Low HLA-DR expression on peripheral blood monocytes predicts bacterial sepsis after liver transplantation: relation with Prednisolone intake

JW Haveman¹, AP van den Berg²,³, JMM van den Berk⁴, G Mesander², MJH Slooff¹, LHFM de Leij², TH The²

¹ Department of Surgery
² Department of Clinical Immunology
³ Department of Gastroenterology and Hepatology
University Medical Center Groningen, The Netherlands
⁴ Department of Internal Medicine, University Hospital Nijmegen, The Netherlands

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ABSTRACT

Bacterial sepsis remains a frequent complication after liver transplantation. We previously reported the results of a pilot study that suggested that low expression of HLA-DR on monocytes is a predictive marker for the occurrence of sepsis. We have studied the value of this marker in an additional cohort of patients, and have analyzed the relation of HLA-DR expression with the use of immunosuppressive agents. 20 adult liver transplantation patients were prospectively monitored during the first 4 weeks after transplantation. All were treated according to standard protocols. The percentage of monocytes expressing HLA-DR was measured by flow cytometry. In addition, the effects of incubation of monocytes with prednisolone in vitro on the expression of HLA-DR was determined in 7 healthy volunteers. Seven patients developed bacterial sepsis after a median 15 (range 10–20) days after transplantation. HLA-DR expression was significantly lower in these patients on days 7, 14, 21, and 28 after transplantation compared with non-septic patients. The percentage of HLA-DR positive monocytes was 30% or less, 3 (1–8) days before onset of sepsis. On day 7 after transplantation, HLA-DR expression on 50% or less of monocytes had a positive predictive value for sepsis of 71%, whereas the negative predictive value was 85%. Patients who developed sepsis received significantly more prednisolone. Incubation with prednisolone in vitro lowered the expression of HLA-DR in a dose-dependent manner. We conclude that low HLA-DR expression on monocytes is a marker for a high risk of subsequent sepsis in liver transplantation patients. This high risk may be (at least partly) related to the dose of prednisolone.
Sepsis remains an important cause of morbidity and mortality after liver transplantation, despite improvements in antibiotic treatment and supportive care (84,85). Immunosuppressive agents probably constitute the most important underlying cause of the increased susceptibility for infections in these patients, but other factors such as poor nutritional status, hepatic failure, surgical trauma, uremia, multiple blood transfusions and viral infections also have a negative influence on the immunocompetence (86). Drug-induced immunosuppression is the only one of these factors that can be easily modified in order to prevent over-immunosuppression with its attendant risk of severe infections. To date, drug dosage still depends on clinical intuition, as no good parameter is available for identification of patients at high risk of developing septic complications.

HLA-DR expression on monocytes might be a useful marker of an increased risk of infection. Low HLA-DR expression on monocytes is associated with an increased risk of infections in patients with trauma (23) or thermal injury (62), patients undergoing major abdominal (24,25) or neurosurgery (87), and in patients with a ventricular assist device awaiting cardiac transplantation (27). We have previously reported that low HLA-DR expression preceded clinical signs of sepsis in a small series of liver-transplant patients (63). Here we present an extension of that study, confirming the usefulness of HLA-DR expression on monocytes. Moreover, we present evidence that low HLA-DR expression and sepsis may be related to the dose of prednisolone.

PATIENTS AND METHODS

Patients
We prospectively monitored 20 adult liver transplant recipients during the first 4 weeks after transplantation. Results in 9 of these patients have been reported previously (63). All patients were treated according to standard protocols, without knowledge of the results of flow-cytometric analysis. Demographic data and details on the operative procedure are shown in Table 1. The nutritional status was determined before
transplantation by clinical examination by two investigators. This method has been shown to be as accurate as laboratory testing (88).

