Neutrophil Function and Pyogenic Infections in Bone Marrow Transplant Recipients

By Werner Zimmerli, Andrea Zarth, Alois Gratwohl, and Bruno Speck

In a consecutive entry trial, the incidence and time course of decreased neutrophil function was assessed in 20 patients treated with allogeneic bone marrow transplantation (BMT). The aim of the study was to assess the prognostic value of low neutrophil function for late pyogenic infections. Chemotaxis, superoxide production, and phagocytic-bactericidal activity were studied before and 2, 6, 9, and 12 months after BMT. Skin window migration was quantitatively assessed 2 months after BMT. Infectious complications were recorded prospectively with preset criteria during 1 year. Six of the 20 leukemic patients had defective neutrophil function before BMT. Two months after BMT all 10 patients with greater than stage II graft-versus-host disease (GVHD), and 6 of 10 patients with ≤ stage II GVHD had at least one decreased function. At this time, patients with subsequent pyogenic infections had lower chemotaxis (P < .05), phagocytic-bactericidal activity (P < .005), and superoxide production (P < .025) than those without. Defective skin window migration and combined defects were predictive for late pyogenic infections. At 9 months all tests were normal in seven patients surviving without GVHD. In contrast, at 9 months three of three patients, and at 1 year two of three with chronic GVHD had still decreased neutrophil function. In conclusion, neutrophil function is frequently impaired during the first months after BMT. Combined neutrophil defects predispose to pyogenic infections and indicate the patient at risk.

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Infectious Episodes

Infections were diagnosed according to preset criteria and definitions summarized below. Each infectious episode between engraftment (leukocytes > 10^9/L) and 1 year was recorded for each individual patient. Infectious episodes during the period of neutropenia (leukocytes < 1,000/μL) were excluded from the analysis.

Sepsis was defined as at least two positive blood cultures with the same isolate or one positive blood culture and a localized infection with the same microorganism.

Pneumonia was defined as infiltrate in a conventional x-ray or computed tomography scan, and clinical signs of infection (fever, cough, or purulent sputum), or purulent sputum with a predominant microorganism. Interstitial pneumonia of unknown etiology was defined as radiologic interstitial infiltrate with negative bronchoalveolar lavage. In the lavage fluid the following etiologies were considered: aspergillus, staphylococcus aureus, Pneumocystis carinii, herpes simplex virus (HSV), adenovirus, influenza and parainfluenza virus, mycobacteria, and pneumocystis carinii.

Urinary tract infection was defined as clinical symptoms, bacterial counts greater than 10^9/mL, and leukocyturia greater than 15 per high power field (at least two criteria).

Febrile episodes (two times >38°C during >12 hours), which remained microbiologically unclear in a patient without specific local symptoms, were classified as fever of unknown origin responsive to antibiotics (FUORA), provided the fever dropped and remained <38°C within 2 days after an empirical antibiotic trial. Other febrile episodes were not classified as infections (eg, GVHD or drug fever).

Laboratory Methods

Reagents. Reagents used and their sources were: bovine serum albumin (BSA), cytochalasin B, cytochrome C (type IV), HEPES, formyl-peptide (FMLP) (Sigma Chemical, St Louis, MO); dextran and Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), and Harleco Diff-Quik (Merz & Dade AG, Düdingen, Switzerland).

HEPES-Hanks' balanced salt solutions with (HBSS) or without calcium and magnesium (mHBSS) were prepared according to standard methods.21

Isolation of PMN. Human blood PMN were purified from EDTA-anticoagulated blood, drawn from the patient and from healthy volunteers as described.22,23

Chemotaxis assay. PMN migration was quantitated using a micropore filter technique in a 48-well microchamber (Neuro Probe, Inc, Cabin John, MD), as described by Harvath et al.24 PMN from the patients and a normal volunteer were always tested in the same microchamber. Results are reported as percent PMN migration through the filter.

