An Increase in Myeloid-Related Protein Serum Levels Precedes Acute Renal Allograft Rejection

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Abstract. Upon interaction with activated endothelium, monocytes and neutrophils form complexes of myeloid-related protein 8 (MRP8) (S100A8) and MRP14 (S100A9), two members of the calcium-binding S100 family that are secreted during transendothelial migration. In a pilot study of 20 renal transplant recipients and a validation study of 36 renal transplant recipients, MRP8/14 serum levels were measured with an enzyme-linked immunosorbent assay for 28 d, associated with C-reactive protein and creatinine serum levels, and grouped according to biopsy-proven acute rejection. Serum levels of MRP8/14 but not C-reactive protein were significantly increased for several days during the first 2 wk for the acute rejection groups in both studies (P < 0.005, on day 6 after transplantation). As determined by using receiver operating characteristic curves, the optimal cutoff for 100% specificity and high sensitivity (67%) for acute rejection on day 6 after transplantation was calculated to be 4.2 μg/ml for MRP8/14 in the pilot study; this value was confirmed in the validation study. Positive MRP8/14 serum levels preceded acute rejection episodes by a median of 5 d. A 3-d course of intravenous methylprednisolone therapy reduced prerejection MRP8/14 serum levels from 5.7 μg/ml to 3.3 μg/ml (P < 0.05). All MRP8/14 serum levels were below the cutoff during urinary tract infections, delayed graft function, or cytomegalovirus infections, and these values did not differ significantly from control values. It is concluded that the MRP8/14 complex is a very early serum marker suitable for monitoring of acute rejection with high sensitivity and specificity.

Acute rejection is a complex inflammatory process influenced by the innate and adaptive immune systems of the renal transplant recipient and the immunosuppressive therapy. Over- and under-immunosuppression are clinical problems that have not been completely solved. A reliable, sensitive, specific early marker for acute rejection is still missing.

Cytokines and chemokines have short half-lives and are designed for communication between individual cells across very short distances in the local microenvironment. Serum concentrations of cytokines are thus very low and difficult to measure and usually exhibit low sensitivity and specificity for rejection episodes. Therefore, although a few authors reported increased serum cytokine levels during rejection (1–4), other authors could not confirm those results with the same or other cytokines/chemokines or their soluble ligands/receptors (5,6). Kiln biopsy, which is still the standard technique, cannot be performed daily, and its significance can be reduced by focal sampling errors or the presence of a mononuclear infiltrate despite stable graft function. In many cases, only tubulitis and endotheliitis (according to the Banff classification) are specific signs of aggressive acute allograft rejection (7).

Monocytes/macrophages and neutrophils play important roles in the initial inflammatory and immunologic responses after transplantation. After activation of these cells by activated endothelium, a complex of two preexisting, cytoplasmic, calcium-binding proteins, i.e., myeloid-related protein 8 (MRP8) (S100A8) and MRP14 (S100A9), is rapidly formed via calcium influx and protein kinase C activation. In the form of heterodimers that self-polymerize along the microtubules, MRP8/14 binds and connects the cytoskeleton and the cell surface, also affecting actin polymerization. Finally, MRP8/14 is secreted by monocytes/neutrophils during transendothelial migration into the extracellular space, i.e., into the serum (8–13).

MRP8 and MRP14 are abundant proteins in neutrophils and monocytes, comprising up to 20% of the cytoplasmic protein content (14). They are members of the S100 family of calcium-binding proteins. S100 proteins play intracellular roles in the nervous system and the immune system and in calcium-dependent signaling, cell differentiation, cell cycle progression, regi-
ulation of kinase activities, and cytoskeleton-membrane interactions (15,16).

MRP8 and MRP14 have molecular masses of 8 kD and 14 kD, respectively, and their three-dimensional structures were recently described (17). In the literature, several synonyms have been used, i.e., L1 antigen light and heavy chains, calgranulin A and B, and calprotectin (18–21). In mice, MRP8 is one of the strongest granulocyte-attracting chemokines (22,23). A null mutation for MRP8 causes early resorption of murine embryos by day 9, demonstrating the importance of this molecule (24).

The MRP8/14 complex has been detected in many acutely inflamed tissues and the serum of patients with acute inflammatory diseases (25–29). MRP8/14-positive monocytes and neutrophils are activated cells that are able to produce large amounts of interleukin-6, interleukin-1, and tumor necrosis factor-α (12). Immunohistologic data provide indirect evidence that monocytes release MRP8/14 during endothelial diapedesis at sites of inflammation (18). It was recently demonstrated that neutrophils/monocytes secrete soluble MRP8/14 in an intercellular adhesion molecule-1 (ICAM-1)-dependent process of transendothelial migration (13).

The aim of this study was to determine whether acute renal allograft rejection, which is always accompanied by increased transendothelial migration of leukocytes into the graft, would be associated with increases in the serum levels of soluble MRP8/14. The specificity and sensitivity of this new marker for acute rejection should be assessed in comparison with those of C-reactive protein (CRP; another inflammatory parameter) and creatinine (a marker for renal dysfunction).

