Modulation of Acute Graft-Versus-Host Disease After Allogeneic Bone Marrow Transplantation by Tumor Necrosis Factor α (TNFa) Release in the Course of Pretransplant Conditioning: Role of Conditioning Regimens and Prophylactic Application of a Monoclonal Antibody Neutralizing Human TNFa (MAK 195F)


Contribution of host-related cytokine release in the course of pretransplant conditioning to early tissue damage and induction of acute graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (BMT) has been shown in experimental models. We performed a clinical phase I/II trial applying a monoclonal antibody neutralizing human tumor necrosis α (TNFa) during pretransplant conditioning as additional prophylaxis in high-risk patients admitted to allogeneic BMT; TNFa serum levels and clinical courses in 21 patients receiving anti-TNFa prophylaxis were compared with data from 22 historical controls. Absence of significant release of TNFa in the period of busulphan (BUS) treatment, but significant induction of TNFa by total body irradiation (TBI) and cyclophosphamide (CY) conditioning were correlated with significantly earlier onset of acute GVHD in patients receiving BUS/CY regimens as compared with BUS/CY-treated patients. Prophylactic application of monoclonal anti-TNFa seemed to postpone onset of acute GVHD from day 15 to day 25 (P < .05) after TBI/CY and from day 33 to day 53 after BUS/CY (P < .10) conditioning. Application of monoclonal anti-TNFa in low and intermediate doses was safe and not associated with an increased incidence of infectious or hematologic complications. Thus, our data provide indirect and direct evidence for involvement of conditioning-related cytokine release in induction of early acute GVHD in the clinical setting and support further investigation of this novel approach in randomized trials.

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Following either CY2 or CY4. Although CY2 (1 in the MAK195F group) or oral busulphan (BU; 4 mg/kg/d on four daily infusions of 40 to 50 mg/kg cyclophosphamide (CY?) and nine in the historical control group) and CY4 application (10 in total dose) followed by either two daily infusions of PGE2 prophylaxis as well (P < .01).

Abbreviation: MDS, myelodysplastic syndrome (RAEB or RAEB-T). CY and BUS/CY conditioning regimens, and was recently closed. Our study was designed to analyze side effects of TNFα neutralization in an early period of allogeneic BMT and to evaluate the role of host-related cytokine release in induction of clinical GVHD. Careful analysis of patients included in this trial and a comparable group of historical controls now provides additional insights in cytokine induction by various myeloablative regimens and its association with kinetics of acute GVHD.

MATERIALS AND METHODS

Patients

Based on our previous data showing an increased incidence of systemic release of TNFα during pretransplant conditioning in patients suffering from chronic myelogenous leukemia (CML) or myelodysplastic syndromes (MDS) with an age ≥40 years, only this clinical high-risk group was considered for inclusion in our phase I/II study on prophylactic neutralization of TNFα. Only patients receiving grafts from HLA-identical or single-antigen–mismatched siblings were included, and autologous back-up marrows were collected before conditioning at least for patients receiving a new dose level of MAK 195F. Twenty-five consecutive patients fulfilling these criteria from August 1990 until January 1994 were enrolled; patient characteristics are summarized in Table 1. The study was performed according to a protocol approved by the ethical committee of Ludwig-Maximilians University of Munich (Munich, Germany), potential risks were fully explained to patients and relatives and written informed consent was obtained. Twenty-two patients suffering from CML or MDS who were ≥40 years old and were receiving HLA-identical or single-antigen–mismatched sibling grafts from January 1984 until July 1990 served as a historical control and were compared with the study group.

Pretransplant Conditioning and Supportive Care

Pretransplant conditioning regimens consisted of total body irradiation (TBI; 3 daily fractions of 4 Gy, lung shielding above 10 Gy total dose) followed by either two daily infusions of 60 mg/kg or four daily infusions of 40 to 50 mg/kg cyclophosphamide (CY2; CY4) or oral busulphan (BU; 4 mg/kg/d on 4 consecutive days), followed by either CY2 or CY4. Although CY2 (11 in the MAK195F and nine in the historical control group) and CY4 application (10 in MAK195F patients and 13 in controls) was equally distributed, because of historical reasons, a higher proportion of MAK195F prophylaxis patients was treated with BUS/CY regimens (Table 1). Since 1989, patients ≥45 years old or those with a history of preexisting liver disease or hepatomegaly at admission received additional prophylaxis with progolandin E2 (PGE2) (Prostavasin, 480 µg/d, continuous intravenous infusion; Schwarz Pharma, Monheim, Germany) starting from the first day of pretransplant conditioning until day 20 after BMT, again resulting in an imbalance toward PGE2 prophylaxis in the MAK195F group. Further supportive care was identical for all patients: They were treated in reverse isolation rooms with air filtration and received oral nonabsorbable antimycotics and antibiotics, intravenous acyclovir and intravenous polyvalent Ig (0.3 mg/kg in 2-week intervals).

