CMV Seropositivity Determines Epoetin Dose and Hemoglobin Levels in Patients with CKD

Michiel G.H. Betjes, Willem Weimar, and Nicolle H.R. Litjens
Department of Internal Medicine, Division of Nephrology, Erasmus Medical Center, Rotterdam, Netherlands

ABSTRACT
Cytomegalovirus (CMV)-seropositive patients with ESRD may have more CD4\(^+\) T cells lacking the co-stimulatory molecule CD28 (CD4\(^+\)CD28null) than CMV-seronegative patients. Increased numbers of CD28null T cells associates with epoetin nonresponsiveness in patients with ESRD, but whether expansion of CD4\(^+\)CD28null T cells in CMV-seropositive patients associates with demand for epoetin is unknown. In a cohort of 129 stable patients with ESRD, CMV seropositivity significantly associated with a lower hemoglobin level in predialysis patients (12.5 versus 11.5 g/dl; \(P = 0.02\)). CMV seropositivity did not associate with average hemoglobin level in hemodialysis patients, but CMV-seropositive patients required significantly more epoetin (median 12,000 versus 6300 U/wk; \(P = 0.02\)). Multivariate linear regression analysis identified CMV seropositivity as the only variable significantly associated with hemoglobin levels in predialysis patients and epoetin dosages in hemodialysis patients. In CMV-seropositive hemodialysis patients, the number of circulating CD4\(^+\)CD28null T cells positively correlated with epoetin dosage. These CD4\(^+\)CD28null T cells were proinflammatory; they were capable of producing large amounts of IFN-\(\gamma\) and TNF-\(\alpha\). In conclusion, expansion of CD4\(^+\)CD28null T cells in CMV-seropositive patients with ESRD associates with increased demand for epoetin.


The dosage of recombinant human erythropoietin (epoetin) in patients with ESRD, necessary to reach the target plasma hemoglobin concentration, is highly variable. This variability in responsiveness has been related to many factors, including increased level of inflammation, hyperparathyroidism, insufficient iron supplementation, vitamin B\(_{12}\) or folic acid deficiency, aluminum toxicity, impurities in the dialysate, underdialysis, smoking, diabetes, race, gender, and the presence of significant residual renal function.\(^1\)–\(^7\)

Recently, we observed that in patients with ESRD, a massive increase may occur in CD4\(^+\) T cells lacking the CD28 co-stimulatory molecule on the cell surface (CD4\(^+\)CD28null T cells).\(^8\) In contrast to CD28\(^-\)CD8\(^+\) T cells, CD4\(^+\)CD28null T cells are rarely observed in cytomegalovirus (CMV)-seronegative individuals, in whom they generally do not exceed 0.5% of the total CD4\(^+\) T cells; however, in CMV-seropositive patients with ESRD, with or without renal replacement therapy (RRT), their numbers may increase substantially to as much as 40 to 60% of total circulating CD4\(^+\) T cells. Further characterization of CD4\(^+\)CD28null T cells showed a highly cytotoxic profile, with IFN-\(\gamma\) production upon CMV antigen and polyclonal stimulation. Interestingly, an association between increased numbers of CD28\(^-\) circulating T cells and nonresponsiveness to epoetin therapy in patients with ESRD has been noted;\(^9\) therefore, we hypothesized that the CMV-related induction of inflammatory CD4\(^+\)CD28null T cells may be negatively

Received April 15, 2009. Accepted August 13, 2009.
Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Michiel G. H. Betjes, Erasmus Medical Center, Department of Internal Medicine, Division of Nephrology, Dr. Molewaterplein 40, 3015 GD Rotterdam, Netherlands. Phone: 31-10-7040704; Fax: 31-10-7035756; E-mail: m.g.h.betjes@erasmusmc.nl
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associated with the responsiveness to recombinant epoetin therapy in patients with ESRD.

