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Symptomatic cytomegalovirus (CMV) infections identified by image cytometry and other parameters for CMV infection

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Abstract. Thirty-eight renal transplant recipients were followed during the first 3 months after transplantation. Once weekly, cultures of urine and buffy coat for cytomegalovirus (CMV) were taken and an immunocytochemical assay for immediate early antigens of CMV (IEA assay) was performed. Thirty patients had evidence of a CMV infection and 11 had a symptomatic CMV infection. All symptomatic patients had one or more positive urine cultures or a positive IEA assay. However, 15 patients with positive urine cultures and 12 patients with a positive IEA assay lacked any signs of symptomatic CMV disease. Moreover, 6 out of 15 patients with positive buffy coat cultures for CMV did not have symptomatic CMV disease. Using a computerized system to quantify IEA-positive granulocytes, we show that the absolute number of positive cells per million correlates very well with the occurrence of symptomatic CMV disease.

Key words: Cytomegalovirus infection, in kidney transplantation – Cytometry in CMV infections

Cytomegalovirus (CMV) infection has been demonstrated in 60%–80% of all renal transplant recipients [3] and is the most common infectious disease in renal allograft recipients [20, 25]. Affected patients display increased morbidity [9, 29], graft loss [16, 24, 26], and mortaility [17, 19, 20].

During a symptomatic CMV infection, immunosuppressive treatment should be reduced while antirejection therapy has to be postponed. Thus, rapid detection of CMV infections is essential for good clinical management. Moreover, early diagnosis becomes increasingly important now that antiviral drugs such as 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (ganciclovir) are available, drugs that might be more effective upon early administration [8, 12].

Recently, several new techniques have been developed for the detection of CMV. A reduction in processing time has been made possible using a combination of inoculate centrifugation and short-term viral culture, followed by immunocytochemical detection with monoclonal antibodies [11, 28]. This technique – detection of early antigen fluorescent foci (DEAFF) – enables a virological diagnosis within 18–72 h, compared to an isolation time of 1–8 weeks for the conventional virus culture techniques. Furthermore, a rapid immunocytochemical detection technique that detects CMV immediate early antigens (IEA assay) in peripheral blood leukocytes has proven to be very valuable for early CMV viremia detection [4, 5]. However, this technique yields a low frequency of positive staining cells per patient sample. This frequency is far too low for routine flow cytometry. An image analysis system interfaced to an automated bright-field microscope can detect frequencies of positive cells as low as $1:10^6$ in 20–40 min [21]. This system has proved to be useful in screening patient samples [22].

In this study we compared the results obtained using the DEAFF technique on buffy coat and urine with those obtained in the IEA assay using quantification of IEA-positive cells by this image analysis system.

Materials and methods

Patients

Thirty-eight patients who had received a kidney allograft were followed for 12 weeks after transplantation. Cyclosporin A (CyA) was given in a dose of 16 mg/kg per day, tapering to 10 mg/kg per day over 3 months according to a standard schedule, depending on CyA blood levels and clinically detected toxicity.

Prednisone was started in a dosage of 20 mg/day and was tapered off to 10 mg/day with in 8 weeks. First and third rejection episodes were treated with three intravenous injections of high-dose methylprednisolone on 3 subsequent days; second rejection episodes were treated with rabbit antithymocyte globulin (ATG).

Diagnosis of active CMV infection required seroconversion or a significant increase in IgG antibody and/or detection of CMV antigenemia by IEA assay or of viremia by DAEFF of buffy coat.

Symptomatic CMV infection was defined as an episode of fever (>38.5°) for 2 days or more, or leukopenia, thrombopenia, hepatitis, intestinal ulceration, or interstitial pneumonia. In addition, an active CMV infection had to have been diagnosed.

Patient samples were taken weekly.

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Detection of early antigen fluorescent foci (DEAFF)

MRC-5 cells (Flow Laboratories, Herts, UK) were grown to confluence in 24-well multidishes (Greiner, Alphen a/d Rijn, The Netherlands) in a humidified atmosphere containing 5% CO_2. Buffy coat cells were isolated from heparinized blood and resuspended in culture medium (Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum (FBS), 0.005% vancomycin, 0.002% gentamycin, and 0.0003% fungizone). Urine samples were filtered through a 450 nm filter, followed by addition of FBS to a concentration of 10%. Buffy coat cell suspensions or pretreated urine samples were then inoculated onto monolayers of MRC-5 cells and centrifuged for 45 min (37 °C, 3000 *g*). After refreshment of the culture medium, the multidishes were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 18 h. For propagation of the buffy coat cultures, a second medium refreshment was performed, after which incubation was continued for 48 h. Thereafter, the monolayers were fixed in 90% acetone at – 20 °C for 20 min and air-dried. Indirect immunofluorescent staining was performed using the murine anti-HCMV monoclonal antibody E13 (Biosoft, Paris, France) and FITC-conjugated anti-immunoglobulin (Tago, Burlingame, Calif.). Fluorescent nuclei were scored on a Zeiss inverted fluorescence microscope.

