected by the complement fixation test correlated best with the onset of CMV shedding in primary infection. There was more variation in the pattern of antibody response in cases of reinfection or recurrent infection , with no single serological test correlating better than the others.

It is concluded that serology is of limited value in the detection of active CMV infection after renal transplantation.

Cytomegalovirus (CMV) infection is common in man: there is serological evidence of previous infection in about half the adult population of the United Kingdom.[1] CMV is usually, and perhaps always, latent following primary infection, which in healthy subjects is usually asymptomatic. CMV is, however, an important pathogen in immunosuppressed patients either as a cause of primary infection or following reactivation of latent virus.[2 3] Recipients of renal[4] or bone marrow[5] transplants, or patients infected with the human immunodeficiency virus (HIV) may become seriously ill and in some cases die of disseminated CMV infection. CMV infection may also have a clinically important role in graft failure following renal transplantation.[6–8]

Many different serological tests have been used to assess the immune response to CMV.[9 10] Many studies have investigated when CMV specific antibody becomes detectable in renal transplant recipients, and general correlations have been made using pooled information on groups of patients.[4 11] In only a few instances have prospective studies on renal transplant recipients been presented in sufficient detail to permit the correlation of serological results using a variety of tests with virus shedding. Particularly notable is the appearance of CMV specific IgM in both primary and reinfection or recurrent infection in renal transplant recipients.[12 13]

We correlated the serological response of renal allograft recipients with the timing of CMV shedding using conventional culture of urine and throat swabs. The complement fixation test and the Labsystems IgM and IgG indirect enzyme linked immunoassay (ELISA) were used to follow the appearance of CMV specific antibody in individual patients.

Material and methods

From March to December 1985 inclusive 52 patients underwent kidney transplantation at the Western Infirmary, Glasgow. Five patients underwent nephrectomy because of acute rejection within four weeks of transplantation and were not included in the study. The final group, therefore, comprised 47 patients (28 men and 19 women) with a mean age of 38 years (range 19–61). Forty patients were recipients of a first kidney while seven received a second. Four patients received donor kidneys from relatives, while the remaining 43 kidneys were taken after the donor's death.

All patients received a combination of prednisolone

and cyclosporine throughout the study. The prednisolone dose was 20 mg a day for the first three months, which was reduced to 15 mg a day for three months, followed by 12·5 mg a day for six months. The cyclosporine dose was 15 mg/kg/day for the first few days, with a reduction to around 5 mg/kg/day by three months, 4 mg/kg/day by six months, and 2–3 mg/kg/ day by one year. The cyclosporine dose was adjusted on the basis of whole blood concentrations measured by radioimmunoassay.

A throat swab and samples of urine and serum were taken from every patient immediately before surgery and subsequently at intervals of three to four weeks whenever possible.

Samples for culture were transported to the laboratory and inoculated with minimum delay on to semiconfluent monolayers of human embryo lung fibroblasts (Flow 2002 cells, Flow Laboratories, Hertfordshire, England) on 25 cm^2 tissue culture bottles (Nunc). Monolayers were overlaid with maintenance medium (Glasgow modified Eagle's medium supplemented with 5% fetal calf serum) and incubated for at least six to seven weeks at 37°C in an atmosphere of 5% carbon dioxide in air. Cultures were usually examined at weekly intervals for the development of any cytopathic effect. In some bottles the characteristic morphological changes of CMV were evident. Isolates were subcultured when the cytopathic effect was near confluent, and identified as CMV by their ability to grow on Flow 2002 cells but not on Vero cells or on baby hamster kidney cells, BHK 21/C13, and also by dot blot hybridisation with a CMV specific nucleic acid probe (Dolan, *et al*, unpublished observations).

