Allogeneic Mixed Lymphocyte Reactions During a Second Round of Ontogeny: Normal Accessory Cells Did Not Restore Defective Interleukin-2 (IL-2) Synthesis in T Cells But Induced Responsiveness to Exogeneous IL-2

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The T-cell–accessory-cell interaction in mixed lymphocyte cultures was investigated in 25 patients following autologous bone marrow transplantation (ABMT) using autologous bone marrow treated in vitro with the cyclophosphamide derivative ASTA Z 7557. In a previous study using the same group of patients, T cells failed to synthesize interleukin-2 (IL-2) and proliferate in response to CD3- and CD2-mediated stimuli even in the presence of exogenous IL-2. To investigate whether this defect in IL-2 synthesis and proliferation was caused by defective cell-to-cell interactions, we analyzed mixed lymphocyte reactions (MLR) using T cells and irradiated non-T cells. When normal T cells from 10 different healthy subjects were challenged with allogeneic normal non-T cells, IL-2 production and proliferation were observed. In contrast, when normal T cells were cultured with non-T cells derived from patients found between 20 and 330 days after ABMT, no IL-2 secretion and no proliferative responses could be seen. The addition of lymphokines such as interleukin-1 (IL-1), interleukin-3 (IL-3), tumor necrosis factor (TNF), granulocyte-macrophage colony stimulating factor (GM-CSF), and interferon-gamma (IFN-γ) did not improve the reactions. Furthermore, when patients’ T cells were incubated with normal, irradiated non-T cells, defective IL-2 synthesis or proliferative response was obtained. However, when IL-2 was added to these cultures, an improvement in proliferative reactions was observed. Taken together, these new data provide additional evidence that T cells early in ontogeny possessed an intrinsic defect in IL-2 synthesis and that physical cell-to-cell contact between patients’ T cells and allogeneic accessory cells induced functional responsiveness to exogeneous IL-2.

MATERIALS AND METHODS

Patients. Peripheral blood from 25 patients with hematologic malignancies who underwent autologous bone marrow transplantation (ABMT) following supralethal chemotherapy and radiotherapy was used. Patients were treated either for acute myeloid leukemia (AML) (17 patients) or for acute lymphocytic leukemia (ALL; 8 patients). All patients were treated at the Medizinische Poliklinik, University of Heidelberg, FRG. The transplant conditioning regimens have been described in detail elsewhere. Collection and reinfusion of bone marrow was performed during complete remission (CR). 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 for subsequent reinfusion. Post-ABMT, all studies were done when the patients were clinically stable. The 10 control subjects were healthy individuals.

Cell phenotyping using direct and indirect immunofluorescence. Flow cytometry analyses (using an EPICS C, Coulter) were performed on the E' T-cell fraction with directly fluorescein (FITC)-coupled monoclonal antibodies (MoAbs) CD2 (OKT11, Ortho Diagnostics, Westwood, MA) and CD3 (Leu-4, Becton Dickinson). Indirect immunofluorescence (IF) was performed with the E' non-T-cell fraction with HLA-DR MoAb (Class II, Becton Dickinson), W2/36 MoAb (Class I, a gift from G. Moldenhauer, DKFZ, Heidelberg), CD14 (Leu-M3, Becton Dickinson, Heidelberg, FRG), and CD20 (Leu-16, Becton Dickinson) and as second antibody a fluorescein-conjugated goat antimouse IgG/lgM (Medac, Hamburg, FRG).