Materials and Methods

General Follow-up Monitoring of Patients

All patients were studied longitudinally after renal transplantation during the first hospitalization. The follow-up period was 28 d for all patients. Relevant clinical parameters, such as creatinine levels, CRP levels, 24-h urine volume, BP, temperature, leukocyte numbers, hemoglobin levels, and platelet numbers, were measured daily in the first 2 wk and at least three times per week thereafter. Twelve-hour morning trough levels for cyclosporine A and tacrolimus were measured at least three times per week. The perfusion of the renal transplant was determined within 48 h after transplantation by using scintigraphy or duplex sonography and was reassessed when clinically appropriate. Close follow-up monitoring for possible complications, such as urinary tract infection (UTI), hematomas, lymphoceles, and cytomegalovirus (CMV) infection, was performed. No regular CMV prophylaxis was administered, but selected patients with a high-risk CMV constellation (donor positive/recipient negative) received oral ganciclovir prophylaxis. Patients with CMV PCR positivity were treated with orally administered ganciclovir, and patients with pp65-positive leukocytes or CMV disease were treated with intravenously administered ganciclovir. The diagnosis of rejection (n = 25) was based on clinical symptoms and was routinely documented by histologic analysis of renal biopsies (n = 22). The decision to perform a renal biopsy was based on at least an increase in serum creatinine levels, compared with baseline values.

### Table 1. Patient data for the pilot study (n = 20), grouped on the basis of acute rejection.

<table>
<thead>
<tr>
<th>Acute Rejection (n = 7)</th>
<th>No Acute Rejection (n = 13)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age (yr)</td>
<td>49 (24)</td>
<td>48 (27)</td>
</tr>
<tr>
<td>Donor age (yr)</td>
<td>47 (27)</td>
<td>39 (32)</td>
</tr>
<tr>
<td>Mismatches (no.)</td>
<td>3 (1)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Cold ischemia time (h)</td>
<td>19 (9)</td>
<td>18 (5)</td>
</tr>
<tr>
<td>Warm ischemia time (min)</td>
<td>32 (16)</td>
<td>28 (19)</td>
</tr>
<tr>
<td>Delayed function (d)</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Rejection day postoperative Banff classification</td>
<td>7 (21)</td>
<td></td>
</tr>
<tr>
<td>4,IA</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4,IB</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4,IIA</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4,IIIB</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MP rescue</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>MMF/AZA</td>
<td>2/4</td>
<td>12/1</td>
</tr>
<tr>
<td>MMF switch</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Delayed graft function</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Donor type (cd/LRD)</td>
<td>6/1</td>
<td>13/0</td>
</tr>
<tr>
<td>Recipient gender (M/F)</td>
<td>3/4</td>
<td>9/4</td>
</tr>
</tbody>
</table>

\(a\) Values are median (interquartile range) or numbers of patients. MP, methylprednisolone; MMF, mycophenolate mofetil; AZA, azathioprine; cd, cadaver; LRD, living related donor; PRA, panel reactive antibodies; CyA, cyclosporine A; FK, tacrolimus; ATG, antithymocyte globulin; CMV, cytomegalovirus; RTx, renal transplantation; CI, calcineurin inhibitor.

\(b\) The use of methylprednisolone boluses for the rejection group and the predominant use of mycophenolate mofetil in the nonrejection group were the only significant differences between the two groups.

Patients in the Pilot Study

Twenty consecutive patients were included in this study after renal transplantation at our transplant center between April and August 1997. The patient data, grouped on the basis of acute rejection, are summarized in Table 1. The two groups, i.e., the acute rejection and nonrejection groups, were not significantly different with respect to recipient age, recipient gender, donor age, donor type (cadaver donor,
or living related donor), cold ischemia time, warm ischemia time, number of patients with delayed graft function (DGF), number of days with DGF, CMV donor/recipient constellation, or complications such as UTI, CMV PCR positivity, lymphoceles, hematomas, infected hematomas, or transplant nephritis.

Calcineurin inhibitor-based, triple-drug, immunosuppressive therapy, including mycophenolate mofetil (MMF) or azathioprine plus methylprednisolone, was used as the standard immunosuppressive regimen for all 20 patients. The use of MMF was significantly greater for the nonrejection group (P = 0.017). Cyclosporine A was administered orally beginning on day 1 with 8 mg/kg body wt; the dose was then adjusted according to trough serum cyclosporine A levels determined using a monoclonal antibody assay. The median trough level for all patients in the first 4 wk after transplantation was 276 ng/ml (interquartile range [IQR], 58 ng/ml). Tacrolimus was also administered orally, at a dose of 0.2 mg/kg body wt, from the day of transplantation. The median trough level in the first 4 wk after transplantation was 14 ng/ml (IQR, 5 ng/ml).

The rejections (seven rejections, of which five were biopsy-proven) occurred a median of 7 d (IQR, 21 d) after transplantation. All rejections were the first rejections after transplantation. Histologic analyses revealed one 4,IA rejection, two 4,IIA rejections, and two 4,IIIB rejections, according to the Banff classification. In one case, a core biopsy was not performed because of thrombopenia. An early acute rejection episode in another patient was treated with three steroid boluses, plus a successful switch from azathioprine to MMF, without biopsy.

**Patients in the Validation Study**

Thirty-six patients were included in the validation study. The patients received consecutive renal grafts at our transplant center between July and December 1999. Eighteen of these patients experienced at least one acute rejection episode during the first 4 wk after transplantation. As in the pilot study, the acute rejection and nonrejection groups in the validation study were not significantly different in most respects (Table 2).