Baseline immunosuppression and rejection treatment
Standard immunosuppression consisted of ‘high-dose’ prednisolone (1.25–1.5 mg/kg/d during the first week, tapering to 0.4–0.5 mg/kg/d at day 28), azathioprine (125 mg/d), and cyclosporine A (target trough levels 200–250 mg/l), combined with a one-week induction course of cyclophosphamide, as reported previously (89) in 12 patients. The remaining patients took part in a randomized trial comparing tacrolimus with cyclosporin A, both combined with ‘low-dose’ prednisolone (1.5 mg/kg on day 1, tapered to 0.3 mg/kg at day 7 and 0.2 mg/kg at day 28). Target trough levels of tacrolimus ranged between 10 and 15 ng/ml (6 patients), whereas trough levels of cyclosporin A ranged between 200 and 300 ng/ml (two patients). The selection of patients for this comparative trial did not depend on the risk of infection after transplantation, or on the expression of HLA-DR on monocytes. In case of abnormal liver tests a Doppler-ultrasound examination was performed to exclude vascular or biliary tract problems. A liver biopsy was taken to confirm the diagnosis of rejection. Firstline rejection treatment consisted of methylprednisolone pulses of 1 g intravenously on 3 successive days; steroid-resistant rejection was treated with antithymocyte globulin (90).

Infection prophylaxis, monitoring, and treatment
All patients received selective bowel decontamination consisting of colistine, tobramycine, and amphotericin B until the bile drain had been clamped and full oral intake had been resumed, as described before (91). In addition, systemic antibiotic prophylaxis was given for 48h, consisting of cefotaxim with tobramycin, or, in case of renal failure, imipenem monotherapy. In cases of fulminant liver failure or retransplantation, amphothericine B was given intravenously in a dose of 0.25 mg/kg for the first 10 postoperative days as a prophylactic measure against fungal infections. All patients received low-dose oral acyclovir against herpes simplex virus infections.
No prophylaxis was given against CMV or PCP. In case of fever or suspected infections 2 or 3 blood cultures were taken, in addition to urine and sputum cultures. Bile, ascites, aspirates from fluid collections or abscesses, and catheter tips were also cultured. Treatment of infections consisted of antibiotics and surgical drainage when necessary. In case of severe infections or CMV disease, prednisolone was lowered to 10 mg/d and azathioprine to 50 mg/d.

**HLA-DR expression on monocytes**

EDTA anticoagulated blood was taken before transplantation, and on days 7, 14, 21, and 28 post-transplantation. Monoclonal antibodies against CD-14 antigen (anti CD-14-PE (phycoerythrine), Immuno Quality Products, Groningen, The Netherlands) were used to set a lifegate for monocytes. The percentage of HLA-DR⁺ monocytes was determined using anti-HLA-DR FITC (fluoresceine isothiocyanate) (Becton Dickinson Immunocytometry Systems, San Jose, CA), with an IgG2a isotype control (isotype control IgG2a FITC, Immuno Quality Products); in the last 11 patients the signal intensity of fluorescence was also determined. For sequential analysis the flow cytometer was calibrated by using QC windows™ microbeads as a standard curve (Flow Cytometry Standards Corporation (FCSC), San Juan, Puerto Rico).

**In vitro monocyte incubation with prednisolone**

To determine the effect of prednisolone on HLA-DR expression by monocytes *in vitro* we incubated whole blood from 7 healthy volunteers, diluted 1:10 with RPMI, with increasing concentrations of prednisolone. After 24h of incubation at 37°C, monocytes were stained by monoclonal antibodies and analyzed by flow cytometry as described above.

**Definitions**

Sepsis, septic shock, sepsis syndrome, and multi-organ failure were defined following the criteria of Bone (92). Sepsis was defined as a systemic response to a microbiologically confirmed infection consisting of hypothermia or hyperthermia (core
or rectal temperature, <35.5 or >38.0°C), tachypnea (respiration >20 breaths/min; if mechanically ventilated, minute ventilation >10 l/min), and tachycardia (heart rate >90 beats/min).