SEROLOGY
Sera were stored at −20°C until tested. A standard complement fixation test was performed but starting at a serum dilution of 1/2. Glycine-extracted CMV cytopathic effect antigen and negative control antigen were purchased from Northumbria Biologicals Ltd, Cramlington, England. Guinea pig complement was supplied from Wellcome Laboratories, Beckenham, Kent.
Assays of CMV specific IgM and IgG were carried out strictly according to the manufacturer's protocol (Labsystems Ly, Pulttitie 8, Helsinki, Finland).
All sera positive or equivocal for CMV IgM were assayed for IgM rheumatoid factor by ELISA (Veitch J, *et al*, unpublished observations).
All CMV IgM positive sera were negative for heterophil agglutinins (Mercia Diagnostics, Surrey, England).

Active CMV infection in a given patient was identified either by isolation of the virus or a significant rise in antibody titre (more than four-fold rise

in complement fixation antibody, positive IgM or a more than 20 unit rise in IgG antibody). Patients were classified before transplantation as seronegative if the complement fixation titre was less than 2, and CMV infection in this group was regarded as primary. There was no evidence of CMV IgM before renal transplantation in any of the patients.

Results

Twenty two (47%) of the 47 patients in the group were seronegative for CMV before transplantation (average age 34, range 18–61) while 25 (53%) were seropositive (average age 41, range 21–58).

Primary CMV infection occurred in 11 (23%) of the patients studied and reinfection or recurrent infection in 20 (43%). Although in most cases virus was isolated within the first few months following transplantation (figs 1 and 2), one patient had serological evidence of primary CMV infection 60 weeks after transplantation. No other data on this patient were available.

CMV SHEDDING DETECTED BY CULTURE
Plaques were first noted after an average of 25 days in culture (range seven to 47 days). Of the 93 CMV positive samples originally identified by morphological appearance in tissue culture, two subsequently became contaminated and 22 (18 urine specimens and four throat swabs from 14 patients) failed to grow well enough to permit subculture and further identification.

Isolation of CMV from urine was more common during primary infection (44 of 108 or 40·7% of samples being positive) than during reinfection or recurrent infection when 39 of 222 (17·5%) of samples were positive (table). Virus isolation from throat swabs, though less common, showed a similar pattern with eight of 102 (7·8%) of samples being positive in primary infection and two of 204 (1%) being positive in reinfection or recurrent infection.

Primary infection (fig 1)
The earliest detection of CMV shedding was in a urine sample of case 3 taken four weeks after transplanta-

Table *CMV excretion during first year after renal transplantation*

	Primary infection	Secondary infection
Total number of urine specimens	108	222
Number of specimens from which virus was isolated	44 (9/10*)	39 (12/20*)
Total number of throat specimens	102	204
Number of specimens from which virus was isolated	8 (5/10*)	2 (2/20*)

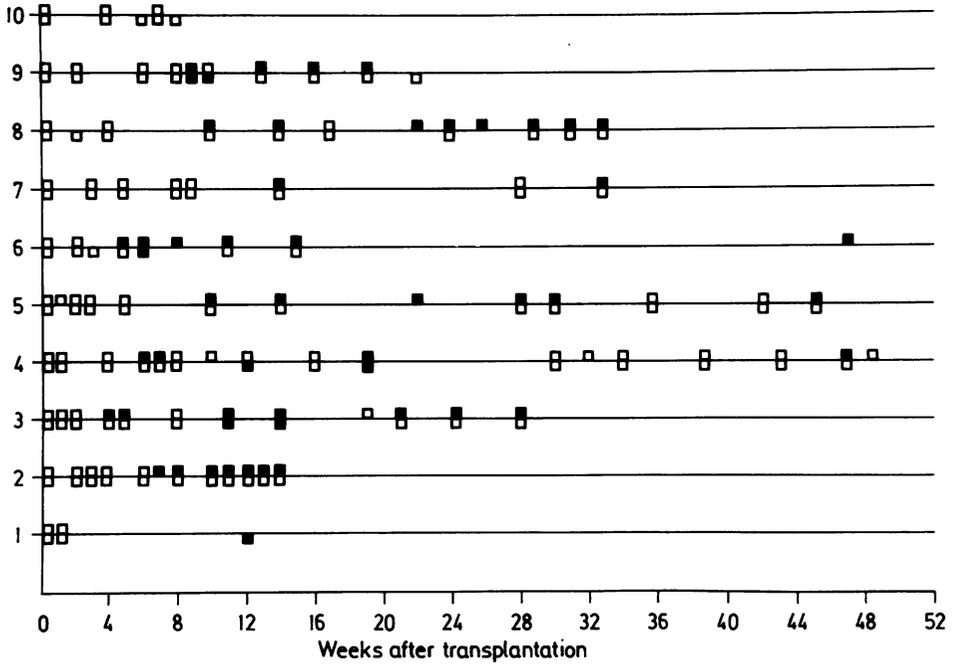*Number of patients from whom CMV was isolated/number of patients sampled.