As in the pilot study, all patients were treated with a calcineurin inhibitor-based, triple-drug, immunosuppressive regimen. Cyclosporine A was switched to tacrolimus for nine patients in the acute rejection group, one patient required OKT-3 administration, and azathioprine was switched to MMF for two patients. One patient experienced two rejection episodes, which were treated with intravenously administered methylprednisolone, within the first 4 wk after transplantation. First rejections (18 rejections, of which 17 were biopsy-proven) occurred a median of 11 d (IQR, 7 d) after transplantation. Using the Banff nomenclature, two rejections were classified as 3, seven as 4,IA, four as 4,IB, and two each as 4,IIA and 4,IIIB. For one acute rejection of a kidney from a living donor, methylprednisolone was administered without biopsy.

**Collection of Samples and Determination of MRP8/14 Serum Levels**

After transplantation, serum samples were obtained daily for the first 2 wk for each patient and were frozen at −20°C. From day 15 to day 28, samples were obtained three times per week. Serum creatinine and CRP level determinations, white blood cell and platelet counts, and hemoglobin measurements were performed in the routine laboratory on the same days.

A sandwich enzyme-linked immunosorbent assay for the detection of soluble MRP8/14 (BMA Biomedicals, Augst, Switzerland) was used (30). Briefly, the test for the MRP8/14 heterodimer is based on the selective recognition of one epitope on MRP14 by one monoclonal antibody and that of another epitope, produced by the formation of the heterodimer by MRP8 and MRP14, by the monoclonal antibody 27E10 (31). The assay exhibited a linear range from 0.3 to 20 μg/ml. Normal values for serum MRP8/14 levels were 0.6 ± 0.2 μg/ml.

<table>
<thead>
<tr>
<th>Rejection Day Postoperative</th>
<th>Acute Rejection (n = 18)</th>
<th>No Acute Rejection (n = 18)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP rescue</td>
<td>18/2</td>
<td>0/1</td>
<td>0.000</td>
</tr>
<tr>
<td>FK rescue</td>
<td>9/0</td>
<td>0/1</td>
<td>0.001</td>
</tr>
<tr>
<td>MMF switch</td>
<td>2/0</td>
<td>0/1</td>
<td>0.486</td>
</tr>
<tr>
<td>OKT-3 rescue</td>
<td>1/0</td>
<td>0/1</td>
<td>1.000</td>
</tr>
<tr>
<td>Delayed graft function</td>
<td>10/4</td>
<td>0/4</td>
<td>0.088</td>
</tr>
<tr>
<td>Donor type (cd/LRD)</td>
<td>12/6</td>
<td>17/1</td>
<td>0.088</td>
</tr>
<tr>
<td>Recipient gender (M/F)</td>
<td>11/7</td>
<td>11/7</td>
<td>1.000</td>
</tr>
<tr>
<td>PRA &gt;30%</td>
<td>1/1</td>
<td>0/1</td>
<td>1.000</td>
</tr>
<tr>
<td>2nd or 3rd RTx</td>
<td>3/2</td>
<td>2/2</td>
<td>1.000</td>
</tr>
<tr>
<td>CyA/FK as initial CI</td>
<td>16/2</td>
<td>14/4</td>
<td>0.658</td>
</tr>
<tr>
<td>MMF/AZA</td>
<td>13/5</td>
<td>15/3</td>
<td>0.691</td>
</tr>
<tr>
<td>ATG induction</td>
<td>1/3</td>
<td>1/3</td>
<td>0.603</td>
</tr>
<tr>
<td>Anti-IL-2R induction</td>
<td>3/1</td>
<td>1/1</td>
<td>0.603</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>4/5</td>
<td>5/5</td>
<td>1.000</td>
</tr>
<tr>
<td>CMV PCR positive</td>
<td>3/0</td>
<td>0/0</td>
<td>0.229</td>
</tr>
<tr>
<td>Lymphocele</td>
<td>2/0</td>
<td>0/0</td>
<td>0.486</td>
</tr>
<tr>
<td>Hematoma</td>
<td>0/1</td>
<td>1/0</td>
<td>1.000</td>
</tr>
<tr>
<td>Candidiasis</td>
<td>1/0</td>
<td>0/1</td>
<td>1.000</td>
</tr>
<tr>
<td>Urine fistula</td>
<td>1/0</td>
<td>0/1</td>
<td>1.000</td>
</tr>
<tr>
<td>Toxic colonic dilation</td>
<td>0/1</td>
<td>1/0</td>
<td>1.000</td>
</tr>
<tr>
<td>AV shunt occlusion</td>
<td>0/1</td>
<td>1/0</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Values are median (interquartile range) or numbers of patients. IL-2R, interleukin-2 receptor; AV, arteriovenous. All other abbreviations are as in Table 1.

The use of methylprednisolone boluses and tacrolimus rescue were the only significant differences between the two groups.
(mean ± SD) for 18 healthy control subjects. All serum samples were measured in duplicate (coefficient of variation, <5%). The intra-test variability coefficient was 6.7%, and the inter-test variability coefficient was 11.7%.

**Histologic Analyses**

Conventional histologic analyses with at least four stains (hematoxylin/eosin, periodic acid-Schiff stain, trichrome stain, and silver methenamine) were performed on paraffin sections. Biopsies were scored using the 1997 Banff diagnostic categories for renal allograft biopsies (7).

