Serum Neopterin After Lung Transplantation*

Marc Humbert, M.D.; Rose Marie Delattre, Ph.D.; Jacques Cerrina, M.D.; Philippe Dartevelle, M.D.; Gérald Simonneau, M.D.; and Dominique Emile, M.D.

Objective: Neopterin (N), a marker for activated cell-mediated immunity, was assayed in the sera of 44 lung recipients early and late after transplantation. The study was a prospective, blind clinical trial designed to evaluate the following: (1) the daily dynamics of the serum neopterin/creatinine (N/C) ratio during the first 3 weeks after transplantation; (2) the correlation between changes in the serum N/C ratio and episodes of rejection or infection; (3) the correlation between the serum N/C ratio and the concentration of serum soluble interleukin 2 receptor (sIL-2R), a marker of T-cell activation; and (4) the potential value of monitoring the serum N/C ratio during noninvasive long-term follow-up of lung recipients.

Methods: Sera from lung recipients were collected every day or every 2 days for the first 3 weeks after transplantation (22 patients) and before fiberoptic bronchoscopy and routine consultation (44 patients). The N concentrations were determined by radioimmunoassay and sIL-2R levels were measured using a sandwich enzyme immunoassay.

Results: Serum N/C is an early and sensitive marker of immune activation in the 21 days following transplantation.

Lung and heart-lung transplantations are now accepted therapies for a wide range of end-stage lung diseases.1,3 Survival rates have consistently increased over recent years due to improvements in lung preservation, surgical techniques, immunosuppressive regimens, assessment of infectious complications, and antibiotic therapy.3 Despite this progress, acute rejection and infections are still frequent after transplantation.4 Moreover, the long-term results are uncertain due to development of obliterative bronchiolitis in 10 to 50 percent of lung recipients.4,5 The mechanisms of this fibrotic obstruction of bronchioles is unclear and may result from repeated insults, mainly rejection and cytomegalovirus (CMV) infections.5 A rational approach to prevent acute and chronic allografted lung alterations would be to diagnose rejection or infection prior to the induction of significant tissue damage and thus prior to the development of subsequent clinical symptoms.

The diagnosis of acute rejection episodes is currently based on detecting perivascular mononuclear cell infiltrates in multiple transbronchial biopsy specimens.6 However, the use of this method is limited due to the fact that samples cannot repeatedly be obtained safely from the graft at short time intervals. Furthermore, assessment of these specimens is time-consuming and requires much expertise. Demonstration of immune cell activation by analysis of biologic markers might greatly facilitate the monitoring of transplant recipients and clinical decision-making.

Activated T lymphocytes are an important effector cell population mediating rejection.7 Stimulation of these cells is associated with the release of interferon-γ. It may be valuable to monitor this cytokine during follow-up of lung recipients: endogenous release can be assessed by assaying various metabolites of interferon-dependent metabolic pathways. Interferon-γ is capable of inducing in macrophages a guanosine triphosphate-specific cyclohydrolase leading to increased excretion of neopterin (N) (6-D-erythro-trihydroxypropylpterin).6,9 Thus, N is a marker for activated cell-mediated immunity. Elevated serum or urinary levels of N have been described in some pathologic conditions, including pulmonary sarcoidosis, infections, and

*From the Laboratoire d’Immunopathologie et d’Immunologie Virale, Institut U131, and the Service de Pneumologie, Hospital Antoine Bécère, Clamart, and the Service de Chirurgie Thoracique et de Transplantation Pulmonaire, Centre Chirurgical Marie Lannelongue, Le Plessis Robinson, France.

Support by grants from the Association Française de Lutte contre la Mucoviscidose.

Manuscript received February 4; revision accepted June 26.

Reprint requests: Dr. Humbert, Service de Pneumologie, Hospital A. Becère, 92161 Clamart Cedex, France

CHEST / 103 / 2 / FEBRUARY, 1993 449
neoplastic diseases. Moreover, N is a useful marker of kidney, liver, and heart allograft rejection. It is mainly excreted in urine at a rate dependent on the glomerular filtration rate. The neopterin/creatinine (N/C) ratio is therefore considered to be a more accurate reflection of the level of N than assaying N directly. We therefore investigated the value of following the serum N/C ratio to the assessment of rejection and infection following lung transplantation.