Fig 1 Timing of isolation of virus from throat and urine specimens of 10 individual patients (cases) with primary CMV infection. Urine sample no virus isolated ⬜; urine sample virus isolated ⬛; throat swab no virus isolated ⊓; throat swab virus isolated ▼.
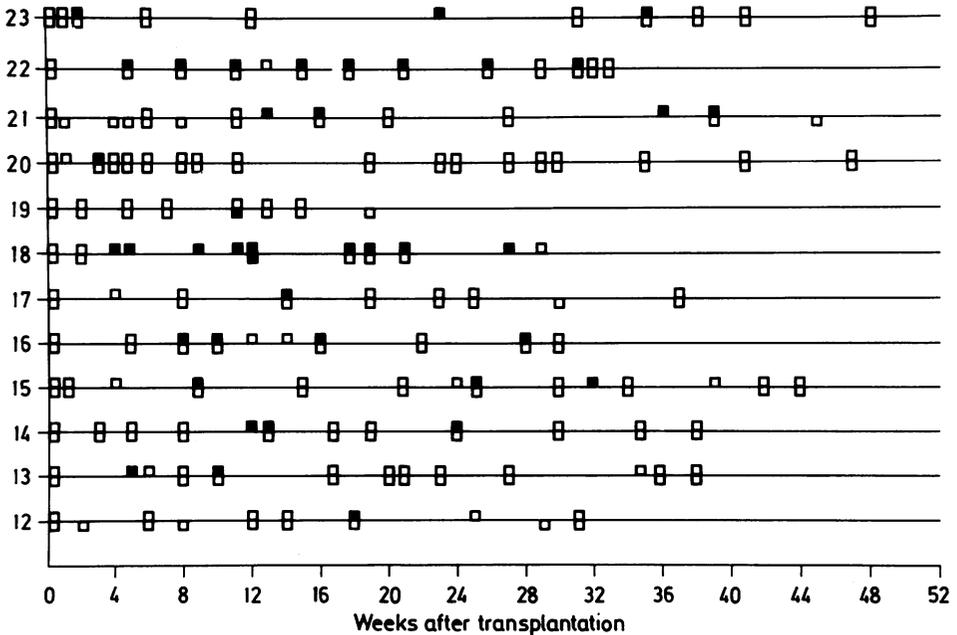

Fig 2 Timing of virus from throat and urine specimens of 12 patients (cases) with secondary CMV infection. Urine sample no virus isolated ⬜; urine sample virus isolated ⬛; throat swab no virus isolated ⊓; throat swab virus isolated ▼.

tion. Fourteen weeks after transplantation CMV was isolated from the urine of eight patients. In most of these patients virus was detected consistently over long periods of time though there was evidence of intermittent shedding in cases 4 and 8. Urine samples were not available beyond the 10th week following transplantation in the remaining two patients: case 1 became anuric 10 weeks after transplantation and case 10 had a bout of rejection and underwent nephrectomy at eight weeks. Five patients (cases 1, 3, 4, 6, 9) excreted virus from the throat which was first detected between six and 12 weeks after transplantation. None of 20 throat swabs taken after 19 weeks yielded virus, though CMV was detected in 16 of 25 urine samples taken at these times. Initial isolation of CMV from the throat was coincident (case 9) or subsequent (cases 3, 4, 6) but in no case preceded excretion of the virus in the urine.

