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Temporal patterns of immunoblot-reactive antibodies to cytomegalovirus in transplant recipients

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Abstract. A total of 234 sera from 44 allograft recipients were compared with 12 sera from 9 immunocompetent patients with symptomatic cytomegalovirus (CMV) infection and with 20 sera of 20 healthy individuals with latent CMV infection. The presence of immunoreactive proteins was not associated with a specific transplant group or with different immunosuppressive regimens but rather with the kinetics of the immune response. Acute phase sera demonstrated early antibodies to proteins p38 and p48, followed by high or still rising antibodies to high molecular weight proteins, particularly p150, and their later decline to persistent lower levels. Convalescent phase sera were identified serologically by the transient appearance of IgG antibodies directed to 22–26 kDa polypeptides. Immunoreactive p44 was present in 85% of all patients with mild disease and in 40% of all patients with severe CMV disease. When tested in parallel, the immunoblot analysis was shown to be a more sensitive indicator of early CMV antibodies in allograft recipients than the ELISA technique.

Key words: CMV immunoblotting – Antibody detection, CMV – Prognostic marker, CMV

Infections with cytomegalovirus (CMV) are a major source of morbidity and mortality in allograft recipients. Even under improved immunosuppressive therapy, CMV-related death still occurs in 1%–3% of all renal transplant recipients and in 15%–20% of all bone marrow transplant recipients [3, 8, 18, 28, 33]. In view of the recent success achieved in the treatment of CMV infections with combined regimens of ganciclovir and hyperimmunoglobulin [1, 5, 11, 26], there is a great demand for early and reliable identification of CMV infection for patient management. Many efforts have been made to replace time-consuming tissue culture methods with, for example, genomic [2, 16, 20, 30, 31] and early antigenic detection techniques [6, 7, 32, 34, 36], which have been shown to correlate quite well with virus isolation. However, none of these techniques has significantly increased the virus detection rate, and the usefulness of some techniques is limited, due to their technical complexity.

Using serological methods, detection of an active infection is based mainly on significant titer rises and, therefore, requires a consecutive serum, as opposed to the detection of specific IgM, which requires only one acute phase serum. Furthermore, seroconversion to CMV in some patients precedes or coincides with virus isolation [19, 22].

Recently, the immunoblot technique was introduced as a diagnostic tool for rapid CMV diagnosis in immunocompetent [9, 14, 17, 23, 25] and immunosuppressed individuals [13–15, 19, 27, 35]. The aim of this study was to examine immunoblot patterns and the appearance of antibody response in various transplant groups, as well as to find specific profiles that may be predictive for the outcome of CMV disease. Finally, the diagnostic value of immunoblotting was compared to the ELISA technique in various groups of CMV-infected individuals.

Materials and methods

Study population

A total of 266 sera from 44 transplant recipients (30 renal, 9 heart, 4 liver, and 1 small bowel; groups 1–5), 9 immunocompetent patients with CMV disease (group 6), and 20 healthy individuals (group 7) were tested prospectively by an indirect ELISA (groups 2–7) and by an indirect immunofluorescence technique (group 1) and were stored frozen at −70°C. Patients with serological and/or clinical evidence of CMV infection were tested retrospectively using the immunoblot technique. Seronegative patients who were recipients of seropositive organs received a dose of CMV immunoglobulins (1.5 ml/kg body weight) once before transplantation and four times every 3 weeks post-transplantation. None of the patients received acyclovir prophylactically. Patients in group 1 were treated with azathioprine (Aza) and prednisolone (Pred), while patients in groups 2–5 received a combined regimen of cyclosporin A (CyA) and methylprednisolone.

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Antigen preparation

CMV antigen for immunoblotting was prepared by growing human diploid fibroblast (MRC-5) to confluence on 150 cm^2 culture flasks (Nunc, Wiesbaden, FRG). The cells were infected with CMV strain AD169 and after 5–7 days the cells were harvested, solubilized in 4 ml of Laemmli sample buffer [13], sonicated with three 15-s cycles at maximum output (Branson Sonifier), and finally boiled for 3 min. Antigens from mock-infected MRC-5 cells served as the control. Sixty µg protein from each preparation was loaded per 1 cm gel and its reactivity was checked with sera known to be positive for CMV-specific IgG and IgM antibodies.

