T-Cell Ontogeny After Autologous Bone Marrow Transplantation: Failure to Synthesize Interleukin-2 (IL-2) and Lack of CD2- and CD3-Mediated Proliferation by Both CD4+ and CD8+ Cells Even in the Presence of Exogeneous IL-2

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T cells generated during a second round of ontogeny after autologous bone marrow transplantation (ABMT) represent a unique model of early T-cell ontogeny in an autologous situation. Since grafted bone marrows were pre-treated in vitro with the cyclophosphamide derivative ASTA Z 7557, circulating T cells had to be regenerated from reinfused hematopoietic progenitor cells. The T-cell population derived from 25 patients post-ABMT was phenotypically characterized: an increase in CD8+ cells, a low percentage of CD4+ cells, and a median of 12% CD56+(NKH1+) cells were found. When the T cells were stimulated with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA), defective interleukin-2 (IL-2) secretion was observed. In addition, proliferative responses of the T cells after activation through the antigen-receptor–dependent CD3 pathway, through the CD2 dependent alternative T-cell pathway, and by the lectin PHA were investigated. Despite the presence of CD2, CD3, alpha/beta chains of the T-cell receptor, and CD25 + IL-2 surface receptors, abnormal proliferative responses were obtained even in the presence of exogeneous IL-2. In experiments where the T-cell population was separated into CD4+ cells and CD8+ cells, both the CD4+ and CD8+ subsets were unable to respond to activating and proliferating signals. Thus, T cells at early stages of ontogeny not only possess an intrinsic defect in IL-2 synthesis but, in addition, were unable to express functional IL-2 receptors in response to mitogenic stimuli.

AFTER ALLOGENEIC bone marrow transplantation (BMT), abnormalities such as imbalance of CD8+ and CD4+ cells, a lack of T-cell proliferative responses and depressed interleukin-2 (IL-2) synthesis have been thoroughly documented.1,2 These abnormalities have been attributed mainly to the low number of circulating CD4+ cells. However, after allogeneic BMT an unusual process of ontogeny takes place that may be influenced by the incidence and treatment of graft-versus-host disease (GVHD) and the occurrence of cytomegalovirus infections.3,4 Therefore, the attempt to dissect mechanisms underlying immunodeficiency is complicated by the clinical situation and the administration of immunosuppressive therapy.

As discovered recently, human T cells will produce a proliferative response when stimulated through the antigen-dependent pathway where specific antigen/T-cell receptor (TCR) complex interactions and accessory cells provide triggering signals to T cells. This mode of activation can be mimicked by anti-CD3 monoclonal antibodies (MoAbs).5,6 An alternative pathway exists via the CD2 receptor which, in the absence of accessory cells, can be triggered in an antigen-independent fashion through the combination of T112 + T113 MoAbs.7,8 The production of IL-2 when T cells are stimulated through the CD3 or the CD2 pathways in the presence of accessory cells or by the combination of phytotemagglutinin (PHA) and phorbol myristate acetate (PMA) (activating the protein kinase C pathway) can substitute for the above signals and induce IL-2 secretion and IL-2 receptor expression.

We have investigated 25 patients who underwent supralethal chemotherapy and radiotherapy followed by the autografting of their bone marrow that had been treated in vitro with the cyclophosphamide derivative mafosfamide ASTA Z 755712–14 before grafting. In this unique situation, T-cell reconstitution results from a de novo ontogenic process. We have analyzed T-cell ability to secrete IL-2, the proliferative responses of the whole T-cell population plus the CD4+ and CD8+ T-subsets to agents stimulating the CD2 or CD3 activation pathways both in the presence and absence of IL-2. This study may be useful for the understanding of the mechanisms regulating the pathways of activation in immunodeficient states.