Enrichment of T and non-T cells from human peripheral monocellular cells. Human peripheral monocellular 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 (RBCs) followed by FH centrifugation. The E’ cell fraction interactions in allogeneic mixed lymphocyte cultures using cells in an early phase of the ontogenic process from recipients of ASTA Z 7557-treated autologous bone marrow.
cytes/macrophages. The interphase recovered after rosetting and (pH 7.2) and consisted of 87% to 98% 
and I cells per well were incubated at Class detected levels >0.05 U of IL-2 in IL-2 volume of 200 zL containing 10% FCS in a 96-well plate were 
also incubated the addition of irradiated non-T cells. Irradiated non-T cells were 
penicillin, and 100 .og streptomycin for I to 4 days with and without 
mmol/L in the MLR. 
non-T 
cells, containing 
(5 2 3+79%) CD2+ cells, 60% to 96% (median 87%) CD3+ cells, 
and the remaining cells were 2% to 21% CD11b+/CD14+ monocytes/macrophages. The interphase recovered after rosetting and 
and FH centrifugation consisted of the E- cell fraction (referred to as 
non-T cells, containing 79% to 99% Class I+ cells and 44% to 92% 
Class II+ cells); non-T cells were irradiated (3,000 rad) to be used in the MLR.
Assay for IL-2 production and activity. Cultures using 5 × 10⁴ T 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 1 to 4 days with and without 
the addition of irradiated non-T cells. Irradiated non-T cells were also incubated alone. After the incubation time the supernatants 
from triplicates were pooled, centrifuged, filtered, and frozen until the bioassay for IL-2 was performed.
IL-2 was measured by using the bioassay previously described by Falk et al.1 T 10⁵ cells from the C57Bl/6-W2 cell line in a final 
volume of 200 μL containing 10% FCS in a 96-well plate were incubated with 100 μL undiluted supernatant (thawed just before use). 
Time of incubation was 20 hours followed by 4 hours of culture with 1 µCi of ³H-thymidine (New England Nuclear, Boston, MA). 
Cells were then harvested onto glass fiber filters with an automatic cell harvester, and radioactivity was measured in a scintillation 
counter. IL-2 was measured by using a probit analysis: units of IL-2 activity were determined from a standard curve performed with 
recombinant IL-2 (Boehringer Mannheim, FRG). Results are expressed as U/mL. IL-2 produced by 10⁶ cells/mL. This assay 
detected levels >0.05 U of IL-2 in 100 μL (>0.5 U/mL of IL-2).
Mixed lymphocyte cultures. Cultures were performed in triplicates (as described above for the assay for IL-2 production and activity) 
with 5 × 10⁴ responding T cells and 1 × 10⁴ irradiated (3,000 rad) stimulating non-T cells (E- cells) from single individuals in 
200 μL of RPMI 1640 with 10% FCS. In “mixing experiments,” 
stimulating non-T cells from normal subjects (5 × 10⁴ cells) were 
mixed with non-T cells from patients (5 × 10⁴ cells) or normal 
subjects (5 × 10⁴ cells) to test the suppressive effect of patients’ 
non-T cells in a mixed lymphocyte reaction (MLR) using as 
responders normal T cells.
Control cultures with T cells alone, T cells + IL-2, and irradiated non-T cells alone were done. When added, recombinant IL-2 
(Boehringer Mannheim, FRG) at 10 U/mL, recombinant IL-1 
(Enzyme) at 100 U/mL, recombinant interleukin-3 (IL-3, 
Behringwerke, Marburg, FRG) at 10 ng/mL, recombinant granulo-
cyte-macrophage colony stimulating factor (GM-CSF; Behring- 
werke, Marburg, FRG) at 100 ng/mL, interferon-gamma (IFN-γ, 
Boehringer Mannheim, FRG) at 10 U/mL, and tumor necrosis 
factor (TNF, Boehringer Mannheim, FRG) at 1,000 ng/mL were 
used. Cells were cultured for 2 to 8 days, and 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 minute (median cpm).
RESULTS
Study of patients’ non-T-cells’ ability to function as stimulators when cultured with normal T cells in an allo-
geneic MLR. Non-T cells derived from five representative 
patients at days 27 to 242 post-ABMT were incapable of 
triggering normal levels of proliferation by control T cells. 
We tested each patients’ non-T cells in three separate 

![Fig 1. MLR with normal T cells plus patients’ non-T cells. In this diagram the median-response curve obtained with normal T cells from one individual when incubated separately for 2 to 8 days with irradiated, normal non-T cells from three single individuals (-O- line) and with non-T cells derived from five patients (-Δ-, -D-, --. -γ-, -Φ- line) is shown. In 200-μL culture medium, triplicates of 5 × 10⁴ T cells from the representative normal subject (from 10 normal subjects tested) were cultured with 1 × 10⁴ irradiated (3,000 rads) non-T cells from single normal individuals (the median curve is shown) and from five patients who were 27, 84, 152, 198, and 242 days respectively post-ABMT. ³H-Thymidine was added during the last 16 hours of culture. Results are expressed as median cpm. The maximum response was found between day 4 and day 6 of culture. No proliferation was observed with T cells or irradiated non-T cells alone (not shown).](image-url)
proliferation due to an inability to secrete a single factor or to respond to a single factor provided by T cells (which in turn lead to factor production by accessory cells), various cytokines were added to the MLR. The factors were used at doses tested to be optimal in other assays. When the factors were added to T cells alone, no effect was seen (data not shown).