Electrophoresis and immunoblotting

SDS-PAGE was performed with the Laemmli buffer system [13] using 3% stacking and 5%–17% linear acrylamide gradient separation gel. Prestained molecular mass markers with 180 kDa, 116 kDa, 84 kDa, 58 kDa, 48 kDa, 36 kDa, and 26 kDa (Sigma, München, FRG) were included in each gel. After electrophoresis at constant voltage (40 V) for 15 h, the gels were soaked in blotting buffer [25 mM TRIS, 200 mM glycine, pH 8.3, 20% (v/v) methanol] and transferred to nitrocellulose sheets (Schleicher & Schüll, Dassel, FRG; BA 85, 0.45 µm) at 250 h at 4 °C using a transblot apparatus (Hoefer Scientific Instruments, Heidelberg, FRG), and the sheets were stored at −30 °C. Before use, individual strips were soaked for 15 min in blocking buffer (50 mM TRIS-HCl, 150 mM NaCl, 5% nonfat dry milk, pH 7.4). Ten µl of each serum was tested at a 1:250 dilution in blocking buffer ovenight at room temperature. After washing three times with 50 mM TRIS-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4, the strips were incubated for 1 h with 2 ml of either biotinylated goat antihuman IgG (H + L) or goat antihuman IgM Fc5µ fragment (Dianova, Hamburg, FRG) at a dilution of 1:2500, followed by an incubation for 1 h with avidin peroxidase conjugate (Dianova, Hamburg, FRG). Bound enzyme was detected by incubation with 4-chloro-1-naphtole (0.3 mg/ml)/H_2O_2 (0.03% v/v) until sufficient color developed.

Serological test assays

For detection of CMV antibodies, an indirect ELISA specific for IgM and IgG was performed as described by the manufacturer (enzygnost CMV, Behring Werke, Marburg, FRG). An in-house, indirect immunofluorescence test was used as described previously [4]. All sera were tested in a paired fashion. A fourfold IgG titer rise, IgM ELISA titer greater than 1:200, and/or IgM IFT titer greater than 1:20 indicated an acute CMV infection.

Immunoblot patterns were analyzed as follows. A primary infections was defined by seroconversion in an individual who was seronegative before transplantation. A recurrent infection (reactivation and/or reinfection) was diagnosed by an increase in the number of immunoreactive bands with or without a change in the intensity of the pretransplant protein bands.

Diagnostic criteria

Patients whose sera gave a positive IgM signal to one of the three serological test assays and/or a fourfold or greater IgG titer difference in paired sera by the ELISA or the IFT were regarded as CMV-infected. In some cases infection was confirmed by histological changes or virus isolation. CMV disease was diagnosed by clinical criteria. CMV disease staging was based on a modified scoring scheme as first described by Smiley et al. [29]. Briefly, the following clinical signs were scored: fever (> 100.4 °F, 2–4 days, 1 point; 5–20 days, 2 points; > 20 days, 3 points), leukopenia (< 4000/µl, 1 point), thrombocytopenia (< 10000/µl, 1 point), interstitial pneumonia (X-ray changes, 1 point; X-ray changes plus symptoms, 2 points; ventilation support, 3 points), hepatitis (ALT > 2.5 times normal level, 1 point; elevated ALT plus icterus, 3 points), gastrointestinal ulceration and bleeding (3 points), cerebral symptoms (lethargia, 1 point; stupor, 2 points; coma, 3 points), retinitis (3 points), arthritis (2 points), allograft dysfunction (fully reversible dysfunction, 1 point; partly reversible dysfunction, 2 points; graftectomy, 3 points), microbial superinfections (3 points). Grade 0 included patients with serological evidence of CMV infection. A total of 3 points was registered as grade 1. Grades 2 and 3 reflected a total of 4–6 points and 7 or more points, respectively, while grade 4 represented CMV-related death.

Statistical analysis

Statistical analysis was performed with the chi square test and the Wilcoxon rank test, respectively. *P* values less than 0.05 were considered significant.

