Increased Serum Levels of Tumor Necrosis Factor α Precede Major Complications of Bone Marrow Transplantation


Acute graft-versus-host disease, interstitial pneumonitis, endothelial leakage syndrome, and veno-occlusive disease are major complications of bone marrow transplantation. Though several new regimens for prophylaxis and treatment of these syndromes have been introduced, the overall incidence has been only slightly reduced over the last few years. We prospectively analyzed tumor necrosis factor alpha (TNFα) serum levels between day −8 and day 100 after bone marrow transplantation in 56 patients transplanted in our unit for a variety of hematological diseases. In 34 patients with uneventful courses, mean TNFα levels rose to a maximum of 76 ± 29 pg/mL. In contrast, 22 patients with major transplant-related complications showed mean increases of TNFα of 492 ± 235 pg/mL (P < .0001). Increases of TNFα occurred before interstitial pneumonitis and severe acute graft-versus-host disease with a latency of 25 to 54 days. Early complications such as endothelial leakage syndrome and veno-occlusive disease were closely associated with increases of TNFα serum levels. Our study suggests two pathways of TNFα release: activation of host macrophages and stimulation of donor cells in the course of acute graft-versus-host disease. Cytokine monitoring should be helpful for prediction and earlier treatment of major transplant related complications.

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MATERIALS AND METHODS

Patients. All patients receiving BMT in our unit from January 1986 until June 1988 were included in this analysis. Fifty-two patients received allogeneic (49 HLA-identical and three HLA-mismatched sibling) transplants; two patients were grafted from syngeneic donors and two patients had autologous grafts. Median age of these patients at the time of BMT was 35 (range 18 to 55) years. Twenty-three recipients were female and 33 were male. Diagnoses and stages of disease were as follows: acute myeloid leukemia (AML): first or second complete remission (CR), n = 15, greater than second CR, n = 3; acute lymphoblastic leukemia (ALL): first or second CR, n = 8, greater than second CR, n = 2; chronic myelogenous leukemia (CML): chronic phase, n = 12; CML advanced stages, n = 8; severe aplastic anemia, n = 2; myelodysplastic syndromes (MDS), n = 6.

Pretransplant conditioning regimens were fractionated total body irradiation (3 times, 4 Gy/d) in 41 patients or busulphan (4 mg/kg/d over 4 days) in 15 patients, followed by high dose cyclophosphamide (50 mg/kg on 4 consecutive days). Attendant risks were fully explained to all patients, donors, and relatives. Written informed consent was obtained from patients and donors.

Prophylaxis and treatment of aGVHD in patients receiving allogeneic BMT. Prophylaxis of aGVHD was performed by continuous infusion of cyclosporin from day −1 until day 28 after BMT followed by oral cyclosporin. Plasma levels were adjusted between 150 and 200 ng/mL in the first 3 to 4 months. After this period, cyclosporin was gradually reduced in patients without evidence of aGVHD. In addition, all patients received prophylactic intravenous methotrexate on days 1, 3, and 6. Acute GVHD was graded according to standard clinical and biochemical criteria. Patients who developed aGVHD grade II or more were treated with corticosteroids and further weekly injections of methotrexate (10 mg/m²) in the absence of myelosuppression. Patients not responding to this treatment received 1 or 2 courses of monoclonal antibody (MoAb) OKT3 (OrthoHoechst OKT 3; n = 7) or rabbit antithymocyte globulin (ATG Fresenius; n = 4).

Serum samples. Sera of 20 healthy volunteers or bone marrow donors were used as control. All sera were collected by peripheral vein puncture or drawing from Hickman catheters and stored at −60°C until use. In patients, sera were obtained weekly after...
admission to the BMT unit (day -8) until day 21. In patients with complicated courses, further weekly samples were obtained until discharge or death. Random samples of patients with uneventful courses were available after day 21 until day 100. Sera of at least 15 patients were analyzed for each time interval in these patients. In total, 363 serum samples were included in this study.

As an example, consider the following text snippet: "Assay of TNFα. TNFα was analyzed by an enzyme-linked immunosorbent assay (ELISA) using two MoAbs recognizing different epitopes of TNFα. Specificity of the MoAbs was proven by immunosorbent assay (ELISA) using two MoAbs recognizing different epitopes of TNFα. Quantitation of recombinant human TNFα (rhTNFα) and natural human TNFα in supernatants of stimulated mononuclear cell cultures by ELISA gives identical results as compared with bioassays using L929 cells. The limit of detection is 5 pg/mL as determined by analysis of rhTNFα. From each sample, at least three dilutions were analyzed. Mean TNFα levels were calculated and recorded.