The current study was a prospective, blind clinical trial designed to evaluate the following: (1) the daily dynamics of the serum N/C ratio during the first 3 weeks after transplantation; (2) the correlation between changes in the serum N/C ratio and episodes of rejection or infection; (3) the correlation between serum N/C and serum-soluble interleukin 2 receptor (sIL-2R), a marker of T-cell activation known to be increased in lung recipients displaying rejection and clinical sepsis; and (4) the potential value of monitoring the serum N/C ratio during noninvasive long-term follow-up of lung recipients.

METHODS

Patient Population

Forty-four patients (26 male, 18 female), aged 9 to 61 years, were studied from November 1989 to April 1991. They underwent single lung (6 patients), double lung (12 patients), or heart-lung transplantation (26 patients) between January 1987 and March 1991. All these patients were studied after the first 3 weeks after transplantation. Twenty-two of them underwent transplantation during the prospective study and were therefore studied during the early postoperative period (first 3 weeks after transplantation). For these 22 patients, the original diagnosis, the age, the type of transplantation, and the preoperative recipient serum N/C ratio are shown in Table 1.

Immunosuppressive Regimen

The immunosuppressive regimen was identical for all patients during the first month after transplantation. It included a preoperative intravenous (IV) injection of methylprednisolone (1 g) and azathioprine (2.5 mg/kg). Postoperatively, IV cyclosporine was given at 2 mg/kg/d and tapered according to whole blood cyclosporine levels as determined by radioimmunoassay (Sandoz) to give a final serum concentration of 250 to 350 ng/ml. Steroid therapy was withheld for 10 days after the surgical intervention, during which the patients were given azathioprine (2 to 3 mg/kg/d), and a 7-day course of rabbit antithymocyte globulin (Merieux, Lyon, France).

After the first month, patients were given cyclosporine orally (10 to 12 mg/kg/day) tapered according to whole blood levels (150 to 200 ng/ml), azathioprine (1.5 mg/kg/d), and supplements of low-dose prednisolone (10 to 20 mg/d).

Allograft lung rejection was treated by a 3-day course of IV methylprednisolone (1 g/d). In cases of severe or recurrent rejection, this treatment was combined with a 5-day course of antithymocyte globulin or a 10-day course of anti-CD3 monoclonal antibody and followed by increased oral corticosteroids administration.

Diagnosis of Complications

Fiberoptic bronchoscopy was performed for either of the two following reasons: (1) as routine surveillance in the absence of abnormal symptoms (on days 15, 30, 45, and 90 and then every 90 days for the first year); or (2) in response to clinical or paraclinical abnormalities consistent with transplanted lung dysfunction. Bronchoalveolar lavage (BAL) samples, transbronchial biopsy (TBB) specimens, and samples for microbiologic studies were taken during each bronchoscopic examination. Before fiberoptic bronchoscopy, the clinical, radiologic, and spirometric status of each patient was determined. Spirometry was performed using computerized equipment (Coulb 2400, Respiratory Function Laboratory). Vital capacity (VC) was measured as inspiratory VC. Forced expiratory volume in