Results

As revealed by clinical data and serodiagnosis, 12 organ transplant recipients contracted primary infection between 3 and 12 weeks (median 6 weeks) post-transplantation, and 30 patients experienced recurrent CMV episodes between 3 and 24 weeks (median 8 weeks) post-transplantation (mean time to disease expression NS). In two patients, the infection status remained unclear due to discordant test results (Table 1). Regard-

Table 1. Infection modus and disease expression in the study population

Group	No. of patients	Primary infection Grade			Recurrent infection Grade			
		0	1–2	3–4	0	1–2	3–4	Sera
Transplant group								
1. Renal (Aza + Pred)	18[a]	0	3	3	0	6	4	36
2. Renal (CyA + Pred)	12	0	1	2	4	1	4	94
3. Heart	9	0	0	1	2	3	3	75
4. Liver	4	0	0	1[b]	3	0	0	23
5. Small bowel	1	0	0	1	0	0	0	6
Subtotal	44[a]	0	4	8	9	10	11	234
Nontransplant group								
6. Clinical disease	9	0	0	6	0	0	3	12
7. Healthy individuals	20	0	0	0	0	0	0	20
Total	73[a]	0	4	14	9	10	14	266

[a] Two patients whose infection status remained unclear

[b] Patient died 5 weeks post-transplantation after 3 weeks of CMV disease

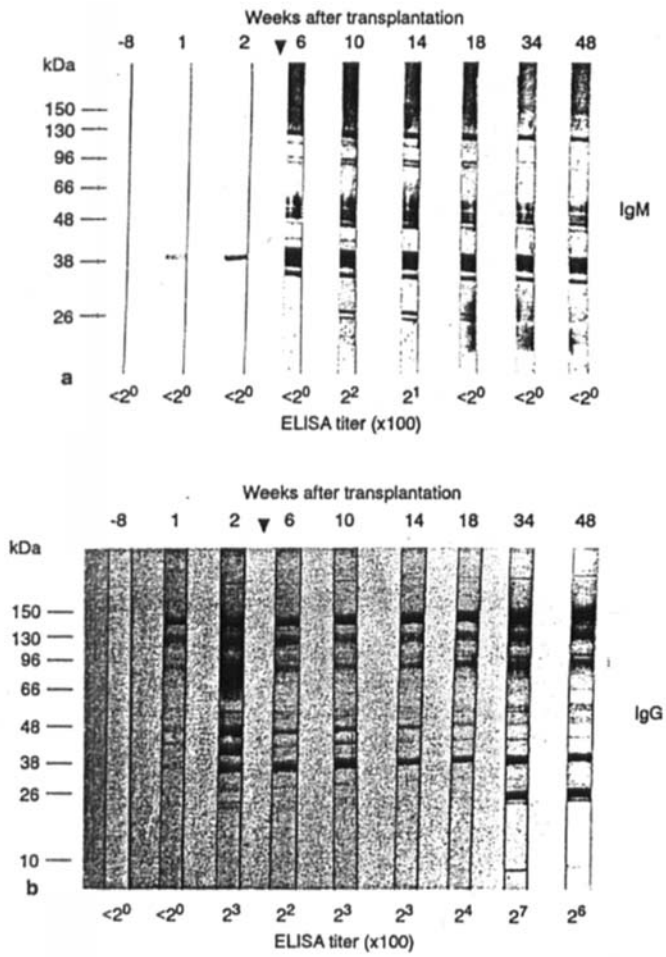

Fig. 1. Antibody immunoblot reaction pattern in a renal transplant recipient with primary infection (transplant dysfunction, 2 pts; fever, 3 pts; leukopenia, 1 pt; grade 2) between the 2nd and 6th weeks after transplantation *(arrow)*. During the 1st and 2nd weeks, the patient received CMV hyperimmunoglobulins. Note the early appearance of p38/48 and p96–p150 during the 6th week and the late appearance of p22–p26 after the onset of clinical syndromes