MATERIALS AND METHODS

Patients

Peripheral blood from 25 patients with hematologic malignancies who underwent autologous bone marrow transplantation (ABMT) after supralethal chemotherapy and radiotherapy was used. Patients were treated either for acute myeloid leukemia (AML) (17 patients) or acute lymphocytic leukemia (ALL) (8 patients). All patients were treated at the Medizinische Poliklinik, University of Heidelberg, FRG. The pretransplant conditioning regimens have been described in detail elsewhere.16,17 Collection and re-infusion of bone marrow was performed during complete remission. The bone marrow suspensions were treated in vitro with 60 to 80 μg of the activated cyclophosphamide derivative ASTA Z 7557 (ASTA-Werke, Bielefeld, FRG) before freezing. Post-ABMT, all studies were done when the patients were clinically stable. The 10 control subjects were healthy individuals.
**Enrichment of T and Non-T Cells From PBMC**

Human peripheral mononuclear cells (PBMC) were isolated from 45 mL of whole heparinized blood by Ficoll-Hypaque (FH) (Pharmacia, Fine Chemicals, Uppsala, Sweden) density-gradient centrifugation. The E+ fraction was obtained by two consecutive rosetting procedures with 2-aminoethylisothiouronium (AET) bromide (Sigma, St Louis, MO) treated sheep red blood cells (RBC) followed by FH centrifugation. The E+ cell fraction (referred to as T cells) was recovered from the pellet after lysis with ammonium chloride buffer (pH 7.2) and consisted of 87% to 98% (median 96%) CD2+ cells, 60% to 96% (median 87%) CD3+ cells, and the remaining CD8+ cells were 2% to 21% CD11b+ /CD14+ monocytes/macrophages.

**Cell Phenotyping Using Direct Immunofluorescence (IF)**

Flow cytometry analyses (using an EPICS C, Coulter) were performed with directly coupled fluorescein (FITC) and phycoerythrin labeled MoAbs: CD1(OKT6), CD2(OKT11), CD3(Leu4), CD4(Leu3), CD8(Leu2), TCR1, CD11b(Leu15), CD25(rIL-2), CD57(Leu7), CD16(Leu11), and CD56(NKH1) MoAbs (from Becton Dickinson [Heidelberg, FRG], Ortho Diagnostics [Westwood, MA], and Coulter Immunology [Hialeah, FL]).

**Assay for IL-2 Production and Activity**

Cultures using 5 x 10^4 cells per well were incubated at 37°C in 5% CO2 in 96-well microtiter plates in triplicates in a final volume of 200 µL of RPMI 1640 medium supplemented with 5% fetal calf serum (FCS) and 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg streptomycin for 21 days post-ABMT. The E+ (T cell) fractions of 25 patients post-ABMT are depicted in Table 1. An excess of CD8+ cells was found: patients' median was 58% (range 29% to 92%) while controls' median was 41% (range 34% to 44%); a very high percentage of CD57+ (Leu7+) cells (patients' median 42%, range 13% to 82%; controls' median 2%).

**Preparation of CD4+ and the CD8+ Cell Populations**

The E+ fraction of the PBMC was separated into CD4+ and CD8+ cell populations. This was performed using a negative selection technique involving MoAbs and the use of immunomagnetic beads (IB). Briefly, for the preparation of the CD4+ cell fraction, the E+ cells were incubated with Leu2 (CD8) MoAb (5 µg/mL) (Becton Dickinson, diaalyzed to remove NaN3) for 30 minutes at 4°C. Sheep antinouse Ig-coated Dynabeads (M-450, Dynal, Oslo, Norway) (IB) were then added at a bead: cell ratio of 10:1 for 30 minutes at 4°C. Subsequently, the beads (attached to CD8+ cells) were removed by applying a flat cobalt samarium magnet (Dynal) to the wall of the tubes. The cell population left after the removal of beads consisted of 81% to 89% CD4+ cells as examined by direct immunofluorescence (IF). Contaminating CD8+ cells were always less than 1% and the remaining cells within

the CD4+ population were CD11b+ /CD14+ (Mo1+ /LeuM3+) cells.

For the preparation of CD8+ cells, an identical procedure was performed using diazylated Leu3 (CD4) (5 µg/mL) MoAb (Becton Dickinson) + IB. The cell population left after the removal of beads (attached to CD4+ cells) consisted of 75% to 90% CD8+ cells. Contaminating CD4+ cells were always less than 1%, the other cells within the CD8+ population being CD11b+ /CD14+ cells.