**Statistical Analyses**

Mean values were stated with the SD. Median values were stated with the IQR. Receiver operating characteristic (ROC) curves were calculated as described by Armitage and Berry (32) and Metz (33). Group comparisons of frequencies were performed with the two-sided Fisher exact test. The two-sided Mann-Whitney U test was used for group comparisons of continuous and rank-scaled variables. P values of ≤0.05 were considered significant. The Wilcoxon test was used for comparisons of paired data. Spearman’s correlation coefficient was used for correlations of rank-scaled variables. Analysis of covariance was used for the calculation of within-subject correlation coefficients for approximately normally distributed data from repeated measurements. All calculations were performed using SPSS for Windows (SPSS Inc., Chicago, IL).

**Results**

*Significantly Increased MRP8/14 Serum Levels Were Observed for the Rejection Group in the Pilot Study, with High Sensitivity and Specificity for Rejection*

The 20 renal transplant recipients in the pilot study were divided into an acute rejection group and a non-acute rejection group, as described in the Materials and Methods section (Table 1). Medians and IQR values for both groups were calculated for MRP8/14, CRP, and creatinine levels for each day of the first 4 wk after transplantation. On day 1 after transplantation and continuing on days 4, 6, 7, 11, and 13, the median MRP8/14 serum levels for the acute rejection group were significantly higher than the corresponding median MRP8/14 serum levels for the nonrejection group (Figure 1A). However, no significant group differences were detected for CRP (Figure 1B) or creatinine (Figure 1C) serum levels on each of days 0 to 28.

ROC curves, which are very useful tools for demonstrating the reciprocal dependence of sensitivity and specificity for all possible cutoff levels for a given test (32,33), were used to determine the optimal cutoff, combining the greatest specificity and sensitivity. The day after transplantation with the greatest specificity and sensitivity was determined by calculating the area under the ROC curve (AUC) for every day after transplantation. Although all AUC values from day 3 to day 9 after transplantation were above 0.75, day 6 was observed to have the highest AUC value, i.e., 0.931. Knowing that all MRP8/14 serum levels increase above 4.2 μg/ml at least 1 d before rejection, which is identical to a sensitivity of 100% (see the Discussion section), we chose 4.2 μg/ml as the lowest and optimal cutoff level to yield 100% specificity. With an AUC of 0.931, this specificity was combined with a high sensitivity of 67% by day 6 after transplantation for the detection of rejections that could be diagnosed by conventional methods between day 6 and day 28 (Figure 2). The other coordinates of the

![Figure 1. Median myeloid-related protein 8/14 (MRP8/14) (A), C-reactive protein (CRP) (B), and creatinine (C) serum levels in the pilot study, grouped according to acute rejection (○, acute rejection group; □, nonrejection group), during the first 4 wk after transplantation. The upper and lower bars represent the 75th and 25th percentiles of all measured values, respectively. *, P < 0.05; **, P < 0.01; ***, P < 0.005 for the acute rejection group versus the nonrejection group.](https://example.com/figure1.png)
ROC curve indicate, for example, that a lower cutoff of 3.2 μg/ml would result in a slightly lower specificity of 92% combined with a higher sensitivity of 83%.

**The Validation Study Confirmed Significant Increases in MRP8/14 Serum Levels for the Rejection Group and the Cutoff Value of the Pilot Study**

Figure 3 presents the MRP8/14, CRP, and creatinine serum levels for the 36 patients in the validation study. As in the pilot study, MRP8/14 serum levels were increased in the acute rejection group from the first days after transplantation, for up to 2 wk (continuously significant from day 2 to day 10 and on days 12 and 14) (Figure 3A). The day with the best discrimination between the acute rejection and nonrejection groups was again day 6 after transplantation ($P = 0.001$). The other inflammatory marker, CRP (Figure 3B), failed to demonstrate a significant difference between the rejection and nonrejection groups ($P = 0.311$ on day 6 after transplantation). Serum levels of creatinine, the marker for renal dysfunction (Figure 3C), were also not able to differentiate significantly between the rejection and nonrejection groups ($P = 0.214$ on day 6 after transplantation).

As in the pilot study, ROC curves were generated for each day after transplantation. High AUC values, between 0.74 and 0.90, could be confirmed for all MRP8/14 ROC curves from day 2 to day 12 after transplantation in the validation study. The highest AUC value, 0.89, of all ROC curves was measured on day 6 after transplantation, as in the pilot study. On day 6, the optimal cutoff of 4.2 μg/ml, which was originally calculated for the pilot study, discriminated between the rejection
and nonrejection groups of the validation study with a specificity of 100% and a sensitivity of 73% (Figure 4). Compared with the pilot study, these values represented a 6% greater sensitivity at the same specificity.

The median of the first day of rejection detected with conventional diagnostic methods and the first day of intravenous bolus methylprednisolone antirejection therapy was 11 d (IQR, 7 d) after transplantation (Table 2). The first positivity of MRP8/14 serum levels, predicting rejection, occurred at a median of 5 d (IQR, 4 d) before the conventional diagnosis of rejection and the beginning of intravenous bolus methylprednisolone antirejection therapy, i.e., a median of day 6 after transplantation.