Analysis of bacterial and fungal infections. Surveillance cultures of nose, throat, mouth, skin, and stool were performed twice weekly. In patients with fever above 38.5°C, urine, sputum, and at least four blood cultures were obtained. Systemic or invasive infections were diagnosed when bacteria or fungi could be isolated from blood or tissue.

Analysis of clinical courses and data. Patients were divided into two groups: patients treated with routine supportive care, antibiotics, and/or antimycotics and immunosuppressive drugs were designated as having no or minor TRC; ie, uneventful courses; and patients requiring any kind of intensive care support were judged to have major TRC. Intensive care support included catecholamine treatment due to renal or circulatory impairment and/or oxygen supply/mechanical ventilation due to respiratory failure.

Major TRC requiring intensive care support in our study were ELS, VOD, IP, severe microangiopathy resembling thrombotic thrombocytopenic purpura (MA), and aGVHD, grade IV. ELS was diagnosed if patients developed increases of body weight (more than 5%) within a few days, due to generalized and/or pulmonary fluid retention that could not be treated by diuretics alone. VOD and aGVHD were diagnosed according to clinical criteria. IP was assumed if respiratory failure occurred simultaneously with typical changes of chest x-rays. Diagnosis of MA included extensive hemolysis and thrombocytopenic purpura requiring de novo transfusions combined with fluid retention as observed in ELS. Necropsy sections were performed in all patients who died. For each patient, maximal serum levels and time courses of TNFα were recorded. TNFα levels were compared in the different groups by Wilcoxon tests. Comparison of overall frequencies was performed by χ² analysis.

RESULTS

TNFα serum levels of healthy controls. TNFα was detectable in sera of 5 of 20 healthy controls. The mean level was 11 pg/mL with a standard deviation of 26 pg/mL (range 10 to 87 pg/mL). Thus, TNFα levels above 100 pg/mL were considered to be pathological within this study.

TNFα serum levels at the time of diagnosis of TRC. A total of 22 patients had major TRC in this study. Sixteen patients had more than one complication in series: in these patients, early ELS was followed by aGVHD and MA (n = 6) or IP (n = 5), or a combination of both (n = 5). For statistical analysis, one diagnosis describing the most serious clinical problem was chosen for each patient: by these criteria, six patients had TRC occurring before engraftment (ELS, n = 5; VOD, n = 1), and 16 patients had later TRC (IP, n = 9; aGVHD, n = 4; MA, n = 3). TNFα levels analyzed at the time of maximal symptoms of TRC allowing clinical diagnosis were pathological in all patients with early TRC, but only in 3 of 16 patients with IP, MA, and aGVHD grade IV.

Time courses of TNFα serum levels. Analyses of time courses of TNFα serum levels, however, revealed significant increases in all patients with IP, MA, and aGVHD IV before diagnosis or development of maximal clinical symptoms. Maximal TNFα serum levels between day -8 and +100 after BMT were significantly different between patients with major TRC and patients with uneventful courses (492 ± 235 pg/mL versus 76 ± 29 pg/mL, P<.0001, Fig 1). TNFα maxima preceded clinical diagnosis or maximal symptoms in patients with IP, aGVHD, and MA with a latency of 24 to 54 days (Table 1). They were closely associated with clinical symptoms in ELS and VOD. Only 6 of 34 patients with uneventful courses had increases of TNFα above 100 pg/mL. In these patients, TNFα maxima were associated with fever without documented infection (n = 1) or occurred in the initial phase of minor aGVHD (n = 5). In most patients with major TRC, TNFα levels were increased for a period of...
Table 1. Maximal TNFα Serum Levels in Major Transplant Related Complications Occurring After BMT