Reinfection or recurrent infection (fig 2)
Of the 20 patients in this group, CMV was isolated from 12. Virus was first detected in a urine sample provided by case 23 two weeks after the transplant operation. By 14 weeks CMV had been isolated from nine other patients. The final patient to shed CMV (case 12) did so in a sample taken 18 weeks after transplantation. There was consistent isolation of virus from the urine of case 18. Ten patients shed CMV intermittently, the number of isolates usually ranging

from one to four a patient. CMV was isolated from the throat of case 18 just once 12 weeks after transplantation. In case 19 no urine isolates were obtained but CMV was detected in a throat swab taken 11 weeks after transplantation. CMV was not isolated from any of the other nine patients in the group.

CORRELATION OF CMV SPECIFIC IgM AND IgG WITH FIRST ISOLATION OF VIRUS
Primary infection (fig 3)
Four patients (cases 1, 5, 8, and 9) were IgM positive on or before the day when CMV was first isolated, and in three of these (cases 1, 5, and 9) IgM titres were positive before isolation of virus. One patient (case 2) had a titre of IgM in the equivocal range when CMV was first detected and throughout the period of observation. Three patients (cases 3, 4, and 6) were IgM negative when virus was first isolated but became positive one to four weeks later. Case 7 had an equivocal IgM response at the time of virus isolation; the next sample at 14 weeks was IgM negative.

Three patients (cases 1, 5, and 8) were IgG positive on or before the time of initial CMV isolation. Three patients (cases 2, 7, and 9) had IgG titres in the equivocal range when virus was first isolated. Case 2 remained equivocal; case 7 was found to be positive at 14 weeks after onset of virus isolation but no samples were available for the intervening period. In case 9 IgG became positive between seven and 10 weeks after
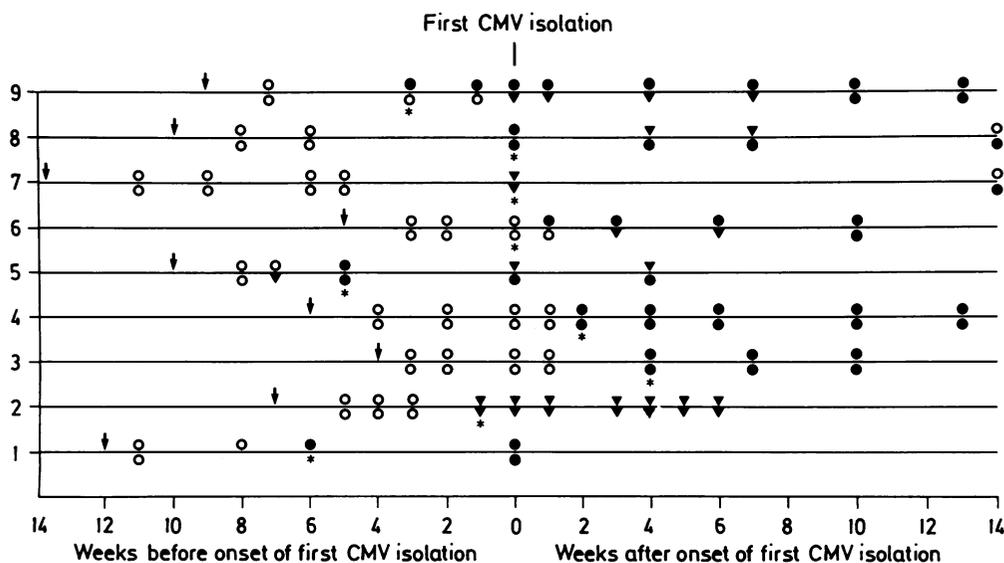
First CMV isolation