**MRP8/14 Is a Superior Marker for Rejection, Compared with CRP**

MRP8/14 exhibited greater specificity and sensitivity for the diagnosis of rejection, compared with another inflammatory marker, CRP. On day 6 after transplantation, CRP measurements yielded only 75% specificity and 67% sensitivity with a cutoff of 1.2 mg/dl. The aggregated median AUC of 0.82 for MRP8/14 ROC curves from day 2 to day 15 after transplantation was higher than the aggregated median AUC of 0.63 for CRP ROC curves.

**Intravenous Methylprednisolone Boluses Significantly Reduced MRP8/14 Serum Levels**

To evaluate whether MRP8/14 levels measured before rejection were higher than those recorded after antirejection treatment, the data on MRP8/14 serum levels for the acute rejection group (n = 18) in the validation study were synchronized according to the first day of intravenous bolus methylprednisolone antirejection therapy. Median MRP8/14 serum levels, which were elevated above the cutoff level beginning on day 5 before rejection, increased to a maximal value of 5.7 µg/ml (IQR, 1.6 µg/ml) on the day before the initiation of intravenous methylprednisolone antirejection therapy. The median MRP8/14 serum level decreased below the cutoff of 4.2 µg/ml during the antirejection therapy and reached a level of 3.3 µg/ml (IQR, 2.2 µg/ml) on day 2 after the last intravenous methylprednisolone injection. The difference in MRP8/14 serum levels measured 1 d before and 2 d after methylprednisolone antirejection therapy was significant (P < 0.05, Wilcoxon test for paired data sets).

The AUC values for the MRP8/14 ROC curves increased continuously during the 3 d before antirejection therapy, from 0.85 to 0.96 and 1.00, and decreased to levels below 0.80 during intravenous methylprednisolone therapy, with further decreases to 0.66 on day 3 after bolus antirejection therapy. On the day before antirejection therapy, 100% specificity and 100% sensitivity for the diagnosis of rejection were achieved with the cutoff of 4.2 µg/ml.

Two acute rejection episodes were observed for one patient, on days 7 and 15 after transplantation. MRP8/14 serum levels increased from 4.8 µg/ml 3 d before the first acute rejection episode to 6.0 µg/ml 1 d before rejection. MRP8/14 serum levels decreased to 3.1 µg/ml immediately after the initiation of intravenous methylprednisolone antirejection therapy and to 2.6 µg/ml on the third day after the 3 d of steroid bolus treatment. On day 14 after transplantation, the MRP8/14 serum level was clearly above the cutoff again, at 5.8 µg/ml. After the second 3-d course of intravenous methylprednisolone treatment, the MRP8/14 serum level again decreased below the cutoff, to 3.6 µg/ml.

The within-subject correlation coefficient (r = 0.06) for MRP8/14 and creatinine serum levels in the two studies, including data for many patients with DGF, was very low, even lower than the same coefficient for MRP8/14 serum levels and CRP serum levels (r = 0.17). However, congruent upward and downward trends for MRP8/14 and creatinine serum levels were observed for patients (six patients, pooled from both studies) with primary graft function and creatinine levels that decreased below 2 mg/dl before subsequent acute rejection. For these patients, the median creatinine serum level increased from 1.8 mg/dl (IQR, 0.9 mg/dl) to 2.2 mg/dl (IQR, 1.1 mg/dl) during the period from 2 d before rejection to the first day of methylprednisolone antirejection therapy, and the median MRP8/14 serum level increased from 4.2 µg/ml (IQR, 0.5 µg/ml) to 6.4 µg/ml (IQR, 6.5 µg/ml) during the same period. Both MRP8/14 and creatinine serum levels decreased immediately after antirejection therapy. On the third day after the 3-d course of intravenous methylprednisolone antirejection therapy, the median creatinine serum level decreased to 1.2 mg/dl (IQR, 0.7 mg/dl) and the median MRP8/14 serum level decreased to 3.3 µg/ml (IQR, 0.5 µg/ml).

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**Figure 4.** ROC curve for MRP8/14 serum levels, with respect to the sensitivity (Sens.) and specificity (Spec.) for an acute rejection within the first 4 wk, on day 6 after transplantation in the validation study. The area to the right of the ROC curve represents the AUC (AUC = 0.9).
CMV Infections, UTI, and DGF Did Not Increase MRP8/14 Serum Levels

DGF and UTI are important inflammatory complications that frequently occur within the first weeks after transplantation. CMV infections most commonly occur after the fourth week after transplantation; however, in some cases infections can occur during the first 4 wk after transplantation. For this special analysis of complications, the data for both sets of patients from the pilot study and the validation study were pooled (n = 56).

One MRP8/14 serum level for each patient was selected for correct statistical assessment, because the complications were evident for each patient for different limited time periods. The day of diagnosis of CMV infections and UTI [median of 24 d (IQR, 7 d) and 17 d (IQR, 10 d) after transplantation, respectively] was selected for the evaluation of MRP8/14 serum levels, because both types of infections were treated with antiviral or antibacterial drugs directly after diagnosis and no increase in MRP8/14 serum levels was apparent for any patient. For all patients without CMV infection or UTI but with DGF or acute rejection, day 6 after transplantation was selected for MRP8/14 serum level analysis. For several patients, one of these complications (for example, DGF) and acute rejection occurred at the same time.