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Maximal TNF Levels (pg/mL)</th>
<th>Time of TNF-Maxima* (Range)</th>
<th>Time of Diagnosis* (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP</td>
<td>9</td>
<td>431 ± 69</td>
<td>31 (11-80)</td>
<td>85 (53-150)</td>
</tr>
<tr>
<td>aGVHD</td>
<td>4</td>
<td>462 ± 250</td>
<td>20 (4-30)</td>
<td>45 (12-60)</td>
</tr>
<tr>
<td>MA</td>
<td>3</td>
<td>472 ± 62</td>
<td>27 (26-85)</td>
<td>80 (80-98)</td>
</tr>
<tr>
<td>ELS</td>
<td>5</td>
<td>558 ± 171</td>
<td>6 (5-12)</td>
<td>8 (5-16)</td>
</tr>
<tr>
<td>VOD</td>
<td>1</td>
<td>680</td>
<td>5 (5-64)</td>
<td>8</td>
</tr>
<tr>
<td>Pts with uneventful courses</td>
<td>34</td>
<td>76 ± 29</td>
<td>13</td>
<td>—</td>
</tr>
</tbody>
</table>

Levels were compared with TNFα in patients with uneventful courses. Mean ± SE of TNFα maxima, median and range of time of TNFα-maxima and time of diagnosis (for IP, VOD, ELS) or maximal clinical symptoms (for MA, aGVHD) of complications are shown.

Abbreviation: Pts, patients.

*Values are days post-BMT.

2 or more weeks. Therefore, mean TNFα levels were different at any time between day +8 and day +60 after BMT from patients with uneventful courses (Fig 2).

Association of TNFα levels with bacterial or fungal infections and fever. At the time of TNFα maxima, only 10 of 56 patients had systemic or invasive infections. In addition, the overall frequency of infections occurring during the aplastic phase was analyzed. A total of 20 patients had documented infections in this period (11 in the group developing major TRC, 9 in the group with uneventful courses, difference not significant). Only 8 of these 20 patients had detectable levels of TNFα at the time of infection, with a mean of 99 ± 66 pg/mL, which is lower than in typical TRC.

Fever and TNFα levels were separately analyzed. During aplastic phase, there was a weak correlation between absolute TNFα levels and extent of fever ($R = .38$, not significant). In this period, 45 patients developed fever greater than 38.5°C, and TNFα levels were pathologic in 23 of them. Moreover, 22 of 23 patients with elevated TNFα levels suffered from fever greater than 38.5°C. Only one patient developed fatal aseptic shock and pulmonary ELS at day +5 after BMT with an acute increase of TNFα to 856 pg/mL in the absence of fever. After engraftment, no correlation of TNFα levels and fever was observed, as only 5 of 20 patients with pathological increases of TNFα had fever greater than 38.5°C. In these patients, absence of fever might be explained by concomitant treatment with corticosteroids due to aGVHD.

TNFα serum levels and aGVHD. When patients receiving allogeneic transplants were grouped according to severity of aGVHD, maximal TNFα levels were significantly lower in patients with absent or grade I aGVHD, as compared with patients with more severe grades (Table 2). Contribution of allogeneic stimulation to cytokine release could also be answered by comparing allogeneic, syngeneic, and autologous BMT. Our study includes only four patients with syngeneic or autologous grafts not allowing statistical analysis. However, one of these patients developed severe ELS after autologous BMT, which was associated with a dramatic increase of TNFα.

Influence of recipient characteristics and increases of TNFα before BMT on development of TRC. Development of TRC and increases of TNFα serum levels were not significantly correlated with age, sex, or diagnosis at the time of BMT. There was a trend to a higher frequency of
increased TNFα levels in patients with more advanced diseases \( (P = .06) \). In a total of 51 patients, serum samples obtained during pretransplant conditioning were available. Twelve patients had increases of TNFα above 100 pg/mL before BMT. This pattern of early TNFα release was more frequently observed in patients with CML and MDS (38%) than in patients with other diagnoses (7%, \( p < 0.01 \)). Increases of TNFα during pretransplant conditioning were predictive for development of TRC within the first 6 months after BMT: 10 of these patients (83%) subsequently developed major TRC as compared with 12 of 39 patients (31%) without increases of TNFα before BMT (\( p < 0.01 \)).

Outcome of patients. Only 4 of 22 patients with major TRC survived beyond 1 year after BMT, while 33 of 34 patients with uneventful courses were alive (\( P < 0.001 \)). Causes of death in patients developing major TRC were ELS/VOD (\( n = 2 \)), mulltorgan failure with adult respiratory distress syndrome (\( n = 8 \)), or fungal septicemias (\( n = 6 \)) in the course of TRC, and early relapse after surviving TRC (\( n = 2 \)). The only death occurring in patients with uneventful courses was due to an early relapse.