Direct immunocytochemical detection technique (IEA assay)

The isolation and staining procedures have been described elsewhere [4, 5, 13]. Briefly, total white blood cells were isolated using the dextran method and the remaining red blood cells were lysed in red cell lysis buffer (0.15 M NH_4Cl, 10 mM KHCO3, 10 mM EDTA, pH 7.4). About 10^6 white blood cells were adhered to slides and fixed with 95% methanol and 5% acetic acid for 15 min. A mixture of two murine monoclonal antibodies against a 70 kDa HCMV-IEA protein were used to incubate the cells for 45 min at 37 °C. After washing, another fixation step was carried out in 4% (w/v) formaldehyde for 15 min. Endogenous peroxidase was inactivated using 1 IU/ml glucose oxidase (Serva, Heidelberg, FRG) in 0.5 M glucose in PBS for 30 min at 37 °C. Next, peroxidase-conjugated rabbit anti-mouse immunoglobulin (1 : 100) was applied for 45 min at 37 °C. Peroxidase activity was visualized using 0.5 mg/ml diaminobenzidine (DAB; Sigma, St. Louis, Mo., USA), 0.05% H_2O_2, 0.05 mM Tris-HCL, 0.01 M imidazole, pH 7.4. Using bright-field microscopy, the number of antigen-positive cells per 50,000 leukocytes were counted. A positive IEA test required at least one positive staining cells. When using automated image analysis with Leyden Television Analysis System (LEYTAS), the slides were counterstained with hematoxylin for 1 min and embedded in fluormount.

Image analysis

The instrumentation consisted of an automated bright-field microscope (AUTOPLAN; Leitz, Wetzlar, FRG) interfaced to a Motorola 68000 microprocessor-based modular image analysis computer (Leitz, Wetzlar, FRG). This type of image analysis microscope uses one objective and two TV cameras, thereby obtaining two magnifications simultaneously: an image with low (20 ×) magnification and high (80 ×) magnification. This system, called LEYTAS, is fully automated and programmed for, e. g., autofocus routine, automated stage movement function, storage of the suspected (labelled) cells into video memories, and elimination of most of the artifacts. In addition, relocation of suspected cells for visual verification using the same microscope is part of the standard routine. A detailed description of the system and its software has been published elshewhere [22].

Determination of CMV antibodies (CMV-ELISA)

This test was performed exactly as described by van der Giessen et al. [10]. Briefly, wells of microtiter plates were coated with CMV late antigens. After washing, serial dilutions of patient sera were added for 45 min at 37 °C. Afterwards, plates were incubated with horseradish peroxidase-conjugated goat anti-human IgG or IgM for 45 min at 37 °C. After washing, wells were filled with substrate (orthophenylene diamine OPD). After 15–20 min of incubation at room temperature, the reaction was stopped with 2N H_2SO_4 and the OD was read. An increase in IgG antibody concentration of 50% of the sample taken 12 weeks after transplantation in relation to the sample taken just before transplantation was considered to be conclusive for a secondary infection. An increase in IgG antibodies above 2 IU, together with IgM antibody levels of 5 IU or more, was considered to be decisive for a seroconversion [10].

Results

Eleven patients (29%) had symptomatic CMV disease in the first 3 months after transplantation. Seven patients had fever, nine leukopenia, and six thrombocytopenia. No interstitial pneumonia occurred and none of the patients died. Five out of these 11 patients had been CMV-seropositive before transplantation. Thirty patients (87%) had a CMV infection, diagnosed by serology or detection of CMV antigenemia or viremia. Only one patient with serologically proven CMV infection without symptoms remained negative in all cultures and IEA assay. Another patient without serological evidence of CMV infection had one positive urine culture but remained negative in the buffy coat culture and IEA assay. For three patients, no second serum sample was obtained. However, these three patients had positive buffy coat cultures and IEA assays. All patients with a CMV infection without symptoms ($n = 19$) had been seropositive for CMV before transplantation. Two of the eight patients without CMV infection had been seropositive before transplantation. Data on the results of urine cultures, buffy coat cultures, and IEA positivity are given in Table 1.