For analysis of the potential confounding of MRP8/14 serum values by concomitant DGF, CMV infection, or UTI, the data were stratified into four groups for each of these complications, enabling differential analyses. The first group included patients who experienced an acute rejection episode coincident with a complication (DGF/acute rejection, CMV infection/acute rejection, or UTI/acute rejection). The second group included patients with ongoing acute rejection, as indicated by clinical parameters and proven by biopsy within the following week, but without one of the aforementioned complications. Complications (DGF, CMV infection, or UTI) without acute rejection were apparent in the third group. Neither acute rejection nor a complication was diagnosed in the fourth group (Figure 5).

The median MRP8/14 serum level for the UTI group (n = 14) was 2.99 μg/ml (IQR, 1.2 μg/ml), which was not significantly different from the median of 2.42 μg/ml (IQR, 1.1 μg/ml) for the control group (n = 22, P = 0.215) (Figure 5A). No patient in the UTI group exhibited MRP8/14 serum levels above the cutoff value of 4.2 μg/ml. However, the median of 6.0 μg/ml (IQR, 1.5 μg/ml) for the acute rejection group (n = 11) was significantly higher than the median for the UTI group (P < 0.0005).

Similarly to UTI, the median MRP8/14 serum level for the CMV infection group (n = 5) was 2.1 μg/ml (IQR, 1.5 μg/ml), which was not significantly different from the median of 2.4 μg/ml (IQR, 1.3 μg/ml) for the control group (n = 28, P = 0.114) (Figure 5B). All MRP8/14 serum levels for the CMV infection group were below the cutoff level of 4.2 μg/ml. The median for the acute rejection group [6.0 μg/ml (IQR, 2.3 μg/ml), n = 13] was significantly higher than the median for the CMV infection group (P = 0.002), and all MRP8/14 serum levels for the acute rejection group were clearly above the cutoff level of 4.2 μg/ml.

The effect of DGF on MRP8/14 serum levels was analyzed using stratification into four groups, as described for CMV infection and UTI. To rule out a possible effect of DGF occurring earlier than day 6 after transplantation, MRP8/14 serum levels were also analyzed on days 2 and 4 after transplantation. However, no differences or trends in these three time points could be observed. All three median MRP8/14 serum levels for the DGF group, i.e., 2, 4, and 6 d after transplantation [2.5 (IQR, 2.4 μg/ml), 2.9 (IQR, 1.4 μg/ml), and 2.6 μg/ml (IQR, 1.3 μg/ml), respectively] were not significantly different from the corresponding medians for the control group with neither acute rejection nor DGF [3.3 (IQR, 1.3 μg/ml), 2.5 (IQR, 1.4 μg/ml), and 2.5 μg/ml (IQR, 1.2 μg/ml), respectively; P = 0.938, P = 0.405, and P = 0.687, respectively]. As shown in Figure 5C for day 6 after transplantation, all MRP8/14 serum levels for the DGF group (n = 12) and the control group (n = 18) were below the cutoff level of 4.2 μg/ml. However, the median MRP8/14 serum level for the acute rejection group (n = 7) was 5.4 μg/ml (IQR, 1.9 μg/ml), which was significantly higher than the median MRP8/14 serum level for the DGF group [2.6 μg/ml (IQR, 1.3 μg/ml), P < 0.0005]. There was no significant difference between the MRP8/14 serum level for the acute rejection/DGF group (n = 6) and that for the acute rejection group (P = 0.283). Therefore, DGF did not increase MRP8/14 serum levels and had no additive effect with acute rejection.

Variable MRP8/14 serum levels were measured during other complications, which occurred for small numbers of patients. All MRP8/14 serum levels for five patients with lymphocele were below the cutoff of 4.2 μg/ml, with a median of 2.4 μg/ml (IQR, 1.6 μg/ml). Four of the 56 patients exhibited median MRP8/14 serum levels of 4.6 μg/ml (IQR, 1.2 μg/ml) on the first or second day after transplantation, despite primary function and without subsequent rejection, which might be explained by surgical trauma. For one patient, acute arteriovenous shunt occlusion on day 3 after transplantation was associated with a MRP8/14 serum level of 4.7 μg/ml. For individual patients, toxic colonic dilatation, transplant pyelonephritis, and esophageal candidiasis were associated with MRP8/14 serum levels of 4.5, 4.3, and 4.2 μg/ml, respectively. One large infected hematoma and an infected lymph fistula were both associated with elevated MRP8/14 levels of 5.3 μg/ml. However, two noninfected hematomas, a surgical revision, a sterile urine fistula, and a case of histologically proven cyclosporine A intoxication were all associated with MRP8/14 serum levels below the cutoff level, i.e., 1.8 (IQR, 2.2 μg/ml), 4.1, 3.0, and 3.3 μg/ml, respectively.