**Proliferation Assay for T Cells**

Proliferation of T cells (for the E+, CD4+, and CD8+ cell fractions) was determined using a 3H-Thymidine uptake assay. Identical cultures, as described above for the assay for IL-2 production and activity, were done using 5 x 10^4 cells in a final volume of 200 µL. The MoAb CD3 (IgG1) was coupled to swollen CNBr-activated sepharose beads (S-CD3) (Pharmacia) and used at a pretested optimal concentration. The MoAbs T11 and T11, were titrated and used at optimal concentration together with 2 x 10^5/mL sheep RBC. PHA (Wellcome, HA15, Dartford, England) at 0.5% vol/vol and recombinant interleukin-2 (rIL-2, Boehringer Mannheim) at 10 U/mL were used. Cells were cultured for 72 hours; during the last 16 hours of culture 1 µCi of 3H-Thymidine was added. Cells were then harvested onto glass fiber filters with an automatic cell harvester, and radioactivity was measured in a scintillation counter. Results are expressed as median counts per min (median cpm).

**RESULTS**

**Analysis of Antigen Surface Expression on T Cells After ABMT**

The phenotypes of E+ fractions (T cells) from 25 patients within 60 days post-ABMT are illustrated in Table 1. An excess of CD8+ cells was found: patients' median was 58% (range 29% to 92%) while controls' median was 41% (range 34% to 44%); a very high percentage of CD57+ (Leu7+) cells (patients' median 42%, range 13% to 82%; controls' median 2%).

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**Fig 1. Phenotype of T cells from patients early after ABMT.** Percentages of CD2+ , CD4+ , TCR+ (alpha/beta chain), CD25+ , CD4+ , CD56+ , CD16+ (NKH1+), CD57+ (Leu7+) cells in the E+ (T cell) fractions of 25 patients post-ABMT are selected from left to right: the patients were between 9 and 60 days post-ABMT. The range obtained with 10 controls is shown by the shaded area (the range for CD4+ being 48% to 66%). Analysis was performed by means of direct immunofluorescence.
median 14%, range 6% to 25%) and a reduced percentage of CD4+ cells (patients' median 18%, range 1% to 49%; controls' median 54%, range 48% to 66%) was observed.

Of note was the percentage of natural killer cells within the T-cell population of patients post-ABMT: a median of 12% CD56+ (NKH1+) (range 3% to 24%) compared with controls (median 8%, range 2% to 11%). In addition, the normal expression of CD25+ IL-2 receptors was interesting: median 4% (range 1% to 19%) for patients and median 6% (range 3% to 12%) for controls. The T cells consisted of 82% to 98% CD2+ cells (median 96%) and 59% to 98% CD3+ cells (median 88%), with a similar percentage of cells expressing the alpha/beta chains of the T-cell receptor TCR1+ (median 82%; range 56% to 98%). Thus, there was no significant difference as compared with normal controls. Furthermore, the percentage of CD1+ cells was higher than for controls (patients' median 4%, range 1% to 13%; controls' median 0.9%, range 0.6% to 1.7%) (not shown).

Two-Color IF Analysis of the T-Cell Population

In Fig 2, histograms of T cells derived from six patients are shown. Patients 1 through 3 (found at 15, 32, and 38 days post-ABMT) showed defective proliferative responses and IL-2 secretion, while T cells from patients 4 through 6 (found at 136, 407, and 422 days post-ABMT) possessed normal proliferative responses and IL-2 secretion.

**CD8+CD11+ cells.** Patients 1 through 4 and 6 had between 7% to 15% CD8+CD11+ cells, whereas patient 5 had 46%. However, as mentioned above, the function of his T cells in our in vitro assays was not affected despite the fact that this population of cells may have a suppressive effect on IL-2 production and response.

**CD8+CD57+(Leu7+) cells.** While patients 1, 2, 4, and 6 had between 21% to 28% CD8+CD57+ cells, patients 3 and 5 had increased numbers of 52% and 54% double positive cells.
CD3-CD16+ cells and CD3-CD56+(NKHI+) cells. All six patients, irrespective of their in vitro T-cell function, had a range of 5% to 20% CD3-CD16+ cells and 3% to 20% CD3-CD56+ cells. This illustrates that the presence of natural killer cells within the patients T-cell population is not directly the cause of the abnormal proliferation obtained in response to PHA or CD3 MoAbs or for the defective IL-2 secretion observed.

IL-2 Synthesis by T Cells Early in Ontogeny After ABMT

When stimulated with PHA + PMA, 1 x 10^5 normal T cells/mL (from 10 healthy controls) have a median production of IL-2 of 3.1 U/mL (range 1.2 to 6.4 U/mL), the peak production being at 2 days following stimulation with PHA + PMA (Fig 3A and B).