Application of MAK 195F

MAK 195F was prepared as a lyophilized powder by KNOLL AG (Ludwigshafen, Germany) and reconstituted immediately before application. Single doses were administered as 15-minute infusions via Hickman catheters every 8 hours. Application of MAK 195F started in the morning of the day of first fraction of TBI in patients receiving TBI/CY conditioning or before first oral uptake of busulphan in patients receiving BUS/CY conditioning. Last dose of MAK 195F was given at 12 PM of the day before BMT resulting in a treatment period between 9 days in patients receiving BUS/CY4 conditioning and 6 days in patients prepared with TBI/CY2 regimens.

The study protocol had a combined phase I/II design: In the first phase, three different dose levels (single doses of 0.5 mg/kg, 1.0 mg/kg, and 3.0 mg/kg) were projected to be analyzed in cohorts of 4 patients. The dose level resulting in prevention of aGVHD in at least two consecutive patients in the absence of considerable toxicity was then chosen to be tested in a further 10 patients. According to this design, 4 patients received 0.5 mg/kg; 14 patients, 1.0 mg/kg; and 3 patients, 3 mg/kg.

Prophylaxis and Treatment of aGVHD

All patients received standard prophylaxis of aGVHD with intravenous cyclosporin starting on day −1 and a short course of methotrexate (MTX) (days 1, 3, and 6) as previously described. There were no major differences in cyclosporin levels and applied MTX dose between MAK195F patients and historical controls. GVHD was graded according to the criteria of Glucksberg as modified by Martin et al. Patients developing aGVHD grade II were treated with a fixed dose of prednisolone (300 mg/d tapered by 10% every 48 hours) and antithymocyte globulin (ATG Fresenius, Bad Homburg, Germany) for 7 days with a daily dose of 3 mg/kg. Second line treatment in patients with refractory GVHD was performed with a 7-day course of 5 mg/d OKT3 (OKT3 Orthoclone, Cilag, Sulzbach/Tannus, Germany).

Diagnosis of Complications and Infections

Veno-occlusive disease (VOD) was diagnosed according to clinical criteria as described by Jones et al. Endothelial leakage syndrome (ELS) was suspected in patients with weight gain ≥5% within 7 days in the presence of normal or low central venous pressure. For diagnosis of infections, at least four blood and urinary cultures were obtained in all patients with fever ≥38.5°C, and chest radiographs were performed immediately and weekly thereafter; infections were categorized as blood borne (at least two positive blood cultures and fever) or organ (positive chest radiograph or skin infiltrates, positive blood or organ cultures and fever) infections. Viral infections were diagnosed in patients with positive antigen tests in blood, urine, stool samples, or biopsy specimens (cytomegalovirus...
(CMV) early antigen/adenovirus antigen/rotavirus antigen) in the presence of symptoms associated with CMV or adenovirus-infections (pneumonia/diarrhea).

**Engraftment and Chimerism**

Engraftment was monitored by routine blood and reticulocyte counts and differential smears. Chimerism was monitored by cytogenetics and isoenzyme analysis in peripheral blood mononuclear cells and BM cells at day 30. Patients showing residual Ph-chromosomes besides normal karyotypes were considered to have mixed chimerism; relapse was diagnosed in the presence of hematologic changes indicative for CML or MDS. Late graft failure was defined by an novo decrease of blood counts (white blood cell count, <1,000/mL; reticulocytes, <10%; and platelets, <20,000/mL) in the presence of complete chimerism.

