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A longitudinal prospective study of cytomegalovirus pp65 antigenemia in renal transplant recipients

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Abstract. Cytomegalovirus (CMV)-encoded pp65 antigen in peripheral blood leukocytes (CMV antigenemia) was investigated in 1017 serial samples from 64 patients for 16 weeks after renal transplantation in a prospective study. In 110 samples from 24 patients, at least one antigen-positive leukocyte was identified. The median number of stained cells was 4 (range 1–1000) per 4×10^5 leukocytes. Twenty-one of 24 patients with serological signs of an active CMV infection were antigen-positive (sensitivity 87.5 %), whereas 3 patients with antigenemia did not show serological signs of infection during the observation period (specificity 92.5 %). Positive results were obtained 19 days (median) before serological response and 9 days (median) before the onset of CMV syndrome. The sensitivity in defining a CMV syndrome was 100 % ($n = 8$). In all patients who presented with CMV syndrome, antigenemia was present prior to the onset of symptoms or on the same day. In contrast, serological monitoring rendered the diagnosis of CMV infection possible at the onset of clinical symptoms in only two of eight patients. We conclude that (1) insufficient results obtained with the CMV antigenemia assay by other investigators are mainly due to technical problems that can easily be overcome by the protocol presented and (2) the detection of CMV pp65 antigen in peripheral blood leukocytes is an excellent tool for rapid and early diagnosis of CMV infection.

Key words: CMV antigenemia, renal transplantation – Renal transplantation, CMV antigenemia – pp65 antigen, CMV, renal transplantation

Introduction

Human cytomegalovirus (CMV) is the most important opportunistic infectious agent in immunosuppressed patients after renal transplantation [19] leading to symptomatic disease and, in 1 %–3 % of cases [14], to fatal outcome. The incidence of active CMV infections after renal transplantation, as shown in the literature, differs significantly, depending mainly on the immunosuppressive regimen and on the frequency of high-risk transplantations. It has been estimated to be 30 %–50 % according to newer studies [9, 13] in which symptomatic CMV disease occurred in 13 %–21 % of all transplanted patients.

The unstable balance between the latently prevalent pathogen and a compromised immune system is strongly modulated by the immunosuppressive regimen [12]. CMV disease and an eventually fatal outcome may occur if antirejection therapy is initiated during an asymptomatic, active infection. Since both events – renal allograft rejection and active CMV infection – are clinically similar and coincidentally peak between 30 and 90 days after transplantation [16], the differential diagnosis of these conditions is one of the major problems in post-transplant monitoring. With the advent of effective antiviral drugs, e.g., ganciclovir, the early and sensitive diagnosis of active CMV infection is the major precondition for patient management and, ultimately, for improvement of graft and patient survival.

The lack of sensitivity in the early diagnosis of CMV infection by conventional methods, i.e., virus cultivation and serology, is well documented. There have been many attempts to optimize CMV diagnosis within recent years, including rapid virus cultivation, antigen detection, and nucleic acid hybridization and amplification [10]. However, until now, no single, standardized test could be relied upon for early, i.e., presymptomatic, diagnosis in the routine management of all patients at risk. One of the most promising approaches in this regard is the direct detection of the CMV-encoded pp65 antigen in peripheral blood leukocytes (antigenemia), originally described by van der Bij and coworkers [3, 5]. Two other groups reported on the successful clinical use of this test [8, 17]. However, the assay still remains controversial as two other investigators failed to confirm those results [15, 21]. We recently published an improved protocol that was based on the alkaline phosphatase-anti-alkaline phosphatase (APAAP)

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technique and that overcame the interpretation problems of the original technique [2]. In the present study, we evaluated the clinical usefulness of the antigenemia assay in a longitudinal and prospective study on renal transplant patients, employing this protocol.

Patients and methods

Patients

Between 1 January 1990 and 30 June 1991, 79 patients received a cadaveric renal transplant at our institution. Fifteen patients were excluded from this study because of incomplete follow-up due to incomplete sampling, graft loss, or CMV-unrelated death. Thus, the complete data of 64 patients were available for analysis.