MRP8/14 Serum Levels Were Not Correlated with Ischemia Times but Were Weakly Correlated with the Number of HLA Mismatches

Pooled groups of patients from both studies with cold ischemia times (median, 18 h; IQR, 10 h) of ≤18 h (n = 28) or >18 h (n = 28) did not differ significantly with respect to either DGF (P = 0.1) or median MRP8/14 serum levels on days 1 and 2 after transplantation [2.8 μg/ml (IQR, 1.9 μg/ml) versus 4.0 μg/ml (IQR, 2.7 μg/ml), P = 0.08; 3.8 μg/ml (IQR,
1.1 µg/ml) versus 3.3 µg/ml (IQR, 3.5 µg/ml), \( P = 0.8 \), respectively]. No significant correlation between cold ischemia time and MRP8/14 serum levels was observed, even on day 1 after transplantation (\( r_s = 0.243, P = 0.231 \)). Patients from both studies who were grouped according to warm ischemia times (median, 30 min; IQR, 14 min) of \( \leq 30 \) min or \( > 30 \) min also did not differ significantly with respect to either DGF (\( P = 1.0 \)) or median MRP8/14 serum levels on days 1 and 2 after transplantation [3.0 µg/ml (IQR, 1.7 µg/ml) versus 4.0 µg/ml (IQR, 3.0 µg/ml), \( P = 0.504 \); 3.7 µg/ml (IQR, 2.7 µg/ml) versus 3.6 µg/ml (IQR, 2.9 µg/ml), \( P = 0.964 \), respectively]. Warm ischemia time was not significantly correlated with MRP8/14 serum levels on day 1 after transplantation (\( r_s = 0.073, P = 0.722 \)).

In the last 3 d before acute rejection was diagnosed by renal transplant biopsy, using the Banff classification, the median MRP8/14 serum levels were 4.4 µg/ml for borderline cases (class 3 rejections), 4.7 µg/ml (IQR, 1.6 µg/ml) for class 4,IA rejections, 5.6 µg/ml (IQR, 3.4 µg/ml) for class 4,IB rejections, and 5.8 µg/ml (IQR, 1.7 µg/ml) for class 4,IIA/B rejections. Therefore, the severity of rejection, as indicated by the Banff categories, seemed to be positively correlated with MRP8/14 serum levels \( r_s = 0.337 \); this trend was nonsignificant, however \( (P = 0.08) \). The number of HLA mismatches in the pooled data was positively correlated with MRP8/14 serum levels above the cutoff value \( r_s = 0.399, P = 0.008, \) day 6 after transplantation.

Discussion

Human renal allograft rejection is a complicated inflammatory process, with many factors influencing the time course and strength of the immune reaction. Similarly complicated immunosuppressive protocols, which can be changed from day to day, attempt to combat the immune response and to maintain a balance between the immune reaction and immunosuppression. However, a useful monitoring marker for rejection is still missing, especially for the time of DGF (within the first 4 wk after transplantation).

In this report, we demonstrate that serum levels of the activation-specific, calcium-dependent, protein heterocomplex MRP8/14, which is released by activated monocytes and neutrophils during contact with activated endothelial cells of the graft during the process of transendothelial migration, are significantly increased for the first 2 wk after transplantation in renal allograft recipients who experience acute rejection within the first 4 wk. The optimal cutoff level is 4.2 µg/ml, for high sensitivity and specificity with a high positive predictive value. These results were obtained in a pilot study and were confirmed in a validation study. MRP8/14 serum levels increased before rejection and were significantly reduced after intravenous methylprednisolone antirejection therapy.

Monocytes/macrophages and neutrophils of the innate immune system play major roles in the initial inflammatory response to the allograft, which may lead to an acute rejection episode. This inflammatory process may be separated into four steps, including, for transplantation of kidneys from cadaveric donors, (1) cytokine release and increased levels of cellular
adhesion molecules in the donor organ after brain death (34), (2) cold and warm ischemia, (3) reperfusion injury, and (4) an immune response to the foreign graft, involving the innate and adaptive components of the immune system.

The median MRP8/14 serum levels were already increased for many patients in the acute rejection group during the first 3 d after transplantation. Therefore, it was assumed that DGF might be an independent factor increasing soluble MRP8/14 levels in the serum. Our data revealed, however, that this was not the case. No significant increase in positive MRP8/14 serum levels was measured for patients with DGF. DGF alone did not increase MRP8/14 serum levels and had no additive effect with acute rejection. Long cold ischemia or warm ischemia times also had no significant effects on MRP8/14 serum levels. Therefore, our data indicated that MRP8/14 serum levels were not significantly affected by nonspecific inflammatory stimuli from the first three steps (as noted above) preceding the allograft-related response. In contrast, a significant positive correlation between HLA mismatches and MRP8/14 serum levels was observed.

The finding of very early activation of MRP8/14-positive macrophages in this study is consistent with our previous observations with 1-h renal transplant biopsies, which indicated very early involvement of activated MRP8/14-positive macrophages and granulocytes in the immune response to the allograft (35). MRP8/14-positive macrophages and neutrophils are also the first cells to arrive at the inflammatory site in many animal models (12).

Several observations led us to concentrate only on measurements of the soluble heterocomplex MRP8/14 in serum samples and not on measurements of the homodimeric MRP8 or MRP14 proteins. MRP8 and MRP14 are preexisting abundant proteins in the cytoplasm. The activation-dependent MRP8/14 complex can be detected, however, solely in acute inflammatory states (12), when monocytes/neutrophils come into contact with activated endothelium (13). It was reported that graft-infiltrating cells positive for the heterocomplex MRP8/14 were typical of acute renal transplant rejection (36,37) and that infiltrating cells positive only for the homodimers MRP8 or MRP14 were more specific for chronic rejection in immunohistologically stained, frozen biopsy sections (38).