RESULTS

To test our hypothesis, first we investigated the relation among epoetin use, hemoglobin concentration, and CMV serostatus. The clinical and demographic characteristics of our patients with ESRD are shown in Table 1. Epoetin resistance is defined by the need for ≥300 U/kg per wk recombinant epoetin to achieve target hemoglobin levels of 11 to 12 g/dl. Only two hemodialysis patients fell in this category, and all other patients were considered to be normally responsive to epoetin therapy.

The average hemoglobin concentration (12.3 ± 1.3 g/dl) was not dependent on the CMV serostatus in the group of hemodialysis patients, but the median epoetin dosage was significantly higher in CMV-seropositive patients compared with CMV-negative patients (median 12,000 U/wk [11 U/kg body wt per Hb/wk] versus median 6300 U week [7 U/kg body wt per Hb/wk]; \( P = 0.02 \); Figure 1). A multivariate linear regression analysis showed that only CMV seropositivity was significantly associated with epoetin dosage (Table 2). The anti-CMV IgG titers did not correlate with hemoglobin levels or epoetin use.

In patients who had ESRD and were not on RRT, the majority were not on epoetin therapy (Table 1), but the average hemoglobin concentration was significantly lower in CMV-seropositive predialysis patients as compared with CMV-negative patients (12.5 versus 11.5 g/dl; \( P = 0.02 \); Figure 1). After multivariate linear regression analysis, only CMV seropositivity remained significantly associated with hemoglobin concentration (Supplemental Table 3). These results identify for the first time an association between CMV seropositivity and the regulation of hemoglobin level in patients with ESRD.

We hypothesized that the CMV-associated appearance of high numbers of proinflammatory CD4^+CD28null T cells may have a pathophysiologic role in the regulation of erythropoiesis and/or erythrocyte breakdown; therefore, we investigated whether the numbers of circulating CD4^+CD28null T cells (identified as shown in Figure 2, A–D) that appear in CMV-seropositive individuals are specifically related to epoetin use. Indeed, a statistically significant correlation was found between the percentage and total number of CD4^+CD28null T cells and the dosage of epoetin given to CMV-seropositive hemodialysis patients (Figure 2, E and F). For example, every 10% increase in circulating CD4^+CD28null T cells increased the median demand for epoetin by as much as 20 to 40% (Figure 2G). For comparison, such a relation could not be found for CD8^+CD28null T cells and epoetin dosage, although CMV-seropositive patients also had an increased percentage of CD8^+CD28null T cells (data not shown).

Finally, we investigate the proinflammatory profile of cir-

Table 1. Demographic and clinical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hemodialysis Patients (n = 84)</th>
<th>Predialysis Patients (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender (%)</td>
<td>68</td>
<td>69</td>
</tr>
<tr>
<td>Age (yr; mean ± SD)</td>
<td>57.4 ± 15.3</td>
<td>47.1 ± 16.9</td>
</tr>
<tr>
<td>Ethnic background Western European/non-Western European</td>
<td>58/26</td>
<td>38/7</td>
</tr>
<tr>
<td>Years on dialysis</td>
<td>2.9 ± 3.2</td>
<td>–</td>
</tr>
<tr>
<td>CMV seropositive/seronegative status</td>
<td>62/22</td>
<td>16/29</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>41</td>
<td>7</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>42</td>
<td>34</td>
</tr>
<tr>
<td>Residual diuresis</td>
<td>46</td>
<td>–</td>
</tr>
<tr>
<td>Kidney disease (%)</td>
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<td></td>
</tr>
<tr>
<td>hypertensive nephropathy</td>
<td>45</td>
<td>27</td>
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<tr>
<td>glomerulonephritis</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>diabetic nephropathy</td>
<td>18</td>
<td>6</td>
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<tr>
<td>polycystic kidney disease</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>reflux nephropathy</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>other</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>unknown</td>
<td>7</td>
<td>9%</td>
</tr>
<tr>
<td>Hemoglobin concentration (g/dl)</td>
<td>12.3 ± 1.3</td>
<td>12.1 ± 1.4</td>
</tr>
<tr>
<td>CRP concentration (mg/L; median [IQR])</td>
<td>4 (2 to 11)</td>
<td>2 (1 to 6)</td>
</tr>
<tr>
<td>Serum albumin concentration (g/dl)</td>
<td>4.3 ± 0.4</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>521 ± 450</td>
<td>145 ± 113</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>30.0 ± 19.3</td>
<td>26.1 ± 18.3</td>
</tr>
<tr>
<td>Parathyroid hormone (pg/ml; median [IQR])</td>
<td>320 (150 to 820)</td>
<td>280 (170 to 260)</td>
</tr>
<tr>
<td>Patients using recombinant erythropoietin (%)</td>
<td>99</td>
<td>35</td>
</tr>
<tr>
<td>Dosage of recombinant erythropoietin (mean ± SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U/wk</td>
<td>10,399 ± 6310</td>
<td>1757 ± 2476</td>
</tr>
<tr>
<td>U/kg per wk</td>
<td>141 ± 96</td>
<td>23 ± 34</td>
</tr>
<tr>
<td>U/kg Hb per wk</td>
<td>12 ± 8</td>
<td>2 ± 3</td>
</tr>
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</table>