**DISCUSSION**

Cytokines have been characterized as the major mediators of immunity in the last years. Interleukin-1 (IL-1) and TNFα are predominantly produced by monocytes/macrophages on activation by a variety of stimuli, including interferon gamma (IFN-γ) and bacterial lipopolysaccharid (LPS). Several reports suggest a pathogenetic role of TNFα in clinical complications such as septic shock and purpura fulminans. A rise of TNFα serum levels has also been reported before rejection of kidney grafts. Here we report a highly significant association of increases in TNFα serum levels with major TRC occurring within the first 6 months after BMT. Differences of TNFα are significant at any time after BMT when compared with patients with uneventful courses.

Our data were obtained by a sensitive ELISA using specific MoAbs. These assays allow detection of immunologically reactive material but frequently lack correlation with bioassays measuring biologically active cytokines. Determination of cytokines in body fluids such as serum, urine, or ascites is complicated by the presence of specific inhibitors for IL-1 and TNFα. So far, no systematic studies addressing the influence of inhibitors on cytokine assays in sera have been reported, and present experiments performed in our laboratory try to analyze these interactions in sera of BMT recipients.

A second concern is raised by lability of TNFα, even in sera cryopreserved at \(-70^\circ C\), which has been attributed to proteases. We recently started a prospective monitoring of cytokine levels in fresh sera of BMT recipients; so far, 20 patients have been analyzed between day \(-8\) and \(+100\) after BMT. Absolute TNFα levels were significantly higher in fresh sera, indicating some loss of activity by cryopreservation, but again we observed a significant correlation of increases of TNFα with aGVHD and occurrence of TRC.

**Table 2. Maximal TNFα Serum Levels in Recipients of Allogeneic BMT: Correlation with Severity of aGVHD**

<table>
<thead>
<tr>
<th>aGVHD (Grade)</th>
<th>n</th>
<th>Maximal Serum Levels of TNFα (±SE, pg/mL)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-I</td>
<td>18</td>
<td>58 ± 22</td>
<td>&lt;.02</td>
</tr>
<tr>
<td>II</td>
<td>20</td>
<td>221 ± 64</td>
<td>NS</td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>306 ± 92</td>
<td>NS</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>483 ± 186</td>
<td></td>
</tr>
</tbody>
</table>

aGVHD was graded according to clinical criteria as described. Statistical analysis between the groups was performed by Wilcoxon tests. Abbreviation: NS, not significant.

Maximal TNFα levels were 56 ± 26 pg/mL in patients without aGVHD (\( n = 6 \)), 480 ± 210 pg/mL in patients with aGVHD grade I (\( n = 7 \)), and 1,672 ± 746 pg/mL in patients with aGVHD grade II or more (\( n = 7 \), \( P < .05 \)). In addition, increases of TNFα levels before BMT again proved to be predictive for development of TRC within the first 4 months after BMT (\( P < .01 \)). Thus, loss of TNFα activity by cryopreservation seems to be a random process and does not interfere with results obtained in the retrospective analysis.

As most clinical studies reported raised levels of TNFα in association with microbial or parasitic infections, we analyzed documented infections in our patients. However, only 21% of patients had documented infections at the time of TNFα maxima, and vice versa, TNFα serum levels increased in only 40% of patients with septicemia without further signs of TRC. Therefore, infections are unlikely to be the major cause of cytokine release observed in our study.

In our analysis of microangiopathy occurring in patients with aGVHD receiving cyclosporin prophylaxis, we postulated cytokine release by monocytes activated via IFN-γ in the course of T cell stimulation. Thus specific immunologic activation in aGVHD might be augmented by monocyte activation with subsequent release of cytokines. Other groups also suggested a role of cytokines as effectors in lesions due to aGVHD. Piguet showed that histological changes of skin and gut in the course of aGVHD could be prevented by prophylactic use of a polyclonal antibody neutralizing TNFα in a mouse model. In addition, this regimen reduced aGVHD-related mortality from 100% to 30% without further immunosuppression.

When TNFα levels were analyzed in patients with aGVHD by other groups, they failed to detect significant levels in more than 50% of patients. This is also confirmed by our study as only 3 of 16 patients with aGVHD or associated complications had pathological levels at the time of diagnosis or maximal symptoms. However, occurrence of raised TNFα levels in all patients before clinical symptoms, as seen in our patients, suggests cytokine release to be an early event during induction of clinical changes. This is further supported by the close correlation of TNFα maxima with clinical grades of aGVHD.