**Statistical analysis**

Data are given as median (range). Patient characteristics were compared with the Fisher’s exact test or in case of continuous data the Mann–Whitney U-test. HLA-DR expression was compared with the Mann–Whitney U-test and with the Wilcoxon signed rank test or Friedman’s test. For detection of correlations we used Spearman’s rank correlation test. A $P$-value of 0.05 was considered to indicate statistical significance.
RESULTS

Patients

Seven of the 20 patients experienced an episode of sepsis during the first 4 weeks after orthotopic liver transplantation (OLT). No significant differences were found regarding demographic, surgical or postoperative characteristics between patients with and without sepsis (Table 1).

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patients with sepsis (n=7)</th>
<th>Patients without sepsis (n=13)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td><strong>Demographic characteristics</strong></td>
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<tr>
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<td>8/5</td>
<td>NS</td>
</tr>
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<td>Age (yrs)</td>
<td>47 (34-60)</td>
<td>36 (18-61)</td>
<td>NS</td>
</tr>
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<td>1/12</td>
<td>NS</td>
</tr>
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<td>Re-transplantation</td>
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<td>2</td>
<td>NS</td>
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<tr>
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<td>0/5/8</td>
<td>NS</td>
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<tr>
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<td>5/5/3</td>
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</tr>
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<td>5</td>
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<tr>
<td><strong>Surgical data</strong></td>
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<td></td>
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</tr>
<tr>
<td>Cold ischemic time (min)</td>
<td>760 (369-960)</td>
<td>730 (376-864)</td>
<td>NS</td>
</tr>
<tr>
<td>Warm ischemic time (min)</td>
<td>58 (36-89)</td>
<td>58 (31-76)</td>
<td>NS</td>
</tr>
<tr>
<td>Operative time (min)</td>
<td>520 (440-900)</td>
<td>703 (515-840)</td>
<td>NS</td>
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<td>Blood loss (l)</td>
<td>9 (7-23)</td>
<td>9 (2-35)</td>
<td>NS</td>
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<td><strong>Post operative data</strong></td>
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<td>prednisolone dose on day 7 (mg/d)</td>
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</tr>
<tr>
<td>Died</td>
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<td>0</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data represents number of patients or median (range). SBP = spontaneous bacterial peritonitis. Graft function: IPF = immediate poor function, PNF = primary non-function. Immune suppressive regimen: 1 = cyclophosphamide induction therapy, high-dose prednisolone, cyclosporine A and azathioprine, 2 = low-dose prednisolone and tacrolimus, 3 = low-dose prednisolone and cyclosporine A. NS, nonsignificant.
Sepsis was first diagnosed 15 (10–20) days after transplantation. Three patients developed sepsis with cholangitis, one of them developed sepsis syndrome caused by *Staphylococcus aureus*; two had sepsis, one of these caused by *S. aureus* and the other by CNS (coagulase-negative *Staphylococcus*). Two patients had peritonitis, one with combined infection of *S. aureus* and *Citrobacter*, and one developed septic shock due to infection with *Serratia* and *S. aureus*. Two patients died in the sepsis group. Patient #7 (see Figure 2) died due to a liver rupture resulting in massive blood loss and catheter sepsis with CNS. Patient #3 died because of fulminant sepsis and multi-organ failure (MOF) caused by *S. aureus*.

**HLA-DR expression on monocytes and sepsis**

Figure 1 shows HLA-DR expression on monocytes in patients with and without sepsis. Patients who suffered sepsis had a significantly lower HLA-DR expression on monocytes on day 7 ($P=0.013$), day 14 ($P<0.001$), day 21 ($P=0.004$), and day 28 ($P=0.010$) after transplantation. In both patients with fatal outcome HLA-DR expression on monocytes was lower than 30%, and remained so until death, whereas in all patients who survived sepsis, expression of HLA-DR on monocytes rose during recovery. On days 21 and 28 it was significantly higher than on day 14 ($P=0.039$ and $P=0.042$, respectively). Figure 2 shows that in all patients with sepsis, low HLA-DR expression was already present 3 days (1–8) before onset of sepsis. Five of the seven patients with less than 50% HLA-DR positive monocytes on day 7 developed sepsis (positive predictive 71%), whereas 11/13 patients with HLA-DR expression on more than 50% of all monocytes on day 7 remained free from septic complications (negative predictive value 85%).