**Analysis of TNFα Serum Levels and Monitoring of MAK195F Treatment**

TNFα serum levels were analyzed using our previously described enzyme-linked immunosorbent assay (ELISA). In historical control patients, serum levels were analyzed once weekly in 12 patients and at least thrice weekly in a further 10 patients. In patients receiving MAK195F prophylaxis, serum samples were obtained every 8 hours before application of MAK195F during pretransplant conditioning and once daily after BMT. Because our TNFα-ELISA uses MAK195F for detection of TNFα bound to a different monoclonal capture antibody, results obtained by this type of ELISA reflect competition between serum MAK195F and MAK195F used for detection (competitive ELISA). Because of this competition, this ELISA is unable to differentiate between free TNFα and TNFα attached to serum MAK195F (TNFα-anti-TNFα complexes) in patients receiving MAK195F prophylaxis. Therefore, based on experiences obtained in studies using MAK195F in patients with refractory GVHD, we evaluated TNFα levels in selected samples of study patients by additional methods: bioactivity was determined by L929 bioassay; to prove binding of TNFα to MAK195F, samples showing high levels in the competitive type of ELISA were incubated with polyclonal rabbit-antimouse IgG (Sigma Chemical Co, Munich, Germany) resulting in precipitation of mouse IgG. After subsequent ultracentrifugation, interphases were collected and TNFα was analyzed by a noncompetitive polyclonal ELISA. This approach precipitates MAK195F and TNFα bound to MAK195F in a pellet, but does not affect recovery of native or recombinant human TNFα (rhuTNFα) in the interphase.

For indirect monitoring of MAK195F serum levels, serum samples were also analyzed by competitive ELISA after titration of samples with a fixed amount of 3,500 pg/mL of rhuTNFα (gift from KNOLL AG, Ludwigshafen, Germany). Recovery of rhuTNFα is decreased depending on the amount of free MAK195F in the sample, as shown by control experiments, and allows linear detection of MAK195F between 1 μg/mL and 100 pg/mL. Recovery of rhuTNFα in an individual sample was calculated as observed concentration (ie, concentration of TNFα in serum samples after addition of rhuTNFα) divided by expected concentration (ie, endogenous TNFα plus amount of rhuTNFα titrated in the sample) × 100%.

**Evaluation of Data**

Characteristics of patients, complications occurring during aplastic phase (days 0 through 20) and thereafter, and parameters characterizing engraftment and aGVHD were listed for historical controls as well as for the three different groups of patients receiving MAK195F prophylaxis. Because recent evidence suggested a strong influence of conditioning regimens on cytokine release and GVHD, further groups were formed according to the type of pretransplant conditioning. If dose effects of MAK195F were absent, data from the whole group receiving MAK195F were summarized for different conditioning regimens and compared for the corresponding group of historical controls. Means and SEM were calculated for continuous parameters. Incidences were compared by Fisher's exact test, continuous parameters by t-tests, and the time course of occurrence of aGVHD was additionally analyzed by Kaplan-Meier plots using the Number Cruncher Statistical Software system.

**RESULTS**

**Historical Controls**

In accordance with the high-risk criteria chosen for inclusion in this analysis, 9 of 11 patients receiving BUS/CY and 11 of 11 patients receiving TBI/CY conditioning developed aGVHD grade II until day 100 in historical controls. However, time of onset of GVHD requiring immunosuppressive treatment with corticosteroids was significantly different with a mean of 15 (± 1) days for TBI/CY-conditioned patients and a mean of 33 (± 6) days for BUS/CY-conditioned patients (Fig 1, P < .005).

In 10 of these patients, sufficient sampling allowed analysis of TNFα serum levels in relation to the various parts of myeloablative conditioning (Table 2): TBI/CY-treated patients showed a first significant increase during TBI. In BUS/CY-treated patients, maximal TNFα release occurred in the period of CY infusion suggesting that CY rather than BUS is involved in cytokine activation. Overall, maximal TNFα release observed during conditioning was significantly higher in patients developing severe GVHD or transplant-related complications (519 ± 448 pg/mL) as compared with those with uneventful courses (50 ± 17) confirming our previously published observations.