Phagocytic bactericidal capacity of PMN. In this assay we used Staphylococcus aureus Wood 46, as previously described.25 The bacterial killing by PMN was calculated from bacterial counts at time 0 and 30 minutes. Each test contained several control tubes (patient's serum without PMN to exclude antibacterial activity of serum, PMN and heat-inactivated serum to verify opsonic requirements, and a mixture of serum from normal volunteers and the patient to test for antiopsonic antibodies).

Superoxide (O_2^-) production. O_2^- production was determined at 37°C spectrophotometrically (550 nm, mmol/L extinction coefficient of 21,000 mol/L^-1 cm^-1) by continuously monitoring the O_2^- dismutase (300 U/mL)-inhibitable reduction of cytochrome C (120 μg/mL) in a double-beam spectrophotometer (model 35; Beckman Instruments, Fullerton, CA) with a temperature regulator set, as described previously.25

Skin window migration. Migration of PMN in vivo was assessed with a skin window technique, as previously described in detail.22,26 The skin suction device (Neuro Probe) was fixed on the patient’s forearm. A constant suction of 360 mm Hg was applied with a valve-controlled pump (Reciprorot 406G, 40W, 50 C/S, Copenhagen, DK). After 1 to 2 hours the blisters were complete. The roofs were then removed with sterile tweezers and scissors, and the lesions (0.38 cm^2) covered with the skin chamber unit. Filled into

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Disease Status</th>
<th>Granulocyte Defect Before BMT</th>
<th>T-Cell Depleted Marrow</th>
<th>GM-CSF D 0-14</th>
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</thead>
<tbody>
<tr>
<td>306</td>
<td>28 y/M</td>
<td>AML</td>
<td>1st CR</td>
<td>N</td>
<td>+</td>
<td></td>
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<tr>
<td>307</td>
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<td>CML</td>
<td>AP</td>
<td>N</td>
<td>+</td>
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<tr>
<td>312</td>
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<td>N</td>
<td>+</td>
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<td>CP</td>
<td>P</td>
<td>+</td>
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</tr>
<tr>
<td>321</td>
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<td>S</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>322</td>
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<td>RT</td>
<td>P</td>
<td>+</td>
<td></td>
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<tr>
<td>326</td>
<td>47 y/F</td>
<td>MDS</td>
<td>RAEBT</td>
<td>N</td>
<td>+</td>
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<tr>
<td>326</td>
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<td>CP</td>
<td>N</td>
<td>+</td>
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</tr>
<tr>
<td>327</td>
<td>33 y/M</td>
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<td>CP</td>
<td>N</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>329</td>
<td>43 y/F</td>
<td>CLL</td>
<td></td>
<td>S</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>330</td>
<td>34 y/F</td>
<td>AML</td>
<td>2nd CR</td>
<td>N</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>335</td>
<td>28 y/M</td>
<td>AML</td>
<td>1st CR</td>
<td>N</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>339</td>
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<td>N</td>
<td>+</td>
<td></td>
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<tr>
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<td>N</td>
<td>+</td>
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</tr>
<tr>
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<td>N</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>349</td>
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<td>CP</td>
<td>N</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>359</td>
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<td>RT</td>
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<td>-</td>
<td></td>
</tr>
<tr>
<td>361</td>
<td>32 y/M</td>
<td>AML</td>
<td>1st relapse</td>
<td>N</td>
<td>+</td>
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</table>
the chambers was 0.8 to 1 mL 70% autologous serum in HBSS. At 2, 4, 6, and 8 hours 0.1 mL of the fluid of the chambers was aspirated, the cell number counted, and the differential counts evaluated. All fluids tested contained greater than 98% PMN as previously observed in normal volunteers.

Definitions of PMN Defects
Chemotaxis, phagocytic-bactericidal activity, and $O_2^-$ production were always simultaneously tested in PMN from the patient and from a normal volunteer. Values at or below the fifth percentile of those from 50 normal volunteers were considered as impaired, if the simultaneous control test was within the mean ± SD of the normal values. In 9% of the tests the volunteer’s and the patient’s values were both greater than 1 SD below the mean. These tests were repeated if possible, or omitted from the analysis. Skin window migration was considered as defective, if cell counts were less than 10% of the geometric mean from previously published normal values.