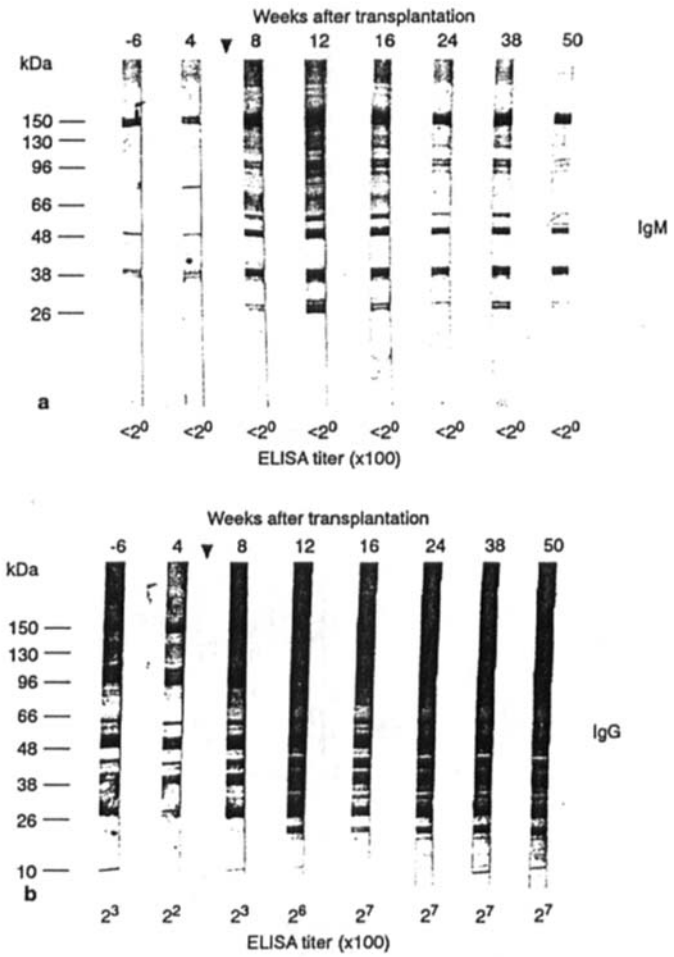

Fig. 2. Timing and pattern of detection of seroconversion in a heart transplant recipient who developed a CMV retinitis (3 pts) between weeks 4 and 8 *(arrow)*. As seen in most patients with latent CMV infection, antigens p38/48 and p96–p150 were present before onset of clinical symptoms. At seroconversion, there was an immediate burst of CMV-reactive antibodies

less of the CMV infection modus, immunosuppressed organ transplant recipients (groups 1–5; Figs. 1–3) and immunocompetent individuals (groups 6, 7; Fig. 4) produced similar IgM and IgG immunoblot reaction profiles. No obvious differences between the transplant groups or between the different immunosuppressive regimens were noted. IgM antibodies reacted against a total of 19 antigens, while IgG immunoglobulins detected 23 antigens ranging from 10000 to 150000 molecular weight. The polypeptides p150, p130, p120, p100, p96, p48, and p38 were the most frequently and the most intensely reacting antigens detected by IgM and IgG immunoblot analysis.

By analyzing consecutive sera of individuals in each group, a reproducible pattern of antibody appearance could be observed (Fig. 5). First detected within 7 days, antibodies to p38 and p48 were retained for about 9 months after the onset of clinical syndromes in primary infection (Figs. 1, 4). Antibodies reacting to high molecular weight proteins p96, p120, p130, and p150 usually appeared within 2–12 weeks after the onset of CMV disease and they increased in intensity over time. During the convalescent phase in most patients, transient antibodies to low molecular mass proteins of 22, 24, and 26 kDa appeared between the 2nd and 9th months after primary CMV infection (Figs. 1, 2).

Acute phase sera from primary infected individuals reacted significantly less frequently and less intensely against CMV antigens p130, p120, p100, p66, p58, p38, and p26 than sera from recurrently infected patients (Fig. 6).

During recurrent infection, IgM and IgG antibodies with specificities against a wide array of CMV proteins appeared within a shorter period and at higher intensity than in primary infection (Figs. 2–4).