**Analysis of Serum Samples in Patients Receiving MAK195F Prophylaxis**

Monitoring of MAK195F treatment. Pharmacokinetics of MAK195F have been defined in a previous study applying MAK195F in patients with refractory GVHD and septicemia...
and showed a biphasic kinetic with a first half-life of 30 minutes and a second of 8 hours. In the present study, serum anti-TNF activity was routinely monitored by an indirect method analyzing recovery of titrated rhuTNFα; recovery of 3,500 pg/mL rhuTNFα is shown in Fig 2 and was most suppressed in patients receiving 3.0 mg/kg. Though infusion of anti-TNF was stopped at the day before BMT, relevant neutralization of rhuTNFα (ie, recovery <95%) could be detected until day 8 after BMT in patients receiving 0.5 mg/kg, until day 12 in patients receiving 1.0 mg/kg, and up to day 20 in patients receiving 3 mg/kg. Trough levels of MAK195F determined in selected samples during pretransplant conditioning were in the range of 0.8 to 5 μg/mL.

Human antimurine antibodies (HAMA) were monitored weekly in all patients receiving MAK195F until day 30 after BMT by commercial tests. Only 1 of 21 patients (receiving a dose of 1 mg/kg MAK195F) developed substantial immunoreactivity tested by three different methods. Although mean TNFα levels detected by our competitive ELISA were 210 ± 108 pg/mL, analysis with a second, noncompetitive ELISA using polyclonal TNFα antibodies for detection showed a mean of 665 ± 299 pg/mL in identical samples. Immunoreactivity was in striking contrast to results of L929-bioassay showing complete absence of bioactivity (all samples <30 pg/mL); Furthermore, immunologic activity detected in the polyclonal and monoclonal ELISA could be almost completely precipitated by pretreatment of samples with antimouse IgG and subsequent separation by ultracentrifugation; TNFα levels in the interphase went down to a mean of 35 ± 19 pg/mL (5%), whereas in the control experiment, ultracentrifugation after preincubation with a rabbit control antibody allowed detection of 392 ± 160 pg/mL (59%) in the interphase portion. These data clearly indicate that TNFα detected by immunoassays is attached to the murine TNFα-antibody in these samples confirming circulation of TNFα-anti-TNFα complexes.

Serum kinetics of TNFα-anti-TNFα complexes during pretransplant conditioning: Influence of conditioning regimen.

Because of formation of TNFα-anti-TNFα complexes in patients receiving MAK195F prophylaxis, the short half life of TNFα is adapted to the antibody’s kinetics, thus allowing prolonged and facilitating detection of TNFα released anywhere in the body. Daily kinetics of these levels gave characteristic patterns depending on the type of pretransplant conditioning used in individual patients (Fig 3, A and B). Patients treated with TBI showed the first sharp
increases of TNF-anti-TNF-complexes after each fraction of TBI. Activity regularly decreased until the next morning, an observation that is in line with the mentioned second half life of 8 hours of MAK195F. Further peaks are observed after CY infusion. This pattern was in strong contrast with patients treated with BUS/CY regimens; in this subgroup, minor accumulation of TNF-anti-TNF-complexes occurred during BUS conditioning, but again, steep increases were induced by CY infusion. Thus, these data clearly indicate differential induction of TNFα production by different principles of cytotoxic and myeloablative treatment preceding BMT.

Clinical Results of MAK195F Prophylaxis

Complications occurring during aplastic phase. Infusion of MAK195F was well tolerated in all patients, and side effects of pretransplant conditioning were unaffected. During aplastic phase, the incidence of raised bilirubin levels as well as overt VOD (1 in the control group, 1 in the MAK195F group) or ELS showed no significant difference (Table 3). Independent of the type of pretransplant conditioning, infusion of MAK195F was associated with a significant reduction of the overall incidence of febrile episodes, and, because of delay in onset of fever, with a reduction of the duration of fever in these patients; reduction of length of febrile episodes was predominantly observed in patients treated by TBI/CY conditioning. This reduction of fever was not accompanied by an increased or altered pattern of infectious complications. Organ infections included 3 bacterial and 1 candida pneumonia in historical controls, and 1 bacterial, 1 aspergillus pneumonia, and 1 pseudomonas sepsis with skin infiltrates in the MAK195F group. In all patients
except one control patient with VOD and candida septicemia, infections resolved after adequate antibiotic and antifungal treatments and engraftment of donor cells.