Statistical Analysis
Comparison of the arithmetic means was interpreted according to Student’s t-test. The differences between geometric means were analyzed with the Mann-Whitney U-test. Comparison of groups was performed by Fisher’s exact test.

Table 2. Patient Characteristics After BMT

<table>
<thead>
<tr>
<th>UPN</th>
<th>GVHD</th>
<th>Granulocyte Defect at 2 mo</th>
<th>Infections between Engraftment and 1 y</th>
<th>Status at Latest Follow-Up (mo after BMT)</th>
</tr>
</thead>
</table>
| 306 | III/chronic | P | FUORA  
Pneumonia (Klebsiella pneumoniae)  
Herpes simplex stomatitis | Alive | Alive, well minimal GVHD (33) |
| 307 | II | N | N | Died of relapse  
(11th mo) | — |
| 312 | O | C | Herpes Zoster | Alive | Alive, well (32) |
| 313 | IV/chronic | M | 2 × FUORA  
Pneumonia (grampositive cocci)  
Herpes simplex | Died of GVHD/IP  
(4th mo) | — |
| 315 | III-IV/chronic | M | 5 × FUORA  
Pneumonia (mixed culture)  
Sepsis (Pseudomonas species) | Died of GVHD/IP  
(5th mo) | — |
| 316 | II-III | C/P/M | 2 × FUORA  
Herpes simplex labialis  
CMV-retinitis | Alive with relapse  
(7th mo) | Died of relapse (29) |
| 321 | I | N | Herpes simplex genitalis | Alive | Alive, well (28) |
| 322 | II | P/S | FUORA | Alive with relapse  
(5th mo) | Died of relapse (13) |
| 325 | IV | C/P/S | 6 × FUORA | Died of GVHD/IP  
(3rd mo) | — |
| 326 | II | N | N | Alive | Alive, relapse (24) |
| 327 | II | S | N | Alive | Alive, relapse (24) |
| 329 | I | N | N | Alive | Alive, well (23) |
| 330 | I | C | N | Alive | Alive, well (23) |
| 335 | II | C/S/M | 3 × FUORA | Died of relapse  
(3rd mo) | — |
| 339 | I-II | C/P | Sepsis (Streptococcus pneumoniae)  
Herpes simplex labialis/genitalis | Alive | Alive, well (18) |
| 341 | I-III/chronic | P/S | 1 × FUORA  
6 × Sepsis (Klebsiella pneumoniae,  
Staphylococcus epidermidis, Proteus mirabilis, Citrobacter freundii, Candida parapsilosis, Pseudomonas aeruginosa)  
CMV-retinitis  
Sterile pericarditis | Alive | Alive, chronic GVHD (18) |
| 347 | II-IV/chronic | S | N | Alive | Alive, minimal GVHD (15) |
| 349 | 0-IV | S | Interstitial pneumonitis | Died of GVHD and rejection (3rd mo) | — |
| 359 | II-IV | P | FUORA | Died of GVHD (3rd mo) | — |
| 361 | IV | C | FUORA  
Pneumonia (aspiration)  
Herpes simplex labialis | Died of GVHD (2nd mo) | — |

Abbreviations: M, defective skin window migration; IP, Interstitial pneumonia.
RESULTS

Patient Population

Table 1 summarizes the patients' characteristics. Nineteen patients received marrow from a HLA-identical sibling. UPN 359 received a HLA-haploidentical marrow from her mother. The median age was 32.5 years (range 17 to 47 years). Eight patients were in complete remission. T-cell depletion of the graft was performed in 16 patients. Five patients received granulocyte-macrophage colony-stimulating factor (GM-CSF) for 14 days on a prospective randomized double-blind study evaluation. Functional tests were performed before GM-CSF treatment and greater than 1.5 months after stopping GM-CSF.