In contrast, T cells derived from patients post-ABMT (five representative patients tested on days 22, 26, 27, 41, and 42, respectively) shown in Fig 3A were unable to be triggered to produce IL-2 by PHA + PMA at days 1 to 4 after stimulation. After ABMT (on days 62, 506, 163, 802, and 636, respectively, post-ABMT), T cells from the same five patients were stimulated with PHA + PMA. In Fig 3B, the secretion of IL-2 by T cells had returned to normal values.

Of importance is the fact that the ability of T cells to secrete IL-2 does not appear to be caused by changes in the percentages of CD8+ and CD4+ cells within the T-cell population (Table 1). However, we are aware that this does not exclude the possibility that a change within the CD4+ or the CD8+ subsets had occurred. But as shown above with six patients, there was no difference in the number of natural killer cells (CD3-CD56+ and CD3-CD16+ cells) present between the three patients with the ability to produce IL-2 and the three others with abnormal IL-2 secretion. In addition, the single patient (patient 5) who had increased CD8+CD11+ cells possessed the ability to produce IL-2.

Proliferation of Early T Cells After Activation Either Via the CD2 or CD3 Pathways With and Without Exogeneous rIL-2

Figure 4 (striped bars) shows the defective in vitro responses of T cells derived from 25 patients to T-cell activating agents at an early stage posttransplantation (the number of days post-ABMT is shown on the left of Fig 4A). T-cell stimuli were used in the presence of exogeneous rIL-2. T cells were triggered either via the antigen dependent pathway using CD3 MoAbs (Fig 4, column A) or via the CD2 dependent pathway by the combination of anti-T112 and anti-T111 MoAbs in the presence of sheep erythrocytes (Fig 4, column B), or with the lectin PHA (Fig 4, column C). The same 25 patients, when tested at a later time after ABMT, showed a normalization of their in vitro proliferative responses (Fig 4, black bars).

Proliferation of the CD4+ and CD8+ T-Cell Subsets to CD3- and CD2-Mediated Stimulation in the Presence and Absence of rIL-2

The non-responding T-cell populations (from five representative patients at days 22, 27, 29, 36, and 42 post-ABMT) were separated into CD4+ and CD8+ fractions and challenged with in vitro stimuli to measure the proliferative responses.

As shown in Fig 5a, both isolated CD4+ and CD8+ subsets did not respond to stimulation either through the lectin- or CD3-, or CD2-mediated T-cell activation pathway even in the presence of exogeneous IL-2, in marked contrast to the responses obtained with normal controls. In addition, when responses had returned to normal (Fig 5b, where the same patients were tested at days 51, 208, 306, 432, and 156 post-ABMT), both the CD4+ and the CD8+ cell fractions possessed the ability to proliferate. In patient 3, the CD4+ fraction was more responsive, conversely, in patient 5, the CD8+ fraction showed more proliferation. Taken together, no significant difference was found. Thus, although an excess in CD8+ cells was still present at the time when the pattern of reactivity became normal, these CD8+ cells and the

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Table 1. Percentages of CD8+ and CD4+ Cells in the T-Cell Population of Five Representative Patients at Two Different Times After ABMT

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days Post-ABMT</th>
<th>WBC*</th>
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<th>%CD4+</th>
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<tr>
<td>5</td>
<td>636</td>
<td>6.300</td>
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</table>

*Normal white blood cell count lies between 4.000 and 10.000 x 10^3/mL.

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On these particular days, T cells were phenotyped and stimulated in vitro with PHA + PMA to measure their ability to synthesize IL-2.
CD4+ cells were able to produce a proliferative response. Therefore, the overall abnormal response in the E+ lymphocytes seems to be unrelated to the imbalance in the numbers of CD4+ and CD8+ cells although we have to take in consideration that changes within the CD4+ and CD8+ subsets could have taken place within this time interval.

DISCUSSION

Using MoAbs directed at unique T-cell surface antigens and known to selectively induce differential pathways of T-cell activation, it is now possible to define in much greater detail whether distinct immunodeficiency states result from blockades or defects at one or the other level of T-cell activation. In the present study we have used a mitogenic CD3 MoAb directed at the CD3-TCR antigen recognition complex of human T lymphocytes,8 a combination of anti-CD2 MoAbs directed at the T-lineage sheep erythrocyte CD2 antigen10,11 and the lectin PHA11 in order to investigate the cellular basis of impaired responsiveness of T cells in patients early after ABMT.