### Table 1 — Recipient Age, Preoperative Diagnosis, Type of Transplantation, and Preoperative Serum N/C Ratio*

<table>
<thead>
<tr>
<th>Patient/Age, yr</th>
<th>Recipient Preoperative Diagnosis</th>
<th>Type of Transplantation</th>
<th>Preoperative N/C Ratio, μmol/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/43</td>
<td>Bronchiectasis</td>
<td>DLT</td>
<td>130</td>
</tr>
<tr>
<td>2/32</td>
<td>Eisenmenger's syndrome</td>
<td>HLT</td>
<td>106</td>
</tr>
<tr>
<td>3/46</td>
<td>Chronic pulmonary thromboembolism</td>
<td>DLT</td>
<td>50</td>
</tr>
<tr>
<td>4/17</td>
<td>Eisenmenger's syndrome</td>
<td>HLT</td>
<td>96</td>
</tr>
<tr>
<td>5/39</td>
<td>Bronchiectasis</td>
<td>DLT</td>
<td>70</td>
</tr>
<tr>
<td>6/24</td>
<td>Eisenmenger's syndrome</td>
<td>HLT</td>
<td>100</td>
</tr>
<tr>
<td>7/31</td>
<td>Cystic fibrosis</td>
<td>HLT</td>
<td>100</td>
</tr>
<tr>
<td>8/52</td>
<td>Fibrosing alveolitis</td>
<td>DLT</td>
<td>240</td>
</tr>
<tr>
<td>9/27</td>
<td>Chronic pulmonary thromboembolism</td>
<td>DLT</td>
<td>110</td>
</tr>
<tr>
<td>10/26</td>
<td>Fibrosing alveolitis</td>
<td>SLT</td>
<td>10</td>
</tr>
<tr>
<td>11/31</td>
<td>Primary pulmonary hypertension</td>
<td>SLT</td>
<td>ND</td>
</tr>
<tr>
<td>12/31</td>
<td>Eisenmenger's syndrome</td>
<td>HLT</td>
<td>ND</td>
</tr>
<tr>
<td>13/42</td>
<td>Bronchiectasis on a single lung</td>
<td>HLT</td>
<td>48</td>
</tr>
<tr>
<td>14/33</td>
<td>Histiocytosis X</td>
<td>HLT</td>
<td>84</td>
</tr>
<tr>
<td>15/22</td>
<td>Primary pulmonary hypertension</td>
<td>HLT</td>
<td>60</td>
</tr>
<tr>
<td>16/47</td>
<td>Emphysema</td>
<td>DLT</td>
<td>50</td>
</tr>
<tr>
<td>17/61</td>
<td>Emphysema</td>
<td>SLT</td>
<td>70</td>
</tr>
<tr>
<td>18/53</td>
<td>Fibrosing alveolitis</td>
<td>SLT</td>
<td>58</td>
</tr>
<tr>
<td>19/49</td>
<td>Emphysema</td>
<td>DLT</td>
<td>115</td>
</tr>
<tr>
<td>20/40</td>
<td>Chronic pulmonary thromboembolism</td>
<td>HLT</td>
<td>35</td>
</tr>
<tr>
<td>21/27</td>
<td>Primary pulmonary hypertension</td>
<td>HLT</td>
<td>ND</td>
</tr>
<tr>
<td>22/27</td>
<td>Lymphangiomatositis</td>
<td>SLT</td>
<td>120</td>
</tr>
</tbody>
</table>

*ND = not determined; DLT = double lung transplantation; HLT = heart-lung transplantation; and SLT = single lung transplantation.
1 s (FEV₁) was read from the largest of three flow volume curves. Data were expressed as the percentage of the values predicted for the patient.

Allograft Lung Rejection: Allograft lung rejection was diagnosed when TBB specimens showed features typical of pulmonary rejection (including perivascular and/or peribronchial mononuclear cell infiltration).

CMV Pneumonia: The diagnosis of CMV pneumonia required the presence of fever, radiologic abnormalities, the absence of other pathogens, and one of the following elements: (1) detection of CMV antigens on BAL cells by immunofluorescent labeling with an anti-CMV early antigen monoclonal antibody (clone E13, final dilution: 1/100, Biosoft, Paris, France); (2) rapid detection of CMV from BAL, plasma, or biopsy samples by viral culture; (3) a cytopathic effect in bronchoalveolar cells or biopsy samples; and (4) seroconversion evidenced by the presence of CMV-specific IgM. The diagnosis was confirmed by a favorable outcome following a 15-day regimen of ganciclovir (5 mg/kg/12 h), leading to a complete resolution of symptoms and hospital discharge.

Bacterial Pneumonia: The diagnosis of bacterial infection required the presence of fever, radiologic infiltrates, the isolation of a bacterial pathogen, and the response to appropriate antimicrobial agents.

Obliterative Bronchiolitis: Obliterative bronchiolitis was diagnosed by serial monitoring of pulmonary function tests. This diagnosis required the irreversible decrease of FEV₁ and a FEV₁/VC ratio under 60 percent of the predicted values despite increased immunosuppressive therapy.

Controls: Control subjects were asymptomatic lung recipients presenting no abnormal clinical symptoms and no histologic changes suggesting rejection. There was no evidence of opportunistic infections, including no positive CMV cultures and no CMV-specific cytopathic effect for any of the control patients.

Analysis of Serum markers

Blood samples were collected every day or every 2 days during the first 3 weeks after transplantation and then before fiberoptic bronchoscopy and routine consultation. This procedure was approved by the institutional review board for human studies. Sera were immediately kept away from light and stored at −20°C until analyzed by a blind procedure. The N concentrations were determined by radioimmunoassay (Henning, Berlin, Germany) and sIL-2R levels were measured using a sandwich enzyme immunoassay (Immunotech SA, Marseille, France), as recommended by the manufacturers. Normal sIL-2R serum values are 75 (±45) pmol/L and the normal upper limit of serum N is 10 mmol/L. As N serum levels increase during renal failure, results were expressed as a N/C ratio as previously described. In normal subjects, this ratio is in the range of 100 μmol/mol.