Nine patients received CMV-hyper-Ig (UPN 307, 313, 315, 316, 321, 322, 341, 347, and 359), 11 patients IV Ig as a cytomegalovirus prophylaxis between day -7 and day 80, as described above. Six patients had a decreased PMN function before conditioning and Ig infusion. Defective PMN function was observed in all categories and stages of disease. \( \text{O}_2^- \) production was at 55% \((n = 2)\), and phagocytic-bactericidal activity at 58% ± 9% \((n = 4)\) of the simultaneous control test.

Patient Characteristics After BMT

Table 2 summarizes the patients' characteristics after BMT. The median time to engraftment (leukocytes > \(10^9/L\)) was 17 days (10 to 42 range). Ten patients had acute GVHD (maximum stage at any time point greater than stage II), six of them died of GVHD and/or interstitial pneumonitis within 5 months after BMT, one survived 12 months with relapse, three survived with chronic GVHD.

PMN Function After BMT

Eleven patients had pyogenic infections between engraftment and 1 year after BMT (Table 2 and below). Eight of those patients had greater than stage II or chronic GVHD. At 1 year 12 of 20 patients were alive, 10 of them without any signs of leukemia. All 10 patients with greater than stage II GVHD, but only 6 of 10 patients without had at least one decreased PMN function at 2 months after BMT \((P = .05\), Fisher's
PMN Function as Predictive Factor for Infection

An isolated impaired PMN function does not necessarily imply an increased susceptibility to pyogenic infections. Therefore, we analyzed whether patients with infectious episodes had lower PMN functions than those without. Table 3 shows the results. For better comparison, the mean values for chemotaxis, phagocytic-bactericidal activity, and superoxide production are expressed as percent of the simultaneous control values. Patients with subsequent pyogenic infections had a significantly lower migration toward endotoxin-activated serum (EAS), phagocytic-bactericidal activity, and FMLP-induced O$_2^-$ production than those without. Furthermore, infections were observed in all patients with impaired skin window migration, and with several decreased PMN functions, respectively. Figure 3 shows skin window migration. The values from patients without subsequent pyogenic infections and those from normal volunteers were not different at any time point (P > .05). In contrast, the geometric mean of leukocytes migrating into the skin chamber were significantly lower (P < .05) at 4, 6, and 8 hours in patients with later infections than in controls. The difference between the patients’ groups was significant at 6 hours.

DISCUSSION

Allogeneic BMT abolishes host defense for approximately 3 weeks. After engraftment the risk for pyogenic infections decreases, but continues for a subgroup of patients. The purpose of this study was to identify this subgroup before infection. We hypothesized that those patients would have one or several acquired PMN defects.

In contrast to some previous studies, we performed a systematic evaluation of multiple functions of PMN at preset time points in consecutive patients. We defined a function as defective if it was at or below the fifth percentile of 50 healthy volunteers. This definition allowed to detect even subtle defects. In order to avoid false low values because of technical problems, we repeated or omitted

Table 3. PMN Function at Two Months After BMT in Patients With and Without Subsequent Infections

<table>
<thead>
<tr>
<th>Function</th>
<th>Patients With Pyogenic Infection</th>
<th>Patients Without Pyogenic Infection</th>
<th>Statistical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemotaxis</td>
<td>$M \pm SD \ (n)^*$</td>
<td>$M \pm SD \ (n)^*$</td>
<td>NS†</td>
</tr>
<tr>
<td>FMLP (10$^{-8}$ mol/L)</td>
<td>84% ± 32% (11)</td>
<td>96% ± 17% (8)</td>
<td>NS†</td>
</tr>
<tr>
<td>EAS (5%)</td>
<td>87% ± 9% (6)</td>
<td>101% ± 11% (6)</td>
<td>&lt;.05†</td>
</tr>
<tr>
<td>Phagocytic-bactericidal activity</td>
<td>74% ± 17% (10)</td>
<td>106% ± 14% (7)</td>
<td>&lt;.005†</td>
</tr>
<tr>
<td>Superoxide-production Cb + FMLP (10$^{-7}$)</td>
<td>67% ± 42% (11)</td>
<td>117% ± 61% (8)</td>
<td>&lt;.025†</td>
</tr>
<tr>
<td>Skin window migration: defective function/number of patients</td>
<td>4/7</td>
<td>0/6</td>
<td>&lt;.05†</td>
</tr>
<tr>
<td>Combined defects</td>
<td>6/11</td>
<td>0/6</td>
<td>&lt;.025†</td>
</tr>
</tbody>
</table>

Abbreviations: EAS, endotoxin-activated serum; NS, P > .05.