In these studies, we demonstrated that positive MRP8/14 serum levels preceded acute rejections detected by conventional clinical methods by a median of 5 d. An explanation might be that conventional rejection parameters do not measure the beginning of the inflammatory process but, rather, assess the end of a process of increasing inflammatory destruction of the transplanted organ, with increasing organ failure (i.e., in the kidney, an increase in creatinine serum levels or a reduction in the daily volume of urine). MRP8/14 serum levels increase, however, at the time when infiltrating leukocytes interact with activated endothelial cells and begin to infiltrate the foreign graft; this seems to be approximately 5 d before increasing destruction decreases organ function parameters. This might explain why strong correlation between the early, specific, monocyte/neutrophil transendothelial migration-related rejection marker MRP8/14 and the rather nonspecific, organ dysfunction marker creatinine could not be observed. First, as described above, there was a time difference of approximately 5 d between the onset of infiltration and dysfunction leading to creatinine serum level increases. Second, up to 50% of renal transplant recipients exhibit DGF, with elevated creatinine levels, independent of ongoing rejection.

The median time between the first positive MRP8/14 serum level and the first acute rejection episode was a median of 5 d but varied between 1 d and >2 wk. This time interval may be influenced by the balance between the strength of the immune reaction and the individual immunosuppressive protocol. The first rejection episode developed later for patients who underwent quadruple-drug induction therapy or prolonged, initially high-dose methylprednisolone therapy than for patients who underwent rapid tapering of methylprednisolone treatment without induction therapy. For some patients, it might take 10 d or more for the innate immune system to obtain sufficient help from the adaptive immune system. An initial increase in MRP8/14 serum levels above the cutoff level might be nevertheless observed.

The value of CRP levels after transplantation is controversial. It was recently reported that CRP serum levels were elevated up to 9 d before rejection (39) and that high CRP serum levels were associated with progressive luminal obstruction in heart transplant recipients (40). CRP serum levels were predictive of allograft failure and were significantly correlated with grade 3 rejection in cardiac transplantation (41). In contrast, for a large number of renal transplant recipients, CRP serum levels were unable to discriminate the causes of renal graft dysfunction, i.e., rejection, infection, or tubular necrosis. Therefore, the specificity of CRP measurements was very low (42). Another problem with CRP measurements is their low sensitivity, as recently described for cardiac transplantation (43).

Our data from both studies confirm these results of low sensitivity and low specificity for CRP measurements. The sensitivity of CRP measurements for the detection of rejection was only 67%, with a specificity of 75% at a cutoff of 1.2 mg/dl on day 6 after transplantation. All AUC values for CRP ROC curves were below the AUC values for MRP8/14 ROC curves. Therefore, low sensitivity and low specificity for rejection seem to be general disadvantages of the application of CRP as a potentially useful monitoring marker.

In contrast, MRP8/14 serum levels did not increase during common infections after renal allograft transplantation, such as UTI or CMV infections. Other complications, such as hematomas and lymphoceles, were also not associated with increased MRP8/14 serum levels. The specificity of elevated MRP8/14 serum levels for rejection might be explained by the fact that MRP8/14 is secreted only during active transcendothelial migration with tissue invasion, which is a prerequisite for rejection but not for bacteriuria or a beginning CMV infection characterized by CMV PCR positivity.

One large infected hematoma, which needed to be removed in a surgical revision, was associated with increased MRP8/14 serum levels, in contrast to a smaller noninfected hematoma. This finding might be explained in this special case by in-
creased transendothelial migration and tissue invasion of monocytes/neutrophils in the large infected hematoma.

In many diseases, such as arthritis and ulcerative colitis, increased MRP8/14 serum levels are associated with disease activity (12). Using a synchronized statistical assessment of rejection episodes, we could prove that increased MRP8/14 serum levels were also closely associated with the acute rejection process. MRP8/14 serum levels increased before the diagnosis of rejection and reacted to a 3-d course of intravenous methylprednisolone treatment with prompt significant reductions. These results are consistent with data from a study of juvenile rheumatoid arthritis. At least one-half of the patients in that study responded to intra-articularly administered triamcinolone with almost complete remission and decreases in MRP8/14 serum levels to the normal range (26).

In addition to this profound effect of steroids, our data provide evidence that a second immunosuppressive drug might influence MRP8/14 serum levels. After the switch from azathioprine to MMF, MRP8/14 levels decreased for two of our patients. This observation, in combination with the fact that significantly more patients in the nonrejection group in the pilot study received MMF, may lead to the interpretation that MMF decreases macrophage activation and MRP8/14 secretion. This interpretation is in accordance with the results of animal studies that demonstrated that MMF was a potent inhibitor of graft infiltration by macrophages and reduced the expression of adhesion molecules (44). Furthermore, treatment of endothelial cells with MMF in vitro inhibited the expression of adhesion molecules, especially ICAM-1 (45). It was recently reported that transendothelial migration of MRP8/14-positive cells involves an ICAM-1-dependent mechanism (13). Therefore, it can be hypothesized that MMF inhibits the process of transendothelial migration of MRP8/14-positive cells into the graft.

In summary, this study demonstrates that MRP8/14 is a useful, very early monitoring marker that can provide clinicians with daily information regarding the balance between factors increasing the risk of rejection and the immunosuppressive drugs combating these factors. This study demonstrates that MRP8/14 is a reliable, sensitive, specific marker with a high predictive value for rejection during DGF and other typical complications of the first 4 wk after renal allograft transplantation.

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