IQR, interquartile range.
calculating CD4⁺ CD28null T cells of patients with ESRD in more detail. We measured both unstimulated and polyclonal-stimulated production of IFN-γ, TNF-α, and IL-10 by CD28⁺ and CD28⁻ CD4⁺ T cells by intracellular cytokine staining. The average percentage of IFN-γ- and TNF-α-positive CD4⁺ T cells was significantly increased in CMV-seronegative hemodialysis patients compared with CMV-seropositive patients (mean fluorescence intensity 6299.4 ± 3.2 versus 150.0 ± 1.7; P < 0.01; Supplemental Figure 3). The increased percentage of IFN-γ- and TNF-α-positive CD4⁺ T cells in CMV-seropositive patients could be largely attributed to the expansion of CD4⁺ CD28null T cells. This observation was most striking for TNF-α, because the total increase in the percentage TNF-α-producing CD4⁺ T cells (on average 20%) was caused by the expanded CD4⁺ CD28null T cell population (Supplemental Figure 3D).

In addition, even without stimulation, the percentage of TNF-α-producing CD4⁺ T cells was increased (1.1 ± 0.2 versus 0.4 ± 0.1% in CMV-seronegative patients; P < 0.01), indicating an increased basal TNF-α production in CD4⁺ T cells from CMV-seropositive patients. On average, 2% of CD4⁺ CD28null T cells spontaneously produced TNF-α compared with 0.6% of CD4⁺ CD28⁺ T cells (P < 0.01).

Semi-quantitative analysis of the cytokine fluorescence signal showed a difference on the single-cell level of TNF-α production between CD4⁺ T cells from CMV-seropositive and CMV-seronegative patients (mean fluorescence intensity 4763.3 ± 427.3 versus 3363.6 ± 314.7; P < 0.01). This could be attributed to a much higher TNF-α production for CD4⁺ CD28null T cells compared with CD4⁺ CD28⁺ T cells (mean fluorescence intensity 6299.4 ± 621.9 versus 4103.5 ± 337.7; P < 0.01; Supplemental Figure 4); therefore, the number of proinflammatory cytokine-producing CD4⁺ T cells is increased in CMV-seropositive patients as a result of the expanded CD4⁺ CD28null T cells. In addition, specifically the TNF-α production per CD4⁺ CD28null T cell is exceptionally high.

DISCUSSION

Both IFN-γ and TNF-α are known for their direct negative effects on erythropoiesis, and TNF-α can shorten the erythrocyte half-life because of an increased phagocytosis by macrophages. Importantly, it has been shown that the suppressive effect of uremic serum on erythropoiesis in vitro could be blocked by addition of anti-IFN-γ and anti-TNF-α antibodies; therefore, the presence of an expanded CD4⁺ CD28null T cell population with an exceptionally high proinflammatory profile offers a pathogenetic concept for the association among CMV seropositivity, anemia, and epoetin dosing.