Other distinctions that could be made involved the degree of disease expression and the immunostatus of infected individuals. Protein p44 was recognized at a higher frequency in patients with mild disease (grades 1, 2) than in patients with grade 3 or grade 4 disease expression

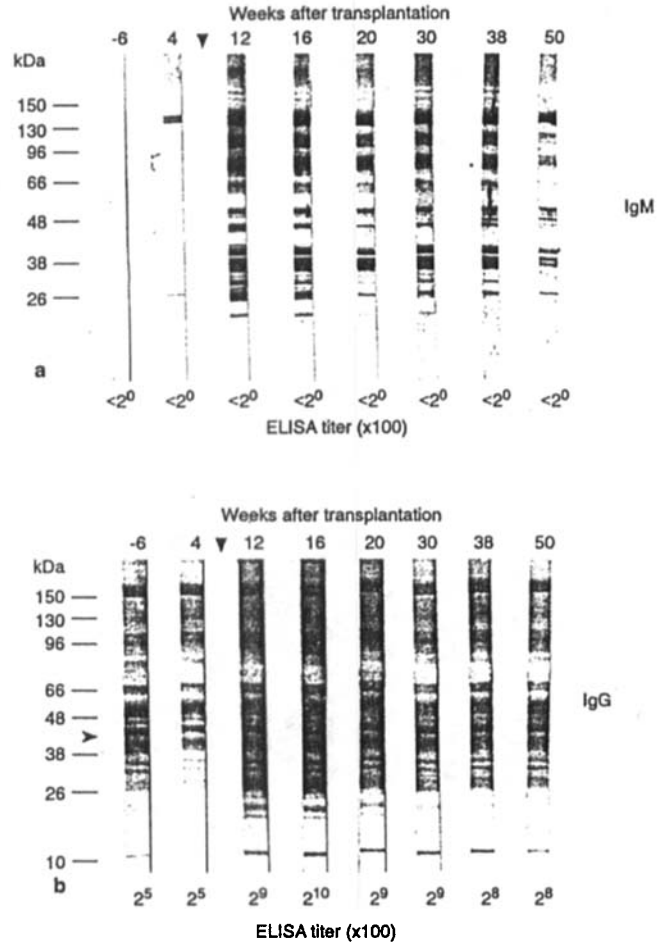

Fig. 3. Immunoblot reaction pattern from serum samples of a renal transplant patient who experienced a severe recurrent CMV infection (transplant dysfunction, 2 pts; fever, 3 pts; leukopenia, 1 pt; grade 2) during week 6 *(arrow)*. Note the rapid onset of IgM and IgG immunoblot reactive antibodies, the eightfold IgG titer rise between weeks 11 and 12, and the lack of ELISA reactive IgM antibodies. *Arrowhead* denotes protein p44

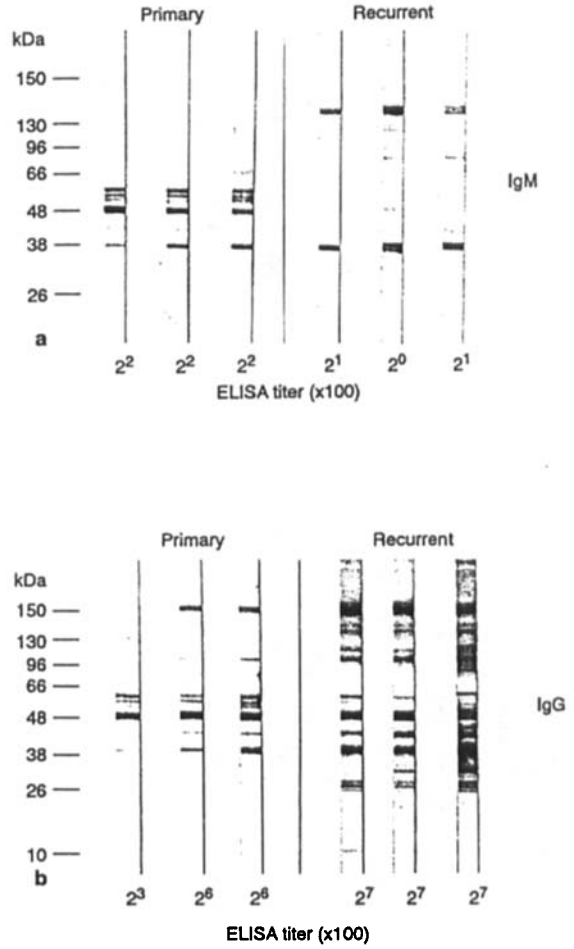

Fig. 4. Immunoreactive pattern in six immunocompetent individuals with symptomatic CMV disease. From left to right: three patients with primary CMV infection (hepatitis) and three with recurrent CMV infection (fever). Note the early appearance of IgM and IgG anti p38/48 and IgG anti p150 in primarily infected individuals, as well as the simultaneous display of antibodies reactive to the whole array of CMV antigens in secondarily infected patients