Immunosuppression in first transplant recipients consisted of triple drug therapy including prednisolone (25–7.5 mg), azathioprine (2 mg/kg body weight), and cyclosporin (3–5 mg/kg body weight); in second or third transplant recipients, it consisted of quadruple therapy, with rabbit antithymocyte immunoglobulin (ATG, 3 mg/kg body weight) in addition. Steroid-resistant rejection episodes (500 mg prednisolone on 3 consecutive days) were treated with ATG or the mouse monoclonal antibody OKT3 (5 mg). In all patients the CMV serostatus of donor and recipient was available at the time of transplantation. Acyclovir was given orally to all patients receiving a kidney from a CMV-seropositive donor according to a recently published scheme [1]. CMV-seronegative recipients receiving a kidney from a seropositive donor were prophylactically treated with CMV hyperimmunoglobulin (Cytotect, Biotest, Dreieich, Germany) for the first 8 weeks after transplantation. Treatment of severe or life-threatening CMV syndrome consisted of ganciclovir, CMV hyperimmunoglobulin, and tapering of the immunosuppressive dose where symptoms did not improve (mostly azathioprine because of leukopenia). All patients received seronegative leukocyte-depleted blood products when indicated.

All patients were monitored weekly during their hospital stay and weekly to biweekly after discharge for at least 12–16 weeks after transplantation.

Titration of anti-CMV IgG and anti-CMV IgM antibodies

CMV-specific IgG and IgM antibody titers were determined using a commercial ELISA (Enzygnost, Behring, Marburg, Germany). The assays where done on serial diluted sera. The endpoint was calculated for the difference in antibody absorption signal, delta A = 0.2 for IgG (antigen-containing well – control antigen-containing well) and delta A = 0.3 for IgM according to the manufacturer's instructions. Regression line analysis was performed with at least three measured values. Prior to use in the CMV IgM assay, the samples were absorbed with antihuman gamma chain antibody (Behring, Marburg, Germany) to absorb rheuma factor. (If present in the sera, this may cause false-positive IgM antibody determinations.)

Detection of CMV pp65 antigen in peripheral blood leukocytes

The detection of CMV pp65 antigen in peripheral blood leukocytes was performed according to a previously published protocol [2] with minor modifications. Briefly, peripheral blood leukocytes were isolated by means of hydroxyethyl starch sedimentation and subsequent hemolyses of the remaining erythrocytes with 0.155 mol/l NH_4Cl, 0.01 mol/l $KHCO_3$ for 3 min at room temperature. Of each sample, 4×10^5 leukocytes were cytocentrifuged on glass slides and air-dried for at least 30 min. After fixation with fresh acetone/methanol (1:1) at 4 °C for 90 s, the presence of pp65 antigen, mainly in the nuclei of granulocytes, was demonstrated by APAAP immunostaining using the monoclonal antibodies (mAbs) C10 and C11 [5] (Biotest, Dreieich, Germany). Both primary antibodies were previously reported to detect the CMV 72 kDa major immediate early antigen. Recent evidence suggests that both mAbs are actually directed against the CMV lower matrix phosphoprotein pp65 [18]. The staining procedure essentially followed the recommendations of the manufacturer of the APAAP complex (Dako, Hamburg, Germany). Incubation with the APAAP complex was repeated once. The enzyme substrate was new fuchsin, and after completion of the immunostaining, the slides were weakly counterstained for 5 min with hematoxylin diluted 1:10 in TRIS-buffered saline. The slides were microscopically evaluated for the presence of stained nuclei and the results were expressed as positive cells per 4×10^5 peripheral blood leukocytes. Positive controls were implemented in each series and consisted of cytospin preparations from known antigen-positive samples. The assay was recently adapted to an automated immunostaining machine (Shandon Cadenza, Astmoor, UK). The processing of 20 blood samples in parallel takes 6 h from the start of sample preparation until the reading of the results, whereas the completely automated immunostaining takes only 3 h. Using automated immunostaining, up to 40 blood samples may be processed by one technician during one working day.

Definitions

Active infection was defined in seropositive patients as the appearance of anti-CMV IgM antibodies or a fourfold rise in CMV IgG titer above the level at the time of transplantation within 16 weeks thereafter. In seronegative patients not treated with CMV hyperimmunoglobulin, primary infection was defined as the appearance of anti-CMV IgM or IgG antibodies. The treatment of seronegative recipients of kidneys from seropositive donors with CMV hyperimmunoglobulin resulted in a mean anti-CMV IgG titer of 800 ($\pm$ 100 SD). Anti-CMV IgM antibodies were not detected. Thus, in this group of patients, active infection was defined as the appearance of anti-CMV IgM antibodies or a fourfold rise in CMV IgG titer above the mean titer due to passive immunization within 16 weeks after transplantation.