*Mean ± standard deviation in percentage of the simultaneous control values. Greater than 100% signifies that the patient’s values were higher than control values. Number of patients in brackets.
†Statistical analysis with the unpaired t-test.
‡$P < .005$, different from simultaneous control values (unpaired t-test).
§$P < .00005$, different from simultaneous control values (unpaired t-test).
| | $P < .01$, different from simultaneous control values (unpaired t-test).
| | $P < .05$, different from simultaneous control values (unpaired t-test).
| | Statistical analysis with the Fisher’s exact test.
pathologic tests in case of simultaneously decreased control values.

The functional tests before conditioning showed impaired PMN function in patients with chronic myelogenous leukemia (CML) (three of seven), acute myelogenous leukemia (AML) (two of nine) and CLL (one of one). Decreased PMN function before BMT did not correlate with defective function after BMT. This observation argues against preexisting host factors which induce PMN defects. Reduced antimicrobial killing in morphologically normal PMN has been described in patients with AML,31 combined defects of phagocytosis, bactericidal capacity, chemotaxis, and $O_2^-$ production in patients with CML.32 Gallin et al33 found normal chemotaxis in CML patients, but decreased FMLP-induced $O_2^-$ production in a subgroup of patients with 31D8 monoclonal antibody negative PMN. These patients lacking 31D8 binding progressed rapidly to accelerated phase or blast crisis.

At 2 months after BMT, 80% of the patients had one or more decreased PMN tests. PMN defects persisted ≥9 months only in patients with chronic GVHD. At all time intervals after BMT, we found a higher fraction of decreased PMN function in patients with greater than stage I GVHD. Our findings confirm previous studies in which a single function3,4 or a small group of patients has been tested at random time points.14,15,17 Because in the present study cyclosporine A has been used in all patients to prevent or to treat GVHD, the impaired PMN function is more likely to be due to GVHD than to its treatment. The pioneer study of Clark et al13 showed a clear correlation between impaired chemotaxis and infection. More recent studies failed to show such a relationship between a defect and infection. Our study shows that multiple functional parameters should be tested to detect patients at risk for infections. In an individual patient the presence of a single functional defect, except skin window migration, did not predict susceptibility to infection. In contrast, all patients with more than one defective PMN function developed later pyogenic infections. This illustrates that isolated defects can be compensated for, whereas even subtle defects may increase the risk for infections, provided that different functions are impaired.

Skin window migration and in vitro chemotaxis were not always impaired in parallel. There are explanations for this apparent discrepancy. First, skin window migration is more complex than migration through a 10-μm membrane; secondly, possible chemotactic inhibitors could play a role in vivo but not in vitro; thirdly, in the skin window test without a chemotactic agent, cytokines such as interleukin-1, which promotes transendothelial migration in the absence of a chemotactic gradient, may play an important role.35,36 Therefore, skin window migration may be a more appropriate test to detect an increased susceptibility to infections than the in vitro chemotaxis.

It is unknown whether impaired PMN function is intrinsic, due to impaired cell maturation, or caused by extrinsic factors.37,38 A decreased production of cytokines after BMT has recently been described.40 Preliminary data from our group show that defective PMN functions can be transiently corrected by exogenous administration of GM-CSF in vitro and in vivo.41

In conclusion, PMN function is decreased in many leukemic patients before and several months after allogeneic BMT. Testing of several PMN functions after BMT allows to recognize patients at risk who require a close follow-up observation and possibly the same management as neutropenic patients during febrile episodes.

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REFERENCES
bone marrow transplantation for chronic myelogenous leukaemia. Lancet 1:665, 1984


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