Statistical Analysis

Standard linear-regression techniques were employed to study the correlation between sIL-2R levels and the N/C ratio. Comparisons within a group and between groups were done with paired and unpaired Student's t tests, respectively. A two-tailed p value less than 0.05 was considered to indicate statistical significance. Values are expressed as means ± SEM.

RESULTS

Early Dynamic Changes of Serum N/C Ratio After Lung Transplantation

The variations of serum N/C ratio were followed for the first 21 days after transplantation in 22 long-heart-lung recipients (311 samples). A preoperative blood sample was available from 19 patients. The mean preoperative serum N/C ratios in these patients (87 ± 11 μmol/mol) were not different from the values observed in a normal blood donor population. The only patient with an elevated preoperative serum N/C ratio (patient 8, N/C = 240 μmol/mol) was febrile prior to transplantation (38°C) and anti-CMV IgM were subsequently detected in the serum sample drawn prior to transplantation. A sharp increase in serum N/C ratio was consistently observed after lung transplantation (Fig 1). The mean serum N/C ratio increased to 359 ± 42 μmol/mol 2 days after transplantation (p<0.001, paired Student's t test). After this initial increase, various different patterns were observed.

In the three control patients with an uncomplicated posttransplantation course, the serum N/C ratio rapidly decreased and reached 160 ± 32 μmol/mol at day 10 after transplantation. In cases of rejection (six events), a marked elevation of serum N/C ratio was always observed. Interestingly, this increase was detectable before the diagnosis of rejection: the mean serum N/C ratio was 815 ± 182 μmol/mol at least 24 h.
A total of 14 infectious events were diagnosed in 13 patients during the first 3 weeks after transplantation. Eleven were of bacterial origin: there were 10 cases of bacterial pneumonia (5 Pseudomonas, 4 Staphylococcus, and 1 Serratia), and 1 case of Staphylococcus parietal suppuration. The other three infections were CMV pneumonia. All these complications were associated with a marked increase of the N/C ratio: the mean serum N/C ratio increased to 677±75 μmol/mol (bacterial infections, 589±64 μmol/mol; CMV pneumonia, 1,011±163 μmol/mol). Interestingly, serum N/C ratios increased early in the course of CMV infection. In these three patients, the ratio increased by more than 30 percent between two consecutive measurements 2 to 7 days before the diagnosis of CMV pneumonia (see Fig 3 for a representative case).

Serum N/C Ratio After the Third Postoperative Week

The serum N/C ratios of 44 patients in the late postoperative period (after day 21) were analyzed. During this period, the most common complications were rejection events and CMV infections. There were 20 cases of CMV pneumonia in 16 patients (diagnosis established between days 26 and 372) and 24 rejection events in 15 patients (diagnosis established between days 30 and 1,152). We compared the results observed in these cases to those from 30 control blood samples taken from 29 asymptomatic patients displaying neither infection nor rejection when the blood samples were collected (studied between days 22 and 1068).

The mean (±SEM) serum N/C ratio was 786±113 μmol/mol in cases of CMV pneumonia. This level was not significantly different from that of patients with bacterial infection (seven events, data not shown), but it was significantly higher than that of control subjects (132±12 μmol/mol, p<0.001) and that of patients undergoing lung rejection (163±25 μmol/mol, p<0.001). There was no statistical difference between the serum N/C ratio in the control subjects and that in the rejection group (Fig 4).

The diagnosis ofobliterative bronchiolitis was established in 12 of the 44 patients studied. This complication was not associated with an increased serum N/C ratio. The mean (±SEM) serum N/C ratio was 145±24 μmol/mol in these patients (Fig 4).

Correlation Between Serum sIL-2R Levels and Serum N/C Ratios

Soluble interleukin 2 receptor is a marker of immune cell activation.14-16 We therefore investigated whether serum sIL-2R levels and the N/C ratio were correlated. We compared these two markers in 438 samples. The N/C ratio and sIL-2R levels correlated, as evidenced by linear-regression variance analysis (r = 0.625, p<0.001). We classed the samples into two
groups: infections (33 cases) and rejection events (31 events), and analyzed the relationship between the N/C ratio and sIL-2R levels independently in each group. The N/C ratio and sIL-2R levels were correlated in both groups. During infection, the coefficient of correlation was $r = 0.516$ (p<0.01), and it was $r = 0.741$ during rejection events (p<0.001). Therefore both infections and rejection events are associated with an increase of both sIL-2R and N/C.