The percentage of HLA-DR positive monocytes and the fluorescence intensity of the positive signal as determined in the 46 samples taken during the second part of the study were strongly correlated ($r=0.60$, $P<0.001$). Signal intensity was low before onset of sepsis in the 2 patients with this complication.
Figure 1. HLA-DR expression on monocytes in patients with and without sepsis during the first month after transplantation. Box plots show the median, 10th, 25th, 75th, and 90th percentiles. Open circles indicate outliers.

Figure 2. HLA-DR expression in seven patients with sepsis. The horizontal axis in the middle of the figure indicates the onset of sepsis. The shaded area indicates mean ±2 SD in non-septic patients.
**Prednisolone, white blood cell, and monocytes counts in relation to HLA-DR expression**

Prednisolone dosage was significantly higher at day 7 after transplantation in patients who developed sepsis compared to those without sepsis (1.33 mg/kg/d (1.25–1.45) vs. 0.60 mg/kg/d (0.18–1.61) respectively, \( P = 0.022 \)). Differences in prednisolone dosage were no longer significant 2 weeks or more after transplantation, probably as a result of our tendency to taper prednisolone in patients with severe infections.

Incubation of monocytes from healthy volunteers with prednisolone *in vivo* did not reduce the percentages of HLA-DR positive monocytes \( (P=0.134) \). However, the amount of HLA-DR per monocyte, as reflected by the intensity of HLA-DR fluorescence signal, decreased in a dose-dependent fashion (Figure 3).

Total numbers of monocytes and white blood cells did not differ in patients who suffered a septic episode from those in patients without sepsis. Furthermore, there were no significant correlations between the white blood cell counts or the numbers of monocytes on the one hand, and HLA-DR expression on monocytes on the other hand \( (r=-0.132, P=0.253, \text{ and } r=-.253, P=0.241, \text{ respectively}) \).

**HLA-DR expression and other complications**

Two patients suffered from a CMV infection. In these patients HLA-DR expression was comparable with patients not experiencing this complication. Patients with other infections or technical complications did not show a lower or higher percentage of HLA-DR positive monocytes. Five patients suffered from acute rejection during the first 4 weeks after transplantation. Three of these patients also experienced sepsis, either shortly after (patient #3, Figure 2) or before onset of rejection. One of these (patient #7) developed rejection 2 days after onset of sepsis when HLA-DR expression was still very low (16%).
DISCUSSION

In this study we show that HLA-DR expression on monocytes is a reliable prognostic marker for sepsis during the first month after liver transplantation. All seven patients who suffered from sepsis had a low percentage of HLA-DR positive monocytes (15%, 7–30) before onset of sepsis. On day 7 after transplantation, expression of HLA-DR on 50% or less of monocytes had a positive predictive value for sepsis of 71%, and a negative predictive value of 85%. These data indicate that monitoring of HLA-DR expression will identify patients at a high risk of developing septic complications. To increase the reliability of this parameter, a repeated measurement of HLA-DR expression on subsequent days might be necessary.