**Engraftment and chimerism.** Hematopoietic engraftment was unaffected by MAK195F prophylaxis in the 0.5 mg/kg and 1.0 mg/kg groups, but seemed to be delayed in patients receiving the highest dose (Table 4). Cyto genetic analysis of the BM performed at day 30 showed residual Philadelphia chromosome-positive cells in one patient of the control group and two patients of the 1.0-mg/kg-MAK195F group indicating mixed chimerism. In two of them, Philadelphia chromosomes disappeared in subsequent controls, but one patient receiving MAK195F developed overt hematologic relapse at day 69. A major concern was observation of late graft failure in two of the three patients treated with 3.0 mg/kg MAK195F; these patients presented with de novo pancytopenia at days 90 and 120 after BMT. One of them ultimately died at day 206 from septicemia without substantial recovery of blood counts, the other patient received a marrow boost from her donor at day 104 and recovered but subsequently died because of progressive GVHD.

**Table 3. Complications Occurring During Aplastic Phase**

<table>
<thead>
<tr>
<th>Complication</th>
<th>Historical Controls</th>
<th>MAK195F Prophylaxis</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin levels ≥13 mg/dL</td>
<td>7/22 (32%)</td>
<td>5/21 (24%)</td>
<td>NS</td>
</tr>
<tr>
<td>ELS</td>
<td>13/22 (59%)</td>
<td>12/21 (57%)</td>
<td>NS</td>
</tr>
<tr>
<td>No. of patients with fever &gt;38.5°C</td>
<td>20/22 (91%)</td>
<td>12/21 (57%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Mean duration (d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>5.8 (1.0)</td>
<td>3.3 (0.7)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>TB/CY</td>
<td>8.1 (1.4)</td>
<td>2.8 (0.8)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>BUS/CY</td>
<td>3.8 (1.0)</td>
<td>3.1 (0.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Infections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive blood culture</td>
<td>8/22 (36%)</td>
<td>4/21 (19%)</td>
<td>NS</td>
</tr>
<tr>
<td>Organ</td>
<td>4/22 (18%)</td>
<td>3/21 (14%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fever: mean days of fever and SEM are shown. Infections: bacterial or fungal infections were analyzed. Organ infections included pneumonia or soft-tissue/skin infections.

Abbreviation: NS, not significant.

**Table 4. Engraftment and Chimerism**

<table>
<thead>
<tr>
<th>Mean days to</th>
<th>Historical Controls</th>
<th>MAK195F 0.5 mg/kg</th>
<th>MAK195F 1.0 mg/kg</th>
<th>MAK195F 3.0 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANC ≥500</td>
<td>20 (1)</td>
<td>19 (2)</td>
<td>20 (2)</td>
<td>26 (1)</td>
</tr>
<tr>
<td>ANC ≥1,000</td>
<td>27 (3)</td>
<td>44 (19)</td>
<td>28 (4)</td>
<td>53 (23)</td>
</tr>
<tr>
<td>Platelet count ≥20,000</td>
<td>30 (6)</td>
<td>39 (20)</td>
<td>23 (4)</td>
<td>52 (24)</td>
</tr>
<tr>
<td>Chimerism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>21</td>
<td>4</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Mixed</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Late graft failure</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Platelet count greater than 20,000; third day of unsustained platelet counts greater than 20,000/mm³: mean days and SEM are shown. Chimerism was evaluated at day 30. Late graft failure: de novo decrease of ANC (<1,000) and platelet count (<20,000) after complete donor cell engraftment.

Abbreviation: ANC, absolute neutrophil count (mm³).

**Table 5. Acute GVHD and Short-Term Survival—Effect of Conditioning Regimens and Additional MAK195F Prophylaxis**

<table>
<thead>
<tr>
<th>Incidence of Acute GVHD</th>
<th>Historical Control</th>
<th>MAK195F Prophylaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade II until day 30</td>
<td>4/11 (36%)</td>
<td>3/15 (20%)</td>
</tr>
<tr>
<td>Grade II until day 100</td>
<td>8/11 (72%)</td>
<td>9/14 (64%)</td>
</tr>
<tr>
<td>&gt;Grade II until day 100</td>
<td>11/10 (100%)</td>
<td>11/10 (100%)</td>
</tr>
</tbody>
</table>

Survival >4 mo: 9/11 (82%) 6/11 (55%) 4/14 (28%) 2/6 (33%)

Differences in the incidence of aGVHD grade II were significant (P < .05) for TB/CY patients at day 30. Differences for severe aGVHD (>grade III) were of borderline significance for both TB/CY and BUS/CY patients at day 100 (P < .10).