The increased basal TNF-α production of CD4⁺ CD28null T cells compared with CD4⁺ CD28⁺ cells may explain the effect of these cells on erythropoiesis in stable dialysis patients. In addition, it is conceivable that at times of inflammation, the cytokine release by CD4⁺ CD28null T cells is stimulated, adding to a blunted epoetin response. Serum levels of inflamma-
tory cytokines, notably TNF-α, are increased in patients with ESRD.13 Whether TNF-α released from CD4⁺CD28null T cells contributes significantly to these increased plasma levels remains to be investigated. The relationship with other TNF-α–producing immune cells, in particular monocytes/macrophages, is likely to be complex, because monocytes and macrophages both can activate T cells and can be activated by, for example, IFN-γ from T cells.

Only two studies have addressed the role of cytokine production by circulating T cells from patients with ESRD in relation to epoetin nonresponsiveness.6,14 Both studies showed an increased percentage of IFN-γ– and TNF-α–producing CD4⁺ T cells in patients with ESRD compared with healthy individuals; however, data are conflicting: Cooper et al.14 found significantly increased numbers of cytokine-producing T cells only in the epoetin nonresponder group, whereas Costa et al.6 found similar increased numbers of cytokine-producing T cells for epoetin responder and nonresponder patients. Notably, both authors described an increased percentage of CD28null CD4⁺ T cells in relation to epoetin responsiveness.

The stable patients with ESRD in this study differ from the patients included in the studies discussed, because they had only moderately increased serum C-reactive protein (CRP) levels and normal average serum albumin concentration and were considered epoetin responsive. We accounted as much as possible for variables that are known to be associated with epoetin responsiveness; however, the possibility of a confounder that is associated with CMV seropositivity and influences the epoetin responsiveness of the patient with ESRD cannot be fully excluded.

The CMV-induced CD4⁺CD28null T cells are likely to be involved in the pathogenesis of cardiovascular disease because CMV seropositivity is associated with the presence of cardiovascular disease15–17 and unstable angina.18 The latter is probably related to the infiltration of plaques by cytotoxic CD4⁺CD28null T cells, which cause plaque vulnerability and rupture19; therefore, CMV-induced CD4⁺CD28null T cells may represent one of the traits d’union that explains the association between anemia/epoetin resistance and cardiovascular disease and mortality in patients with ESRD. This association has consistently been observed for patients who have ESRD and are or are not on dialysis.20–26 Whether an expanded CD4⁺CD28null T cell population in CMV-seropositive patients with ESRD is indeed associated with cardiovascular disease is being investigated in a large cohort of patients.

In conclusion, this study strongly suggests a novel pathophysiologic relation among CMV seropositivity, expansion of proinflammatory CD4⁺CD28null T cells, and negative regulation of hemoglobin levels in patients with ESRD.

**CONCISE METHODS**

**Study Population**

Patients were recruited for the study from our outpatient clinic for renal transplantation. All patients had ESRD, defined as an estimated GFR of <16 ml/min using the Modification of Diet in Renal Disease (MDRD) formula, with or without RRT. Patients who had a hemoglobinopathy, malignancy, or active infection or were taking immunosuppressive drugs...
were excluded. The clinical and demographic data of the patients are shown in Table 1. Patients who were on RRT were treated with hemodialysis thrice weekly for 4 to 5 h, using an arteriovenous fistula or graft as access. Water for dialysis was prepared by the use of reverse osmosis according to European guidelines.27 The bacteriologic quality of the dialysate was measured at regular intervals according to European guidelines (endotoxin levels <0.25 IU/ml and <100 colony-forming units/ml). The targeted sKt/V was ≥3.6/wk. Residual diuresis per 24 h, measured between two dialysis sessions, was taken as a surrogate measure for residual renal function. No patient included used aluminum-containing drugs, and all hemodialysis patients received supplements of folic acid, vitamin B12, and intravenous iron. Human recombinant epoetin was given to achieve a plasma hemoglobin level between 11.0 to 12.0 g/dl. Darbepoetin dosages were converted to units by multiplying micrograms by 200. Patients included gave informed consent, and the local medical ethical committee approved the study. It was conducted according to the principles of the Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations.