Fig 3 *Correlation of serological response to CMV with the first virus isolation in nine patients (cases) with primary CMV infection. Timing of transplantation ↓; time of > 4 × rise in complement fixation antibody *; negative for CMV IgM by ELISA ○ ; equivocal for CMV IgM by ELISA ▼ ; positive for CMV IgM by ELISA ● ; negative for CMV IgG by ELISA ○ ; equivocal for CMV IgG by ELISA ▼ ; positive for CMV IgG by ELISA ● .*

virus was first isolated. The three patients negative for IgG at the time of first CMV isolation (cases 3, 4, and 6) became positive between two and 10 weeks later.

Reinfection or recurrent infection (fig 4)

Of the 12 patients excreting CMV, three (cases 14, 17, and 19) were IgM positive on or before the time of first isolation of virus. In two (cases 14 and 19) the rise in IgM occurred at least four weeks before isolation of virus. Two patients (cases 15 and 22) had IgM titres in the equivocal range at the time of initial CMV isolation while five (cases 12, 16, 20, 21, and 23) were IgM negative. Three patients in this group (cases 12, 16, and 21) remained IgM negative throughout. No serological data were available on cases 13, 18, and 20 around the time of initial CMV isolation but from subsequent results case 20 must have been IgM negative at that time.

Six patients (cases 14–17, 19, and 21) had a significant increase in IgG titres at the time of initial CMV isolation. In four (cases 14, 16, 19, and 21) the rise in IgG occurred between two and seven weeks

before virus was first isolated. Of the remaining six patients, three (cases 20, 22, and 23) seroconverted within four weeks of initial virus isolation. In one (case 12) IgG was equivocal at the time of isolation. The data on two patients (cases 13 and 18) did not allow a correlation to be made of a rise in CMV IgG titre with the timing of first virus isolation.

CMV COMPLEMENT FIXATION ANTIBODY IN PRIMARY INFECTION (fig 3)

All 10 patients with primary CMV infection had a more than four-fold rise in complement fixation antibody. The timing of this can be related to first virus isolation: in three (cases 2, 5, and 9) the rise occurred at least one to five weeks beforehand; in two (cases 3 and 4) two to four weeks afterwards; and in four (cases 1, 6–8) this relation could not be determined precisely. The rise in complement fixation antibody was detected when CMV IgM was first positive in six patients (cases 1, 3, 4, 5, 8, and 9), was equivocal in two patients (cases 2 and 7), and negative in one (case 6). In five patients (cases 1, 3, 4, 5, and 8) the rise in complement fixation
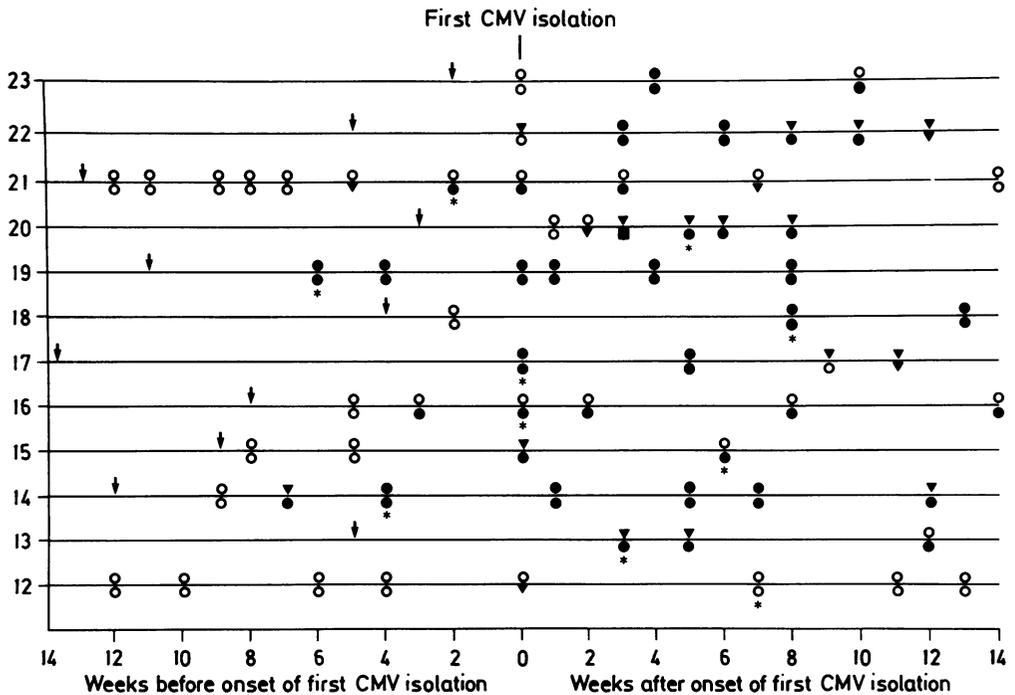