**GVHD and short-term survival.** Because of the strong influence of conditioning regimens on the kinetics of acute GVHD observed in the control group, effects of MAK195F prophylaxis were analyzed in a twofold approach. When patients were grouped according to the conditioning regimen used (Table 5 and Fig 4) reduction of early GVHD by MAK195F prophylaxis was more significant in the TB/CY-treated group, but still detectable in BUS/CY-treated patients. Median time to onset of early acute GVHD was prolonged from day 15 to 25 in TB/CY-treated patients and from day 32 to 53 in BUS/CY-treated patients. In both
groups, delay of early aGVHD grade II translated in a trend to overall suppression of severe GVHD (grade III/IV). With regard to dose effects of MAK195F, single doses of 1.0 and 3.0 mg/kg seemed to be superior because of a lower incidence of early GVHD grade II and overall severe GVHD (Table 6).

Independent of the type of conditioning and the dose of MAK195F, chronic GVHD was unaffected by MAK195F prophylaxis; 8 of 15 evaluable control patients and 6 of 15 patients receiving MAK195F suffered from extended chronic GVHD requiring prolonged immunosuppressive treatment. In addition, no differences with regard to viral infections in the first 4 months after BMT were detected (data not shown).

To exclude an additional bias by the higher proportion of patients receiving PGE2-prophylaxis in the MAK195F group, the incidence of aGVHD was analyzed in relation to PGE2. In historical controls, all four patients receiving PGE2 developed GVHD, three of them grade III or IV, while 2 of 18 patients without PGE2-treatment had no signs of GVHD. In the MAK195F group, five of seven (71%) without PGE2 and 9 of 14 (65%) with PGE2 suffered from GVHD requiring immunosuppressive treatment. Thus, additional modulation of GVHD by PGE2-prophylaxis is unlikely to explain the effects observed in the MAK195F group.

Short-term survival was comparable in BUS/CY-treated patients, but might have been improved by MAK195F in TBI/CY-treated patients. Early deaths in the control group were almost exclusively caused by GVHD and interstitial pneumonitis (7 of 22; 32%), whereas in the MAK195F group two of four patients receiving 0.5 mg/kg and one patient of the 3.0 mg/kg group, who required a marrow boost because of graft failure, but only 2 of 14 (14%) of the 1.0 mg/kg group died because of GVHD. However, even in this group, the reduction of overall early transplant-related mortality is not significantly different because one additional patient died in the absence of aGVHD at day 40 from liver and cardiac failure associated with severe hemosiderosis after a 10-year history of RAEB.

At present, 10 of the 22 historical control patients (45%) are alive with a median follow-up of 60 months (range, 39 to 100 months); Causes of deaths after 4 months were severe GVHD (n = 1, day 157), progressive multigloukencephalopathy (n = 1, day 236), relapse (n = 2, 48 and 55 months) and bronchiolitis obliterans (n = 1, 84 months). In the 0.5 mg/kg MAK195F group, one patient is alive at 40 months, one patient died at day 314 from a cerebral vascular accident. In the 1.0-mg/kg group, 9 of 14 patients survive with a median follow-up of 20 months (range, 10 to 36 months).

Beyond 4 months, one patient died because of late respiratory syncytial virus pneumonia (day 241), one patient presenting with early relapse after mixed chimerism, from recurring disease. None of the three patients receiving 3.0 mg/kg MAK195F is presently alive because the third patient with normal hematopoietic recovery developed bronchiolitis obliterans and pulmonary fibrosis 11 months after BMT and died from progressive respiratory failure.

At present, none of the surviving patients, including the historical control group and patients treated with anti-TNFα, have evidence of clinical or cytogenetic relapse.

**DISCUSSION**

Though the pathophysiologic role of conditioning-induced release of proinflammatory cytokines in induction of early complications and GVHD after allogeneic BMT has been proven in experimental models, our study is one of the first to provide indirect and additional direct evidence in the clinical setting. (1) Indirectly, delayed onset of aGVHD in historical controls and in study patients receiving BUS/CY instead of TBI/CY conditioning and diminished release of TNFα in the period of BUS treatment indicates an association between host-related cytokine release and induction of aGVHD. (2) These interactions are directly supported by our phase I/II study using a MoAb-neutralizing human TNFα during pretransplant conditioning because our data strongly suggest that neutralization of host-related TNFα release is able to postpone onset of aGVHD and to reduce more severe courses.