Conditions after allogeneic transplantation,27 with the use of immunosuppressive therapy, the presence of GVHD, or of cytomegalovirus infection, usually make it difficult to study T-cell ontogeny. However, studies about IL-2 production and response after T-cell-depleted allogeneic BMT where no post-immunosuppressive therapy and GVHD were present, provide a better insight into the process of T-cell reconstitution.5 Only a few studies have been published concerning T-cell ontogeny after autologous transplantation.22 In the present study, ABMT using bone marrow treated in vitro with the cyclophosphamide derivative mafosfamide (ASTA Z 7557) before grafting15,16 offered a unique opportunity for the study of T-cell ontogeny in an autologous system and, in addition, enables us to gain insight into the mechanisms and molecular basis of T-cell activation in humans.

An excess in CD8+ cells and low numbers of CD4+ cells have been uniformly documented after allogeneic, syngeneic, and autologous BMT.25,26,27 Defects of IL-2 synthesis found in such situations have been attributed to the low proportion of CD4+ cells25 and the excess of CD8+ cells.27 Because some authors demonstrated that anti-CD3–induced T-cell proliferation was completely restored by addition of exoge-
neous IL-2, the basic defect in this immunodeficient state was thought to be the defective IL-2 synthesis. In contrast, others have described that the addition of exogeneous IL-2 did not restore proliferation, thus implying that the basic defect was not only the inability of T cells to synthesize IL-2 but the inability to respond to IL-2.

As reported in previous studies, we found an imbalance of CD4+ and CD8+ cells (Fig 1). In agreement with Lopez-Botet et al., the lack of proliferative responses of the T cells to CD3+ and CD2-mediated activation could not be restored by exogeneous IL-2 (Fig 4) nor be explained by the lack of membrane receptors (the T cells possessed normal percentages and density of CD2+, CD3+, and TCR + cells), or by the percentages of CD56+ natural killer cells that lied between 3% and 24% (median 12%), (Fig 1). However, in contrast to these authors, we found normal levels of CD25 + IL-2 receptors on these T cells (Fig 1). Whether at this stage of differentiation, T cells are incapable of producing func-
tional IL-2 receptor or the IL-2 receptors are lacking the gp75 molecule is a possibility that needs to be investigated.

Moreover, this study provides evidence that failure of IL-2 synthesis in T cells early in ontogeny (Fig 3) does not appear to be directly related to the imbalance in CD4+ and CD8+ cells. Thus, when our patients' T cells acquired the ability to synthesize IL-2 (Fig 3B), the T-cell population still consisted of an excess of CD8+ cells and a decrease in CD4+ cells (Table 1). However, we are aware that there may be subsets of cells within the CD4+ and the CD8+ populations that could have changed in the course of time. Furthermore, the percentages of natural killer cells (CD56+) does not appear to be directly responsible for the lack of IL-2 secretion (Fig 1). In addition, the histograms in Fig 2 show similar numbers of CD3-CD56+ natural killer cells present in three patients at a time when IL-2 secretion and response were absent, and in three patients when these T-cell functions had normalized. Additionally, the abnormal T-cell function does not appear to correlate to the percentages of CD8+CD11+ cells (Fig 2). In order to exclude the possibility that abnormal accessory cell function was the cause of the defective IL-2 secretion, stimulation of T cells with PHA in the presence of PMA that directly triggers the protein kinase C pathway was performed. These experiments showed that the T-cell defect cannot be attributed solely to an accessory cell dysfunction. Clearly, this finding does not exclude that a defect within the protein kinase C pathway may exist.

To our knowledge, this is the first report on the proliferative responses of the CD8+ and CD4+ subsets in early ontogeny after ABMT. We were unable to demonstrate that an imbalance in CD8+ and CD4+ cells was directly responsible for the defective proliferation of T cells. Both subsets were unable to undergo CD2- and CD3-mediated proliferation even in the presence of exogenous IL-2 (Fig 5a). Therefore we are still left with the unresolved question of the mechanisms underlying the T-cell defects observed: it is probable that T-cell unresponsiveness to single mitogenic stimuli may be related to abnormal intracellular signalling processes.

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T-cell ontogeny after autologous bone marrow transplantation: failure to synthesize interleukin-2 (IL-2) and lack of CD2- and CD3-mediated proliferation by both CD4- and CD8+ cells even in the presence of exogeneous IL-2

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