Fig 4 *Correlation of serological responses to CMV with the first virus isolation in 12 patients (cases) with secondary CMV infection. Timing of transplantation ↓ ; time of >4 × rise in complement fixation *; negative for CMV IgM by ELISA ○ ; equivocal for CMV IgM by ELISA ▼ ; positive for CMV IgM by ELISA ● ; negative for CMV IgG by ELISA ○ ; equivocal for CMV IgG by ELISA ▼ ; positive for CMV IgG by ELISA ● . In case 12 renal transplantion took place 18 weeks before virus was first isolated.*

antibody occurred when the CMV IgG was positive, in two (cases 2 and 7) when it was equivocal, and in two (cases 6 and 9) when it was negative.

In no case were CMV IgM or IgG titres positive before the four-fold rise in complement fixation antibody.

CMV COMPLEMENT FIXATION ANTIBODY IN
REINFECTION OR REACTIVATION (fig 4)

Ten of 12 patients shedding virus had a more than four-fold rise in complement fixation antibody: in three patients (cases 14, 19, and 21) this rise occurred two to six weeks before first isolation, in two (cases 16 and 17) at the same time, and in three (cases 12, 15, and 20) between five and seven weeks afterwards. In two patients (cases 13 and 18) it was not possible to relate the timing of initial virus isolation to that of the rise in complement fixation antibody.

In four patients (cases 14, 17–19) IgM was positive when the rise in complement fixation antibody was detected, but equivocal in two (cases 13 and 20), and negative in four (cases 12, 15, 16, and 21). A four-fold rise in complement fixation antibody was not found in two patients (cases 22 and 23), though they were positive for IgM.

In nine patients (cases 13–21) the rise in IgG titres had occurred at or before the rise in complement fixation antibody and clearly preceded the complement fixation antibody rise in four (cases 14–16, and 20). In case 12 there was no significant increase in IgG, though there was a four-fold rise in complement fixation antibody. The two patients (cases 22 and 23) who did not have a four-fold rise in complement fixation antibody had an increased IgG titre three and four weeks, respectively, after the first CMV isolation. With the exception of a serum sample taken from case 4, all samples had rheumatoid factor within the normal range (<800 U/ml).

Discussion

The presence of antibody to CMV was taken to indicate previous infection and to identify those patients at risk of reactivation of endogenous virus.[3][14] Following receipt of a renal allograft 31 of the 47 (66%) patients developed evidence of CMV infection as defined by CMV specific serology or virus isolation. Eleven (36%) of these were primary infections and 20 (64%) were reinfections or reactivations. In the latter, unless a CMV isolate is available from the renal allograft recipient immediately before transplant and restriction endonuclease analysis carried out, it is impossible to distinguish between reactivation of endogenous CMV and infection from the grafted kidney.