However, both statements need to be discussed in the light of controversial data reported in the literature and of limitations of our study design. Though different incidences and kinetics of aGVHD between BUS/CY and TBI/CY regimens have not been reported in earlier studies on BUS/CY regimens, our observations are in line with experimental evidence published by Xun et al and results of a recent randomized clinical study from Seattle. In that clinical trial, probabilities of aGVHD were significantly different between BUS/CY- (35%) and CY/TBI-treated patients (48%), and kinetics of onset seem comparable with results obtained in our study. Though the authors explained the difference by impaired delivery of cyclosporin because of renal impairment in CY/TBI-treated patients (which was not observed in our patients), differences in conditioning-related cytokine induction might be an alternative explanation of the Seattle data.

Our data on TNFα-serum levels and the more precise kinetics of circulating TNFα-anti-TNFα complexes in the course of pretransplant conditioning facilitate a more detailed analysis of TNFα-induction by the various principles used for myeloablative/cytotoxic pretreatment. Although Xun et al’s studies indicate a strongly reduced induction of TNFα and interleukin-6 (IL-6) by BUS/CY as compared with TBI/CY regimens, they allow no further clear differentiation between BUS and CY. However, our data indicate absence of significant TNFα release in the course of BUS treatment, but prove strong induction by TBI as well as by infusion of CY. Although induction of cytokines by irradi-
tion has been analyzed up to the molecular level so far, reports on induction by cyclophosphamide are missing. Our observation is supported by reports on increases of interferon levels after CY conditioning. In addition, a recent study on amplification of GVHD by cyclophosphamide in mice, which showed a strong correlation of severity of GVHD with the interval between CY infusion and donor T-cell transfer, at least further suggests a pathophysiologic role of CY-induced cytokine release in activation of donor cells. Of course, further studies analyzing cytokine polymerase chain reaction in blood cells and tissues are needed for a definitive confirmation of cytokine induction by CY.

Though attachment of TNFα to TNF-antibody by formation of complexes is proven by the differences between immunoreactive and bioactive TNFα and by coprecipitation of TNFα with mouse-Ig, the finding of cytokine-anticytokine complexes needs further comment. TNFα-anti-TNF complexes showing similar characteristics have been observed in several clinical studies in patients suffering from sepsis and in children with cerebral malaria; in patients with myeloma, complex formation between IL-6 and an anti-IL-6 antibody and serum measurements have even been used to calculate daily IL-6 production. The biologic relevance of this finding is unknown. Like soluble TNF receptors, TNFα-anti-TNF complexes might serve as a buffer or slow release repository for TNFα, which should still protect from acute TNFα-induced damage. However, complexes are most likely degraded with kinetics comparable with the native antibody that is suggested by the daily decreases of complexes between evening and the next morning observed in our trial.

Because the different potential of BUS and TBI for induction of TNFα was unknown at the time of initiation of our studies, a major limitation of this study is the imbalance of conditioning regimens between MAK195F prophylaxis and historical controls, resulting in small number of patients, especially in the TBI/CY group. Despite these limitations, clinical results obtained are in line with hypotheses suggested by our serum studies. Inclusion of TNFα-inducing CY in both types of conditioning regimens might explain the observation that effects of prophylactic neutralization of TNFα using MAK195F are more pronounced in TBI/CY-treated patients, but still detectable with borderline significance in those receiving BUS/CY. This holds true for reduction of fever as part of the acute-phase response accompanying aplastic phase, which occurred in both groups of conditioning regimens. However, reduction in duration was more prominent in TBI/CY-treated patients, a finding that is in line with prolonged febrile episodes in TBI/CY-treated controls also observed in the Seattle study. This also holds true for the effects of MAK195F prophylaxis on onset and severity of aGVHD that seemed to be more dramatic in the smaller TBI/CY-treated group. Clearly, the suggested effects of MAK195F prophylaxis, i.e., delay of onset and reduction of severity of aGVHD need confirmation in a large randomized study using stratification for different conditioning regimens.