CMV syndrome was defined according to the criteria of van den Berg et al. [3]. These are unexplained fever (> 38 °C) for 3 or more days and serological signs of active infection in conjunction with one or more of the following symptoms: arthralgia, leukopenia ($< 3 \times 10^9$/l), thrombopenia ($< 100 \times 10^9$/l), hepatitis (ALAT > 50 U/l), and pneumonia without evidence of any other cause.

Statistical analysis

The Mann-Whitney U-test and the Wilcoxon matched-pairs signed-ranks test were applied where indicated. The two-tailed probability of error is given. Sensitivity was calculated as true-positives/(true-positives + false-negatives) and specificity as true-negatives/(true-negatives + false-positives).

Results

Clinical outcome

Of the 64 patients investigated in this study, 24 (38%) showed serological signs of an active CMV infection within 12–16 weeks after transplantation. Thirteen of the 29 seronegative patients had a primary infection (45%) and 11 of the 35 seropositive patients developed a secondary infection (31%). Of the 29 seronegative patients, 19 had received a graft from a seropositive organ donor, and 12 of the 19 (63%) were actively infected, whereas the remaining 10, in contrast, had received a graft from a seronegative donor and only 1 of those 10 showed seroconversion (10%).

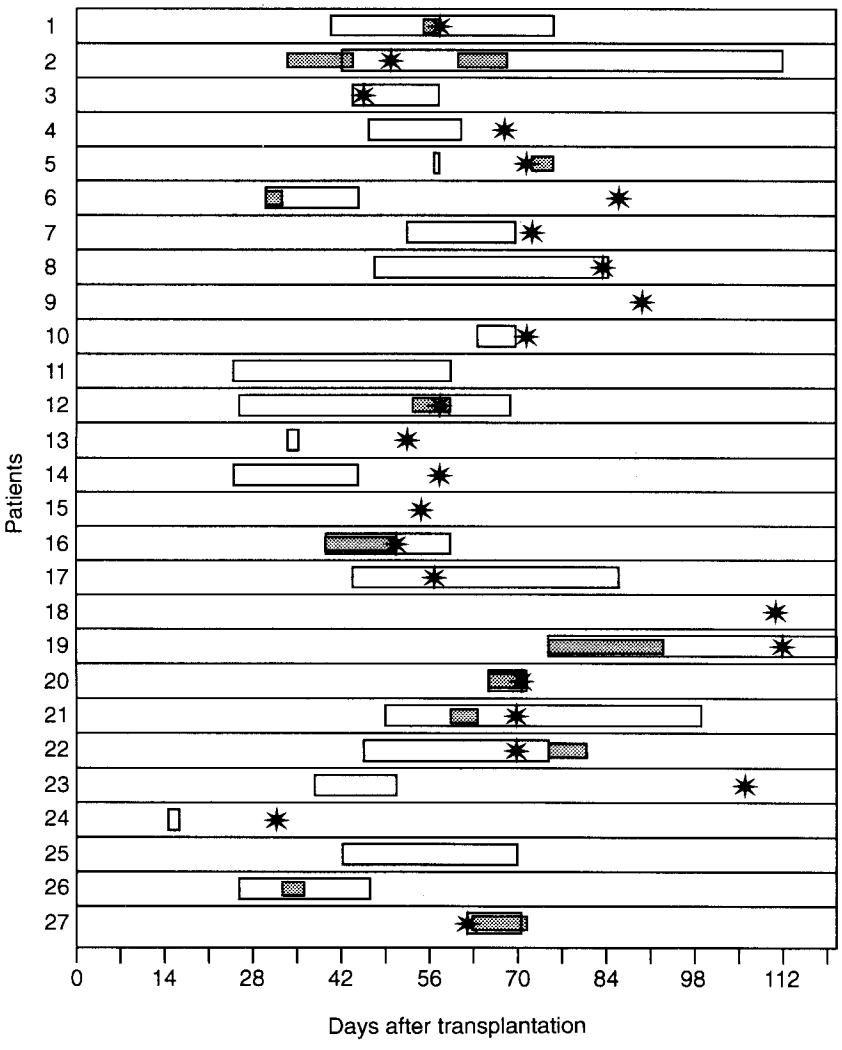

Fig. 1. Time course of antigenemia (□), CMV-related symptoms (▦), and onset of serological response (*) in 27 patients with either antigenemia or serological response for at least 12 weeks after renal transplantation. Three patients (nos. 11, 25, 26) were antigen-positive but did not show serological signs of infection, whereas 3 patients (nos. 9, 15, 18) were antigen-negative but showed serological response

Minor CMV-related symptoms occurred in four patients and another eight patients (13%) fulfilled the criteria for a CMV syndrome. Of these 12 patients, 6 underwent a primary and 2 a secondary infection. Thus, the incidence of symptomatic infection (minor symptoms and CMV syndrome) was 19% ($n = 12$). All 12 symptomatic patients had received a kidney from a seropositive donor and 10 of them were CMV-seronegative at the time of transplantation. One actively infected patient died due to multiple organ failure. However, postmortal histopathological examination did not reveal any morphological sign attributable to CMV infection in any of the clinically affected organs. The question of whether other factors, e.g., ganciclovir toxicity, led to fatal outcome remains unclear.