As shown in Figure 1, OLT patients who developed sepsis did not have lower HLA-DR expression before transplantation. Moreover, demographic and surgical characteristics did not differ between both groups. Although large studies have shown that a poor pretransplant nutritional status (86,93,94) and spontaneous bacterial
peritonitis (95) are associated with high post-transplant infection rate and mortality, it remains difficult to determine the risk of sepsis in the individual patient. Monitoring of HLA-DR could be helpful in adapting immunosuppression to the needs of the individual patient. Sepsis occurred in 6 of the 12 patients on the high-dose steroid, CsA-based regimen, and in only one of the 8 patients on low-dose prednisolone, generally tacrolimus-based immunosuppression. The baseline conditions, difficulty of surgery, and graft function were comparable in these groups. This leaves immunosuppression as the most probable determinant of cause of the difference in the incidence of sepsis. There is no strong evidence that CsA as a primary immunosuppressant is, by itself, associated with a higher risk of infection than tacrolimus (96). The high doses of prednisolone coadministered in the cyclosporin A regimen may be of greater importance. Patients with sepsis received significantly more prednisolone on day 7 after transplantation. Moreover, incubation of whole blood with prednisolone in vitro led to a dose-dependent decrease of HLA-DR expression on monocytes. Concentrations used in this experiment are comparable with concentrations in OLT patients, i.e. $10^{-8} – 10^{-6}$ (96). This suggests that decreasing of steroids in patients with low HLA-DR expression could lower risk of sepsis and might improve prognosis. In this respect data from Oehling et al. are highly relevant. These workers showed that oral glucocorticoid therapy in bronchial asthma was associated with an increased frequency of respiratory tract infections. In addition, HLA-DR expression on monocytes was lower in patients receiving glucocorticoids compared with those who did not receive oral glucocorticoid therapy (96). Although intensive steroid treatment undoubtly increases the risks of sepsis, we do not think that low HLA-DR expression was just a marker for intensive steroid therapy, because half of the patients in the high-dose steroid group had persistently normal HLA-DR expression, whereas several patients in the low-dose prednisolone group had low expression.

Volk et al. reported that prednisolone and azathioprine could be lowered in transplant patients with sepsis and low HLA-DR expression without precipitating rejection (28). The fact that two of our patients suffered from acute rejection shortly after sepsis, one of them at a time when only 16% of monocytes were HLA-DR
positive, suggests that it may not be realistic to expect that a single parameter might be able to indicate the risks of both infection and rejection.

A causal relationship between low monocyte HLA-DR expression and severe bacterial infections remains to be elucidated. HLA-DR serves as a scaffold on which monocytes present peptides derived from exogeneous antigens to CD4⁺ T cells. T cells do not play an important role in the defense against bacteria except for those that can survive intracellularly, and it is difficult to envisage how reduced antigen presentation would predispose to sepsis. Alternatively, low HLA-DR expression might ‘just’ be a very early and sensitive marker of as yet subclinical infection. In agreement with this hypothesis, Randow et al. have shown that repeated LPS stimulation induces monocyte deactivation and down-regulates HLA-DR expression (65). This theory is difficult to reconcile with the predictive value of low monocyte HLA-DR expression for sepsis in patients undergoing elective major surgery (24,25). Thus, we favor the hypothesis that low HLA-DR expression is a marker of generalized monocyte dysfunction. In agreement with this, monocytes expressing low amounts of HLA-DR have been shown to have reduced capacity for synthesis of pro-inflammatory cytokines like TNF-α, IL-1, IL-6, IL-8, and IFN-γ (30). What causes this monocyte dysfunction and down-regulation of HLA-DR remains to be determined. Both IL-10 and TGF-β have been shown to down-regulate HLA-DR expression (72,74). Our study shows that corticosteroid treatment may be another important factor.

If low HLA-DR expression on monocytes is indeed an indicator of a high risk of sepsis, prompt reduction of steroids or treatment aimed at improving monocyte function may prevent development of sepsis. Both IFN-γ and GM-CSF have been shown to improve monocyte function in vitro and in vivo, but IFN-γ might precipitate rejection. In contrast, GM-CSF has been administered to liver transplant recipients with severe infections, apparently without causing rejection, and might therefore an attractive agent for immunomodulation (97). Randomized studies are required to test this hypothesis.