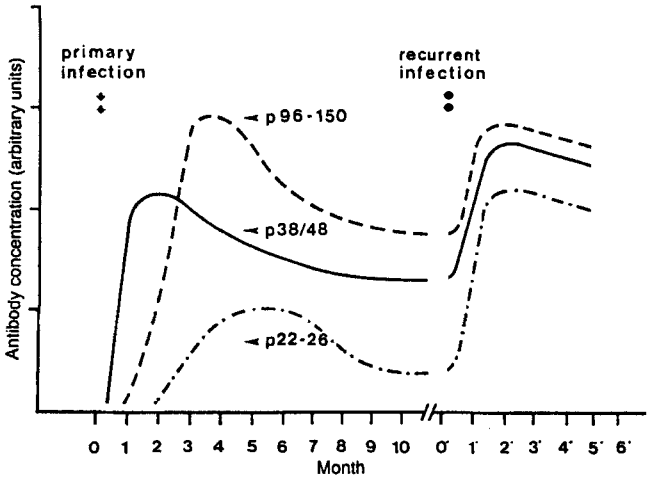

Fig. 5. Schematic diagram of the appearance of CMV-specific proteins reactive with IgG antibodies in primary and secondary CMV infections

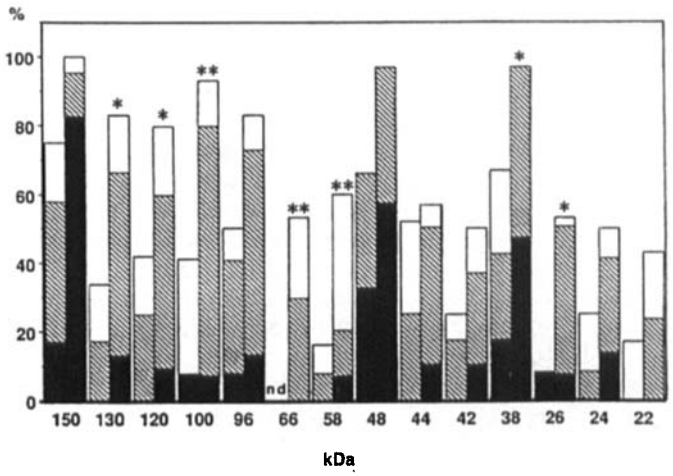

Fig. 6. Reactivity of individual CMV polypeptides with acute phase sera of allograft recipients with primary (*left column*, $n = 12$) and recurrent (*right column*, $n = 30$) infection. ■ Intense; ▨ moderate; □ weak reaction; *nd* not detected. * $P < 0.05$; ** $P < 0.01$

Table 2. Comparison of immunoblotting and the indirect ELISA for the surveillance of 26 allograft recipients (groups 2–5) with serological evidence (grade 0) or clinical evidence (grades 1–4) for CMV infection

		Serological evidence Grade (*n*) 0 (9)	1 week after onset of disease		4 weeks after infection		
			Grade (*n*) 1–2 (5)	3–4 (12[a])	Grade (*n*) 0 (9)	Grade (*n*) 1–2 (5)	Grade (*n*) 3–4 (11)
Immunoblot	IgM	9	5	10	9	5	11
Immunoblot	IgG	6	4	10	8	5	11
ELISA	IgM	3	2	4	5	4	8
ELISA fourfold	IgG	5	3	6	8	5	9

[a] One liver transplant recipient who died the 3rd week after onset of clinical CMV disease

(12/14 vs 5/12, $P < 0.05$). In general, immunosuppressed patients with grades 0–2 disease showed more intense IgG reaction against p38 than did immunosuppressed patients with severe disease expression.

Of 17 patients with clinical CMV disease, 15 were already positive by IgM immunoblot analysis within 1 week after the onset of clinical syndromes, as opposed to the IgG immunoblot assay, which detected 14 of these patients (Table 2). The IgM ELISA only recognized six sera and the IgG ELISA detected nine sera of these patients by the 1st week of clinical CMV disease. This indicates that the IgM ELISA showed the highest rate of false-negative results. However, when consecutive sera were tested, more positive ELISA results were obtained, leading to a much better correlation between these two methods (Table 2).