General comparison of CMV antigenemia and CMV serology

Since the implementation of the CMV antigenemia assay, more than 5000 blood samples have been processed in our laboratory. Of these, 1017 samples were analyzed in the present study. In 110 samples from 24 patients, at least one antigen-positive leukocyte was identified. The median number of stained cells was 4 (range 1–1000) and the immunostaining was mainly restricted to the nuclei of granulocytes. On rare occasions the nuclei of mononuclear cells showed positive signals. On very rare occasions a strong staining of the cytoplasm, i.e., cell membrane, while sparing the nucleus of large mononuclear cells (presumably monocytes), was observed. These rare, circular, stained cells were not counted as being positive. Our recently published protocol [2] proved to be highly successful. The identification of CMV antigen-positive leukocytes was clearly possible as a result of clear-cut immunostaining and the complete absence of background staining.

Twenty-one of 24 patients with an active infection, as defined by serological response, demonstrated CMV antigenemia (sensitivity 87.5%), whereas 3 patients who were CMV antigen-positive did not show serological signs of active CMV infection during the observation period of at least 12 weeks (specificity 92.5%). Those three patients who were false-negative in the antigenemia assay showed secondary infections, as judged by a fourfold IgG titer rise on days 89 and 110 in two patients and by the repeated detection of anti-CMV IgM anti-

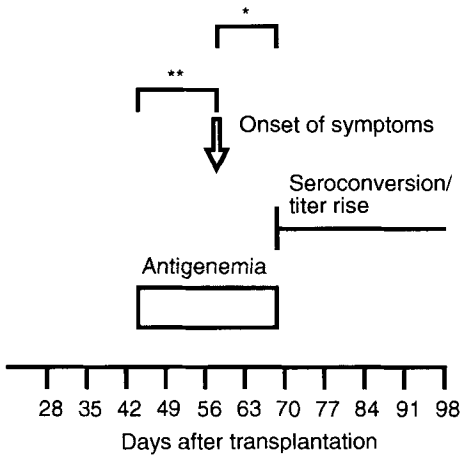

Fig. 2. Time course of antigenemia, onset of CMV-related symptoms, and serological response in 12 patients with symptomatic CMV infection after renal transplantation (median, Wilcoxon test). * $P < 0.05$; ** $P < 0.005$

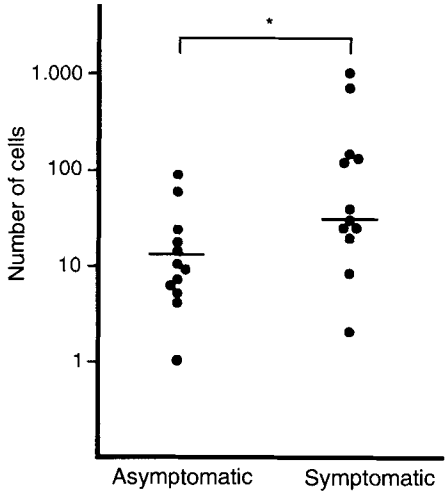

Fig. 3. Maximum number of pp65 antigen-positive cells per 400000 leukocytes in asymptomatic ($n = 12$) and symptomatic ($n = 12$) patients with active CMV infection after renal transplantation (Mann-Whitney U-test). * $P < 0.05$

bodies beginning on day 55 after transplantation in the third patient, respectively.