**DISCUSSION**

The aim of this study was to determine the dynamics of the serum N/C ratio after lung transplantation, and the variations of this ratio during major and frequent posttransplantation complications (allograft rejection, CMV pneumonia, and obliterative bronchiolitis). Overall, the serum N/C ratio was a sensitive but nonspecific marker of immune activation in the first 21 days following transplantation. We also showed that an increased N/C ratio later after lung transplantation was related to infectious complications, mainly those due to CMV.

The serum N/C ratio in 19 patients was normal before transplantation. This finding is interesting, as it suggests an absence of systemic immune activation in patients displaying a wide range of end-stage chronic pulmonary diseases, including bronchiectasis, idiopathic pulmonary fibrosis, lymphangiomyomatosis, and histiocytosis X.

The patterns observed during the first 3 postoperative weeks differed from later patterns. Even in the absence of early complication, there was a fourfold increase in the serum N/C ratio in our patient population immediately following lung transplantation. At least three explanations may account for this finding. First, allogenic-stimulated T cells are known to secrete interferon-γ, and such allogenic-mediated interferon-γ production would lead to an increased excretion of N. Secondly, a 7-day course of polyclonal antilymphocyte globulins was administered to each of the patients. Antilymphocyte globulins are known to activate T cells *in vitro* and *in vivo*. This activation is characterized by the release of a wide range of cytokines, including interferon-γ. Thirdly, modification of N/C ratio may result directly from surgery itself or anesthesia.

The serum N/C ratio increased at least 24 h before diagnosis of rejection events could be established in the early postoperative period. This observation is interesting, as it demonstrates early T-cell and macrophage activation during allograft rejection. Moreover, the serum N/C ratio increased up to 7 days before the diagnosis of CMV infection occurring during the first 3 weeks after transplantation. Cytomegalovirus is one of the major problems in clinical transplantation, and it is suspected of being involved in subsequent lung rejections that may lead to obliterative bronchiolitis. As our results show that N is a sensitive marker of CMV infection, it may be useful to monitor the N/C ratio in D+/R− mismatched pairs (seronegative recipients, R−, transplanted with a lung from a seropositive donor, D+) in order to detect CMV infections as early as possible.

Obliterative bronchiolitis is one of the foremost threats to the long-term survival of lung recipients. Evidence of *in vitro* bronchoalveolar lymphocyte reactivity to allogenic cells both before the onset of obliterative bronchiolitis and in patients with obliterative bronchiolitis indicates that immune mechanisms may play a role in the development of this complication. However our study failed to demonstrate an increase of the serum N/C ratio or serum sIL-2R level in patients with obliterative bronchiolitis.

Cytomegalovirus pneumonia was usually associated with an increased serum N/C ratio regardless of the time after transplantation. In contrast to early rejection and CMV pneumonia, rejection events occurring a long time (3 weeks or more) after transplantation...
were not associated with an increased serum N/C ratio. Early and late rejections may therefore involve different immune mechanisms. This could be related to the progressive development of a partial tolerance of the T lymphocyte compartment following transplantation, which would result in a decreased production of interferon-γ, one of the major T-lymphocyte products.

The absence of an increase in the serum N/C ratio during late rejection events parallels the paucity of clinical symptoms associated with this late complication. Thus late rejection cannot be diagnosed from the analysis of peripheral markers such as N or sIL-2R and multiple TBB are still required. This finding also suggests that evaluation of in situ markers of immune cell activation may be more informative than peripheral markers concerning the pathophysiology of late rejection. The concentration of N in BAL fluid was also evaluated. All samples studied were below the limits of assay sensitivity because of saline solution dilution (data not shown). In contrast in situ hybridization studies of BAL cells have shown that macrophages express the interleukin-1β gene during late rejection events. Macrophages may thus play a role in the pathogenesis of late rejection and in the subsequent development of an obliterator bronchiolitis.

Our results indicate that, in the early postoperative period, N is not a specific marker of rejection or infection. In contrast, later after transplantation, an increased serum N/C ratio is only found in the case of infection, mostly CMV pneumonia. Monitoring the N level might allow earlier diagnosis of CMV infections, particularly in D+ / R− mismatches where CMV infections are most common. Antiviral treatment that might prevent subsequent lung injury may thereby be started earlier.

ACKNOWLEDGMENTS: The writers thank N. Devilliere (Laboratoires Behring) for the kind gift of Neopterin-RIAc.

REFERENCES

454

Serum Neopterin after Lung Transplantation (Humbert et al)