Three patients had a positive IEA without positive urine cultures or buffy coat cultures. Twenty-six patients had one or more positive urine cultures; 11 of them had a symptomatic CMV infection. Twenty-three patients had one or more positive IEA tests; 11 of them had a symptomatic CMV infection. Out of 15 patients with positive buffy coat cultures, 9 were symptomatic for their disease. Thus, all 11 symptomatic CMV infections were accompanied by a positive urine culture and IEA assay; 9 patients had positive buffy coat cultures.

The probability that a patient with an abnormal test would have symptomatic infection (positive predictive value) was 60% for the buffy coat culture versus 42% for the urine culture and 48% for the IEA test.

Table 1. Results of direct immunocytochemical detection technique (IEA assay) and cultures of urine and buffy coat of 30 patients with CMV infection

	Urine culture-positive/ no. of patients	Buffy coat culture-positive/ no. of patients	IEA-positive/ no. of patients
CMV infection; no symptoms	15/19	6/19	12/19
Symptomatic; CMV infection	11/11	9/11	11/11
All patients	26/30	15/30	23/30

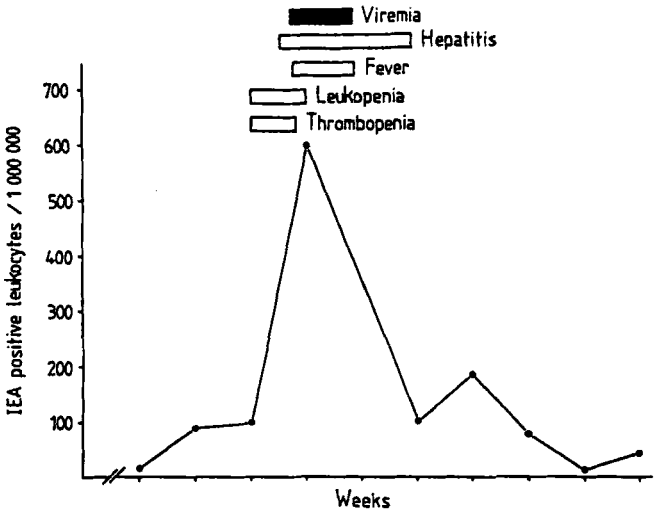

Fig. 1. Follow-up of number of IEA-positive leukocytes, clinical symptoms, and viremia

Of the 23 patients with positive staining cells for IEA antigens, 17 were followed by serial assessment of the number of IEA-positive cells counted by LEYTAS. Ten of them also had positive buffy coat cultures for CMV. Of these ten patients, three had no clinical symptoms. These three all had a low amount of IEA-positive cells counted by LEYTAS (maximal 239 per million), while patients with symptomatic CMV disease had at least 400 positive-staining cells per million (Table 2). Urine cultures were positive in nine of the ten patients. The patient with a negative urine culture did not have symptomatic CMV disease (Table 2).

Analyzing the patients with symptomatic CMV disease, it was observed that the symptoms due to the CMV infection coincided with the peak of the IEA-positive leukocytes. At the same time, viremia was detected using the DEAFF technique. The peak of IEA-positive leukocytes was frequently preceded by low numbers of IEA-positive cells. A representative sample is shown in Fig. 1. The maximum number of positive-staining cells did not differ between primary and secondary symptomatic CMV infections. Six of the seven symptomatic CMV infections described in Table 2 should be classified as mild, according to the criteria used by Peterson et al. [19]. Patient 10 had moderate severe disease.

The seven patients without viremia all had low amounts (maximum 80 per million) of positive-staining cells determined by LEYTAS. All seven had positive urine cultures for CMV. Only one of the seven patients had a symptomatic CMV disease (Table 3). Using a unpaired Student's *t*-test, a highly significant ($P = 0.004$) correlation was found between the number of positive-staining cells determined by LEYTAS and symptomatic CMV disease.

Discussion

After renal transplantation, 60%–90% of the recipients may show evidence of cytomegalovirus infection [1, 18]. Many such patients remain clinically asymptomatic. Our incidence of symptomatic CMV infection of 29% corresponds with the observations of others [2, 14, 27] and with our earlier findings [7]. According to the criteria used by Peterson et al. [19], 10 of the 11 CMV infections were classified as mild, while only one patient had to be considered as having a moderate severe disease. None of the patients had an interstitial pneumonia due to CMV or died because of a CMV infection.