The timing of first detection of CMV shedding in urine did not differ between patients with primary infection and those with reinfection or reactivation. Virus was first detected on average eight weeks after transplantation, which agrees with findings of previous studies.[2][3][15] CMV was isolated from all patients with primary infection who could be followed up for an adequate time; shedding continued for many months in this group. The duration of CMV shedding in patients with reinfection or recurrent infection was more variable; some shed virus consistently over many months while others, the majority, did so intermittently or not at all. Virus isolation from the throat was less common than from urine, and in no case preceded isolation from the urine. In case 19 with reinfection or recurrent infection with CMV, the virus was isolated on one occasion from the throat but not the urine.

Standard culture procedures were used for virus isolation and notably the cytopathic effect developed first on average 25 days after inoculation (range seven to 47 days). The discarding of apparently negative cultures seven weeks after inoculation had elapsed may therefore lead to potential isolates being missed. In some cultures changes characteristic of a cytopathic effect appeared transiently but subsequently regressed. Due to the intermittent nature of CMV shedding, particularly in the group of patients with reinfection or reactivation, it is necessary to culture serial samples of urine.

Another approach providing clinically useful information is to follow up the immune response to CMV. Of the 10 patients with primary CMV infection, seven became CMV IgM positive and two IgM equivocal. The relation between the timing of seroconversion and the first detection of virus shedding differed among patients. In some, seroconversion occurred several weeks before virus was detected, and in others afterwards. Of the 20 patients with reinfection or recurrent infection, nine became IgM positive; again the relation between the timing of seroconversion and the first detection of virus shedding differed among individual patients. Therefore in the context of renal transplant recipients the presence of CMV specific IgM in a single sample cannot be used as evidence that the infection is primary. A definite serological response was detectable by at least one of the tests at or before virus isolation in nine of the 10 patients with primary infection from whom virus was isolated. The serological response to CMV and the timing of virus isolation in a small number of transplant recipients have been correlated individually.[16] Although no attempt was made to distinguish primary infection from reinfection or recurrent infection, there were differences in the patterns of the immunological responses among patients.

There were significant differences among individual patients in the detailed correlation of rising antibody

titres detected by complement fixation and ELISA. These differences may result from the characteristics of the assays used together with the kinetics of synthesis of different subclasses of the CMV specific antibody produced by individual patients. Individual strains of CMV differ antigenically, which may be another factor contributing to the differential immune response patterns seen.[17 18]

Considerable effort has gone into developing reliable tests for CMV specific antibodies. Numerous approaches have been tried, including the enzyme linked immunosorbent assay (ELISA),[19] radioimmunoassay (RIA),[9] M-antibody capture (MACRIA),[20] latex agglutination[21] and immunofluorescence.[22] The first three methods compared well overall in performance and were considerably more sensitive than immunofluorescence.[9] Though evaluation and comparison of immunological methods has been carried out on banks of sera,[9 10 23] previous published work carried out on the detailed temporal relation between the onset of CMV excretion and the serological responses in individual patients has not been extensive. We find that the serological response, even when measured by a variety of methods, is an imperfect guide to the onset of virus shedding. In a previous study the serological response to CMV after renal transplantation in primary infections was compared with that in reinfection or recurrent infection.[11] No attempt was made, however, to correlate the serological response with the time of virus shedding and the titres were presented as geometric mean titres of the patient groups, precluding analysis on a patient by patient basis.

Direct detection of CMV either in blood or body fluids provides an alternative approach to the problem of diagnosis. Because routine culture takes so long to produce results several rapid culture techniques have been developed and have proved useful.[24 25] Potentially even more rapid is the ability to detect virus or cells infected with virus in clinical samples directly by cytology, immunostaining, or nucleic acid hybridisation.[26–28] Cytological changes have been detected in some cases but have not correlated well with overall results of virus isolation.[29] Nucleic acid hybridisation is becoming increasingly refined and has been applied to the diagnosis of CMV infections but has not so far been extensively evaluated in clinical use.

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