Major objectives of our phase I/II trial were definition of an optimal dose of MAK195F and analysis of biologic effects with regard to hematopoietic engraftment and infectious complications occurring during aplastic phase. TNFα is a major cytokine involved in antibacterial and antifungal defense, and experimental data using neutralizing TNF antibodies in models of chronic infections like peritonitis showed a deleterious effect of prolonged neutralization of TNFα. Despite persistence of TNFα-neutralizing-serum activity up to the second week after BMT, our data argue against a relevant interaction of MAK195F prophylaxis with antimicrobial defense during aplastic phase, as we did not observe an increased incidence or altered pattern of bacterial or fungal infections. However, this concern might still be true for a longer application of TNFα antagonists, and the data stress the need to define optimal windows for these novel drugs.

A second concern with regard to neutralization of TNFα was related to the interactions of TNFα and hematopoiesis. Though TNFα has been shown to suppress growth of normal committed hematopoietic progenitor cells and preclinical studies performed in our institution showed no interference of MAK195F with growth of granulocyte-macrophage colony-forming units in BM samples of healthy donors, collection of back-up (recipient) BM grafts was mandatory for our phase I/I study. No acute rejection of donor BM has been observed in our trial. Observation of two patients with mixed chimerism after engraftment in the 1.0 mg/kg MAK195F group might be unrelated to anti-TNFα-prophylaxis because both patients, as well as 1 patient from the historical control with mixed chimerism, received BUCY2 conditioning, which itself has been associated with insufficient eradication of host hematopoiesis in some patients. Though a recent report suggested a costimulatory role of TNFα in proliferation of CD34 cells, contribution of high-dose (3.0 mg/kg) MAK195F prophylaxis to poor graft function occurring 3 and 4 months after BMT is rather speculative as late graft failure can also be explained by GVHD in one patient and by marrow stromal damage associated with prolonged use of busulphan before BMT in the other. However, because the increase of dosage on MAK195F from 1.0 to 3.0 mg/kg did not result in complete prevention of aGVHD despite stronger and more prolonged neutralization of TNFα, our data suggest that 1.0 mg/kg is safer and as effective as 3.0 mg/kg and do not support investigation of higher dosages in the future.

Recently, results of a randomized study using pentoxifylline as an alternative strategy to modulate host-related cytokine release have been published. The study was unable to confirm the promising data of a previous phase II study. Neutralization of TNFα using MoAbs is different from modulation of cytokine production using pentoxifylline. Our group has been unable to prevent murine GVHD using pentoxifylline in the same model where neutralizing antibodies were effective. Though these heterogenous results obtained with different strategies of TNFα modulation seem confusing, the most likely explanation is that clinically achievable PTX levels are too low to suppress TNF production sufficiently, whereas anti-TNF antibodies can be applied in sufficient amounts to neutralize all circulating and locally released TNFα. This is also supported by data from studies measuring TNFα levels during prophylaxis with daily dosages.
of 2,000 mg PTX in patients, because TNF serum activity was still detectable in patients developing GVHD.\(^{30}\) Thus disappointing data from studies using PTX prophylaxis do not argue against prophylactic neutralization of TNF\(_\alpha\) using MoAbs.

Finally, the concept of application of cytokine antagonists during pretransplant conditioning or in the early posttransplant period as an alternative approach to improve clinical outcome in allogeneic BMT has to be discussed in a more general background. If this approach is definitively able to prevent at least early aGVHD while sparing delayed acute or chronic GVHD, it seems attractive from two points of view. First, control of late aGVHD is less difficult to achieve as patients have already recovered from nonspecific tissue damage. This is suggested by our observation that a lower proportion of patients receiving anti-TNF\(_\alpha\) suffered from progressive severe GVHD. Second, sparing of delayed and chronic GVHD might broaden therapeutic range of allogeneic BMT as graft-versus-leukemia effects should be better preserved than in patients receiving T-cell depletion.\(^{31}\) So far, there is no evidence of an increased incidence of relapse in our high-risk group, but of course a longer follow up is needed to answer the role of early cytokine release in eradication of residual leukemia. Because increased relapse rate after T-cell depletion might be overcome by adoptive transfer of donor cells at a later period of BMT,\(^{32}\) cytokine antagonists will have to compete with this alternative approach to improve long-term results in the future.

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Modulation of acute graft-versus-host-disease after allogeneic bone marrow transplantation by tumor necrosis factor alpha (TNF alpha) release in the course of pretransplant conditioning: role of conditioning regimens and prophylactic application of a monoclonal antibody neutralizing human TNF alpha (MAK 195F)

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