Rapid detection of CMV infection is essential in clinical transplantation. During a febrile episode with declining transplant function, the clinician has to choose between giving rejection therapy or, in the case of an active CMV infection, decreasing the dosage of the immunosuppressive therapy and postponing antirejection therapy. Early diagnosis has become more important since the introduction of ganciclovir for treatment of CMV infections. This drug appears to be more effective when started early after the onset of CMV disease [8, 12]. The described immunocytochemistry using antibodies directed to immediate early antigens of CMV, followed by computerized quantification of the number of positive cells per million leukocytes, takes a processing time of 8–10 h. The DEAFF technique takes at least 24–48 h. Serology gives results only weeks after the start of the infection [15].

Table 2. Patients with positive IEA tests and one or more buffy coat (DEAFF) samples

Patient	Type of active CMV infection	No. of DEAFF-positive buffy coat samples	No. of DEAFF-positive urine samples	No. of IEA test-positive samples	Maximum no. of positive staining cells per million
1	Primary	3	8	5	2600
2[a]	Secondary	2	0	5	60
3	Secondary	1	4	5	1000
4	Secondary	4	9	9	5200
5	Primary	1	8	6	400
6	Primary	4	8	10	2650
7[a]	Secondary	1	7	4	112
8	Primary	2	8	8	600
9[a]	Secondary	4	4	7	239
10	Primary	2	6	4	1100
Mean		2.4	6.2	5.9	

[a] No clinical symptomatic CMV infection

Table 3. Patients with positive IEA test without positive buffy coat (DEAFF) samples

Patient	Type of active CMV infection	No. of DEAFF-positive urine samples	No. of IEA test-positive samples	Maximum no. of positive staining cells per million
1[a]	Secondary	3	1	6
2	Secondary	6	2	35
3	Secondary	7	4	80
4	Secondary	2	2	30
5	Secondary	1	3	20
6	Secondary	2	1	25
7	Secondary	8	2	< 1
Mean		4.1	2.1	

[a] Clinical symptomatic CMV infection

Our study clearly demonstrates that routinely taken cultures of urine and buffy coat are frequently positive after renal transplantation without any signs of symptomatic CMV infection.

In the literature, positive buffy coat cultures correlate best with disease activity [6, 24]. In our study, 6 of the 15 patients with positive buffy coat cultures did not have symptomatic CMV disease. Also, the direct immunocytochemistry for IEA antigens and the urine culture for CMV were not very specific for symptomatic CMV disease. Both tests were positive in 40%–60% of the patients without any signs of a symptomatic CMV infection. Yet, these tests may be of value when one has to rule out symptomatic CMV infection. Since results of direct immunocytochemistry for IEA antigens are obtained quickly, this test seems more appropriate than the urine culture for CMV.

To increase the correlation between the direct immunocytochemistry and symptomatic CMV disease, the number of positive-staining cells per number of total leukocytes may be of value [23]. Since the frequency of IEA-positive cells is low, manual counting of positive cells is time-consuming, tedious, and only correct when enough cells are counted. By computerizing the counting of positive-staining cells per million, these disadvantages can be avoided. Computerized counting can be performed by any commercially available image cytometry instrument. In our hands, a high number of positive cells is always accompanied by a symptomatic CMV disease. This peak of IEA-positive leukocytes was frequently preceded by low numbers of IEA-positive cells detected by LEYTAS. Thus, sequential determinations of the number of positive cells can be used for monitoring CMV infection activity. For example, symptoms like fever and thrombocytopenia can be caused by rejection, CMV, or the concomitant occurrence of rejection and CMV. Using the computerized counting technique, the clinician will be able to recognize a symptomatic CMV infection. The modular image analysis computer (MIAC) technique might also be used for monitoring disease activity during or after treatment with ganciclovir.

Only one patient had a low number of positive-staining cells while having a symptomatic CMV disease. Because we performed the diagnostic test routinely only once a week, we could have missed the peak of positive cells. This might possibly have been prevented by taking samples more frequently during clinical suspection of symptomatic CMV disease.

In conclusion, in order to diagnose a symptomatic CMV disease, buffy coat culture is more valuable than direct immunocytochemistry or urine cultures with serology. However, computerized counting of granulocytes staining positive for IEA by immunocytochemistry appears to be an even better technique for detecting symptomatic CMV disease and may be used for monitoring disease activity.

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