Time course of CMV antigenemia and antibody response in actively infected patients (Fig. 1).

The first CMV antigen-positive leukocytes occurred at a median of 43 days (range 15–75 days) after transplantation and remained detectable for a median of 20 days (range 1–91 days). In the 21 actively infected patients who developed antigenemia, the first pp65-positive leukocytes were detected at a median of 19 days (range 0–68 days) earlier than antibody response, beginning at a median of 67.5 days after transplantation (range 32–112 days). The difference between the onset of antigenemia and serological signs of infection was statistically significant ($P < 0.0001$, Wilcoxon test).

Time course of CMV antigenemia and antibody response in symptomatic patients

Antigenemia occurred in symptomatic patients (Fig. 2) at a median of 44 days (range 26–75 days) after transplantation and continued for a median of 25 days (range 1–91 days). In all patients with CMV syndrome antigenemia was present prior to the onset of the CMV-related symptoms or at the same time (sensitivity in defining CMV syndrome 100%). In contrast, there were only two patients who presented with CMV syndrome and positive serological signs on the same day. Clinical symptoms attributable to CMV syndrome occurred at a median of 9 days (range 0–29 days) after the first CMV antigen-positive test result. The difference between the onset of antigenemia and the onset of clinical symptoms was statistically significant ($P < 0.05$, Wilcoxon test). Serological signs of active infection were observed at a median of 67.5 days (range 32–112 days) after transplantation and at a median of 3 days (range 8–37 days) after the onset of disease. The difference between the onset of disease and serological signs of infection was statistically significant ($P < 0.05$, Wilcoxon test).

Quantification of antigenemia

The median maximum number of CMV antigen-positive leukocytes (per 4×10^5) was 33.5 (range 2–1000) in symptomatic patients and 12 (range 1–88) in asymptomatic patients ($P < 0.05$, Mann-Whitney U-test). Since both groups of patients showed a large, overlapping range of maximum cell counts (Fig. 3), the prediction of whether a given patient would develop CMV disease was not possible. However, no patient with counts of more than 100 antigen-positive leukocytes was free of symptoms or remained asymptomatic during follow-up. In individual patients the absolute count of positive leukocytes correlated with clinical disease activity. Initiation of ganciclovir therapy was in all cases ($n = 12$) accompanied by a rapid decline in the number of antigen-positive cells. In one patient who initially responded well to ganciclovir but developed disease relapse 4 days after withdrawal of antiviral therapy, a rapid rise in antigen-positive cells was observed simultaneously with the reappearance of clinical symptoms. After a second course of antiviral therapy, symptoms definitely disappeared, accompanied by a steady decline in the number of pp65-positive cells.

Discussion

The rapid, early and reliable diagnosis of active CMV infection is a major prerequisite for proper patient care after renal transplantation. Recently, the direct detection of CMV pp65 antigen in peripheral blood leukocytes was proposed as a useful marker of symptomatic CMV infection after renal transplantation [3]. However, two other groups failed to confirm these results [15, 21], possibly due to some major technical drawbacks of the originally published peroxidase technique which, in our hands, led to

high background staining and interpretation problems. We recently published an improved protocol employing the APAAP procedure [2] and now report on the evaluation of this assay in a longitudinal prospective study of 79 renal transplant patients.

The assay yielded a sensitivity and specificity of 87.5% and 92.5%, respectively, with active CMV infection defined by serological response and a sensitivity of 100% in the diagnosis of CMV syndrome. We consider the specificity to be even higher because the three patients with antigenemia but without serological response showed CMV DNA in peripheral blood leukocytes at the same time it was demonstrated by the polymerase chain reaction [6]. In contrast, we were unable to demonstrate CMV DNA in blood leukocytes of healthy blood donors [7]. Thus, false-negative serological testing, i.e., lack of antibody response, appeared to be very likely in those patients, one of whom was monitored for more than 16 weeks and developed anti-CMV IgM antibodies about 5 months after transplantation. These findings demonstrate that antibody response may be delayed significantly in immunocompromised patients. The high specificity of the presented antigenemia assay might be due to the APAAP procedure, where nonspecific staining was actually absent, as recently demonstrated in a comparative study of different methods of detecting antigenemia [2].