IP is a typical complication of the first 2 to 6 months after allogeneic BMT. So far toxicity (total body irradiation) and viral (cytomegalovirus) infections have been discussed as the major pathogenetic mechanisms. Cytokine activity before occurrence of respiratory changes as observed in our study might indicate local activation of pulmonary macrophages.
Again, Piguet reported a necrotizing alveolitis associated with increased local production of TNFα in mice with aGVHD.\textsuperscript{17} A pathophysiological role of TNFα for development of IP would also fit with the data of Niederwieser, who detected excessive IFN-γ release in the course of IP and cytomegalovirus infections after human BMT.\textsuperscript{20}

Thus, release of TNFα in patients with IP and MA could be explained by activation of donor cells as a consequence of aGVHD-associated and/or viral stimulation. Our study, however, also revealed increases of TNFα early after BMT while patients are still aplastic, and a close correlation of early complications as ELS and VOD with cytokine release was observed. So far, toxic mechanisms have been discussed in the development of ELS as cyclosporin for pulmonary capillary leakage syndrome,\textsuperscript{3} while VOD was observed more frequently in patients with pre-existing liver disease.\textsuperscript{4} As in MA, endothelial cells are the targets of pathogenetic mechanisms in both syndromes. Contribution of TNFα, as suggested by our data, would fit with experimental results showing TNFα as the predominant cytokine modulating endothelial functions.\textsuperscript{21} In VOD, local activation of Kupffer cells might result in damage of small hepatic venules, which is the characteristic histopathological lesion in this disease.

As only one patient in our study developed VOD, the association with release of cytokines clearly needs confirmation by a systematic analysis of a larger series of patients. However, this observation suggests stimulation of host macrophages to be a further pathway of cytokine release in the setting of BMT. Tissue macrophages and related cells are not eliminated by pretransplant conditioning and persist up to 4 months after BMT, as demonstrated for alveolar macrophages\textsuperscript{22} and Langerhans cells.\textsuperscript{23} In addition, macrophages retain their ability to release cytokines after differentiation from blood monocytes.\textsuperscript{24} Participation of host macrophages in cytokine release and induction of TRC is strongly supported by a significantly higher risk of TRC in patients with increases of TNFα before BMT, as seen in our analysis.

As cytokine activation is independent of HLA-restriction, release of TNFα indicated by positive serum levels might result from a complex interaction of donor cells and recipient macrophages. Host macrophages might be stimulated by pretransplant conditioning or endotoxin as mucosal barriers are most severely damaged in the early aplastic phase. TNFα release by host macrophages should change reactivity of donor cells directly and by induction of HLA-antigens in recipient tissues. Vice versa, IFN-γ and IL-2 released by activated donor cells might also stimulate recipient macrophages in the course of aGVHD and thus potentiate clinical changes. These interactions are of particular interest in patients with CML and MDS. Some BMT centers observed an increased incidence of TRC in patients transplanted for CML, and in our experience, the risk of TRC is increased in patients with MDS. As increases of TNFα before BMT indicating activation of host macrophages were predominantly observed in patients with these diseases, our data suggest abnormal reactivity of host macrophages, which might contribute to an increased cytokine release after BMT.

Finally, the close association of all major TRC with increases of TNFα raises further questions concerning the role of endothelial cells in these syndromes. They might be the primary targets in complications associated with aGVHD, as they are in early complications occurring during aplastic phase. ELS resembles acute endothelial damage observed during immunotherapy with interleukin-2\textsuperscript{25} and might result from direct toxicity of cytokines. Delay between TNFα maxima and complications occurring after engraftment indicates a more complex interaction of cytokines and target cells that might involve enhanced turnover of endothelial cells and macrophages, as well as induction of organ-specific immune reactions. There is increasing evidence for the central role of endothelial cells in initiating local immune responses by expression of various adhesion molecules and production of a variety of further cytokines.\textsuperscript{26}

In summary, our data indicate a major pathophysiological role of TNFα in complications occurring during the first 6 months after BMT. Further studies are needed to reveal the exact pathways of cytokine release with regard to participating cells and involvement of related cytokines. Cytokine monitoring should help in early detection of major TRC and allow an earlier and more differentiated treatment in the future.

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REFERENCES