Isolation of Peripheral Blood Mononuclear Cells
Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood samples before hemodialysis and stored at −80°C.28 Before use, PBMCs were quickly thawed in RPMI 1640 (GibcoBRL, Paisley, Scotland) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 20% heat-inactivated AB+ pooled human serum (standard culture medium) with DNase (0.1 mg/ml; Roche Diagnostics GmbH, Mannheim, Germany). Finally, the cells were resuspended in standard culture medium at 6 × 10⁶/ml.

Flowcytometric Analysis of CD28 Expression
To determine the percentages and amount of the various T lymphocytes, we performed a whole-blood staining as described previously29 but with some minor adjustments. Briefly, to examine expression of CD28 within the various T lymphocyte subsets, we used peridinin chlorophyll protein–labeled anti-CD4 or -CD8 in combination with Amcyan-labeled CD3, allophycocyanin-labeled anti-CD45RO (all from BD Pharmingen, Erebodegem, Belgium) and FITC-labeled anti-CCR7 (R&D Systems Europe Ltd., Abingdon, UK) and phycoerythrin (PE)-labeled anti-CD28 (BD Pharmingen). Analysis was performed using the FACS Canto II (BD Pharmingen) and FACSDIVA software (BD Pharmingen).

Intracellular Cytokine Staining
The intracellular cytokine assay was performed as described recently in detail.30 Briefly, PBMCs (3 × 10⁶/tube) were stimulated with standard culture medium or with a mixture of phorbol myristate acetate (50 ng/ml; Sigma Aldrich, Zwijndrecht, Netherlands) and ionomycin (1 μg/ml; Sigma Aldrich) for 6 h in the presence of the cytokine secretion inhibitor Golgiplug (BD Pharmingen). To analyze cytokine-producing T cells, we stained the cell surface of PBMCs with AmCyan-labeled anti-CD3, Pacific Blue–labeled anti-CD4 (BD Pharmingen), allophycocyanin-Cy7–labeled anti-CD8 (BD Pharmingen), and FITC-labeled anti-CD28 (BD Pharmingen). Dead cells were excluded from the analysis using 7-AAD (viability marker; BD Pharmingen). After fixation and permeabilization, cells were stained with PE-labeled anti–IFN-γ (BD Pharmingen), anti–TNF-α (BD Pharmingen), or anti–IL-10 (BD Pharmingen). Percentages of cytokine-positive cells were determined by analyzing the samples using the FACSCanto II (BD Pharmingen), similar as described already. To estimate the average amount of antibodies bound per cell, we included PE-labeled beads (QuantiBRITE PE; BD Pharmingen). Using the lot-specific fluorochrome/protein ratio for the different PE-labeled cytokines, the geometric means obtained for the cytokines can be converted into average numbers of antibodies bound per cell.

CMV Serology and CRP Level
Serum IgG antibodies to CMV were measured with an enzyme immunoassay (Biomerieux; VIDAS, Lyon, France) and expressed as arbitrary units per milliliter (AU/ml). Following the manufacturer’s guidelines, a test result exceeding 6 AU/ml was considered positive for the presence of CMV-specific IgG antibodies. The CRP level was measured with a fluorescence polarization immunoassay (TDxFLEx analyzer; Abbott Laboratories); 95% of healthy individuals had a CRP level of ≤8 mg/L. All other laboratory test were performed using standard clinical chemistry assays.

Statistical Analysis
Differences between groups were analyzed with the t test when the variable was normally distributed and otherwise by the Mann-Whitney test. Categorical data were analyzed by the χ² test. All statistical tests were two-sided. Univariate and multivariate linear regression analysis was performed on log-transformed epoetin dosage expressed as U/kg body wt per Hb/wk. Statistical analysis was performed using SPSS 10.1 software, and P < 0.05 was considered statistically significant.

ACKNOWLEDGMENTS
The Malpighi Foundation financed this study.

DISCLOSURES
None.

REFERENCES

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