The question of whether clinical symptoms in patients 2 (first episode), 5, and 22 were indeed caused by CMV, despite the absence of antigenemia during the disease episode, was judged according to the aforementioned definitions. Additional evidence was provided by the detection of CMV DNA by the polymerase chain reaction and β_2-microglobulinuria [20] before and during the symptomatic course (data not shown). However, it is not possible to prove that active CMV infection was the only factor that contributed to the clinical symptoms. In patient 5, the pre-emptive treatment with ganciclovir may have caused clearance of pp65 antigen from the blood and, as a consequence, a negative result of the antigenemia assay during the symptomatic episode.

The biological significance of the rare pp65 antigen-positive mononuclear cells that were stained in a circular ring pattern in some patients without evidence of active CMV infection remains unclear. The staining may represent nonspecific binding of immunoglobulin molecules to Fc receptors on cellular membranes. However, one would then expect more patients to show those cells and in a higher frequency. Prospective studies on the expression of CMV pp65 antigen and other viral antigens in different blood cell populations during the post-transplant course may elucidate the clinical significance of this phenomenon.

The incidence of CMV infection during this study appeared to be unmodified by the combined acyclovir-hyperimmunoglobulin prophylaxis compared to the incidence reported in the literature [19]. This may be related to the relatively high proportion of patients treated with quadruple therapy. On the other hand, these results demonstrate that the value of prophylactic measures against CMV infection in renal transplant patients should be readdressed in clinical trials including pre-emptive ganciclovir or foscarnet therapy in high-risk patients.

The detection of pp65-positive cells preceded both the onset of serological response and, in most cases, the beginning of CMV-related symptoms and, thus, facilitated early antiviral therapy when indicated. The quantification of the number of pp65-positive cells was useful in the therapeutic monitoring of individual patients. The results obtained in this study are in agreement with those of the three groups that have thus far reported on the successful use of the antigenemia assay in different clincial settings [3–5, 8, 11, 17]. The authors reported a similar sensitivity and specificity of about 90% and found pp65 early in the course of infection. However, two other groups reported contradictory results [15, 21].

Wunderli et al. [21] diagnosed only 11 of 40 active CMV infections in 104 transplant patients by antigenemia, resulting in a sensitivity of only 27.5%. The authors used the originally published protocol [5], consisting of immunoperoxidase staining. As mentioned earlier, for us this protocol led to significant background staining due to endogenous peroxidase activity. It is possible that the authors interpreted specific staining as nonspecific in certain cases and, in doing so, decreased the sensitivity of their assay. Furthermore, they investigated only 1.5×10^5 leukocytes per sample which, in our opinion, is not enough for optimum sensitivity.

Miller et al. [15] found no antigenemia at all in 18 actively infected transplant patients, 10 of whom showed CMV syndrome. The authors came to the conclusion that their failure to demonstrate CMV antigenemia was due to the investigation of 1- or 2-day-old specimens rather than freshly isolated blood samples. We routinely process specimens successfully 24 h after collection. These are received from distant transplantation centers by overnight courier. We have no evidence that the pp65 antigen is unstable in situ, provided the granulocytes are morphologically intact.

The varying results obtained with the antigenemia assay in different studies show that the successful performance of this test requires some attention to important technical points. The number of leukocytes investigated per sample has to be sufficiently high because the frequency of pp65-positive cells during infection may be extremely low (< 1/100000). The optimal fixation of granulocytes seems to be the most crucial condition during sample preparation. On the one hand, pp65 is very sensitive to strong fixatives [2]; on the other hand, granulocytes, in particular, need a maximum of fixation in order to preserve cellular morphology. Finally, the immunocytochemical detection technique should be sensitive and specific. International standardization of the test procedure and quality control exercises should be performed in the near future to obtain comparable results between different laboratories. The widespread use of the antigenemia assay in post-transplant monitoring programs to guide early and adapted antiviral therapy may further improve the clinical course after renal transplantation.

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