Discussion

The patient groups in our study produced antibodies to individual CMV antigens in a pattern similar to that displayed by immunocompetent patients with natural infection. Similar observations have already been made for renal [13, 19, 27] and bone marrow transplant recipients [35] but have not been reported for heart, liver, or small bowel transplant recipients. Proteins p150, p48, and p38 were the antigens most frequently detected by IgM and IgG in primarily and recurrently infected organ transplant recipients and in immunocompetent individuals, as has already been observed in immunocompetent individuals [9, 17], renal transplant patients [13, 19, 27], and HIV-infected individuals [15].

Each single serum exhibited different patterns of antibody reactivity. However, on the basis of the appearance of immunogenic polypeptides, we found a specific sequence of antibody appearance in primary CMV infection that was characterized by: (1) early presence of antibodies to antigens p38 and p48; (2) very high or still rising antibody titers to high molecular mass proteins, particularly p150, and their later decline to persistent lower levels, indicating the latent carrier state that becomes established after primary CMV infection (Fig. 2); (3) a transient response during the 3rd–9th months to antigens p22, p24, and p26; and (4) a simultaneous and more intense display of antibodies to the above-mentioned proteins in all patients undergoing recurrent CMV infection (Fig. 5).

The 38 kDa and 48 kDa proteins, which were highly immunogenic to both IgG and IgM when tested with immunoblot analysis, may be antigenically related or even identical to immediate early or early viral antigens of similar size. These antigens are recognized by murine monoclonal antibodies CCH2 and DDG9 (results not shown) [37] and by acute phase sera of immunocompetent [9] and renal transplant recipients [14]. In accordance with previous results by other groups, we regularly detected a 150 kDa protein that is known to be a major viral immunogen [13–15, 17, 23, 25, 27]. The transient appearance of antibodies to p22–p26 observed in all groups supports previous observations on the immunoreactivity to a 22 kDa protein in immunocompetent patients [9] and to a 28 kDa protein in renal transplant recipients, indicating the convalescent phase of CMV infection [14].

A protein that caught our interest was protein p44. Significantly fewer patients with severe symptoms (grades 3, 4) immunoreacted with this protein than did patients with grades 0–2 CMV disease (40% vs 85%, respectively). Such observations have not yet been described in systems using human sera. Therefore, antibodies to p44 can be regarded as prognostic markers for disease progression. However, the diagnostic significance of this observation awaits further studies.

Given the increased sensitivity of the immunoblot technique for detecting IgM, it is possible that this antibody class was detected at lower levels than with the conventional ELISA. This would explain the extreme persistence of IgM antibodies in the transplant group. In previous studies IgM antibodies were seen in immunocompetent patients for as long as 5 months [22], while in renal transplant patients IgM persisted for longer than 12 months [10, 21, 22, 24]. The reason for this extreme IgM persistence is unclear, but it is tempting to assume that the switching from IgM to IgG production is altered in immunosuppressed patients.

Of 17 patients who seroconverted within 4 weeks after onset of clinical syndromes, 11 and 8 patients were seronegative during the 1st week by IgM and IgG ELISA, respectively. During this interval, the IgM immunoblot assay detected antibodies in 15 patients. In these instances, it appears that immunoblotting was a more sensitive indicator of early CMV antibodies than was the ELISA technique. These discordant results may reflect differences in antigen preparation, antigen presentation, and/or differences in the antibody detection system (Table 2).

Obviously, the immunoblot technique is superior to other serological test assays for the surveillance of transplant patients. It is more sensitive to antibody detection and allows early detection of CMV infection. Since storage of the nitrocellulose sheets poses no problem, they can be prepared in advance. Serum samples can be analyzed immediately and final results can be obtained within 24 h, allowing the clinician to receive information rapidly. Since none of the techniques currently used in most laboratories is able to detect CMV infection at a very early stage, a network of diagnostic methods – including the immunoblot assay – should be established. By detecting CMV infection as early as possible, patient management can be improved.

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