Addition of these cytokines to the MLR did not improve the reactions observed (Fig 2, columns B through F). Preincubation of patient non-T cells (from days 20 to 242 post-ABMT) with the cytokines and subsequent culture with normal T cells did not improve the reactions (data not shown).

**Induction of IL-2 synthesis in normal T cells by patients’ non-T cells.** When $5 \times 10^4$ normal T cells were incubated with $1 \times 10^4$ normal, allogeneic, irradiated non-T cells from a single donor and IL-2 production measured at days 1 through 4 following the start of culture, a characteristic response curve was obtained where the peak production was after 2 days (median of 2.1 U/mL; Fig 3). No spontaneous IL-2 secretion by T cells alone or by irradiated (3,000 rads) non-T cells alone (to exclude IL-2 production by contaminating T cells) was detectable (not shown). In contrast, when patients’ non-T cells were used to stimulate normal T cells, no IL-2 secretion could be measured (Fig 3). Thus patient accessory cells were unable to induce normal T cells to produce normal levels of IL-2.

**Secretion of IL-2 by patients’ T cells when stimulated with normal or patients’ non-T cells.** Irradiated, allogeneic, normal non-T cells were unable to induce IL-2 synthesis in patients’ T cells (Fig 3). Similarly, when patients’ T cells were incubated with other patients’ non-T cells (from 10 representative patients between days 20 and 242 post-ABMT), no IL-2 production was seen (Fig 3). Therefore normal-functional non-T cells failed to induce IL-2 synthesis in patients’ T cells. It is clear that the failure of IL-2 synthesis in these patients is not the result of defective accessory-cell function alone.

**Effect of addition of IL-2 on the proliferative responses of patients’ T cells to normal non-T cells in the allogeneic MLR.** Figure 4 shows proliferative responses of T cells from 25 patients (all taken at a time when they had not yet recovered their ability to respond in a MLR), a time interval ranging from days 20 to 330 post-ABMT, to three single, different, allogeneic, irradiated, normal non-T cells (Allo1, 2, and 3) after 5 days of culture.

Both the responses of normal T cells (data not shown) and patients’ T cells (Fig 4) in the allogeneic MLR using single, normal, non-T cells (Allo1, 2, and 3) can be increased by exogenous IL-2. This suggests that the presence of allogeneic accessory cells through cell-to-cell interactions can induce IL-2 receptor function.

**Effect of exogenous IL-2 on the responses of normal T cells and patients’ T cells to other patients’ accessory cells.** In Fig 5, addition of exogenous IL-2 improved the proliferation obtained in the MLR when nonresponding patients’ T cells were cultured with nonstimulating accessory cells from other patients (left side, black columns). Similar-

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**Fig 2.** Response of normal T cells to patients’ non-T cells with and without the addition of exogenous cytokines. This is the characteristic pattern of reactions obtained in mixed lymphocyte cultures when T cells from one normal individual (from 10 normal subjects tested) were stimulated with irradiated (3,000 rads) non-T cells from 10 different patients for 5 days. In column A, the white columns display the response of normal T cells when incubated with non-T cells alone from patients 1 through 10 who were at days 20 to 242 post-ABMT. The black column displays the median response obtained with single non-T cells derived from three normal subjects (black column). In column B, IL-1 (100 U/mL) has been added to the T-cell–non-T-cell cultures; in column C, IL-3 (10 ng/mL); in column D, TNF (1000 ng/mL); in column E, GM-CSF (100 ng/mL), and in column F, IFN-γ (10 U/mL). Results are expressed as median cpm. When T cells alone were incubated with IL-1, IL-3, TNF, GM-CSF, or IFN-γ, no effect was observed (data not shown).

**Fig 3.** IL-2 synthesis in normal and patients’ T cells when stimulated with irradiated non-T cells. $5 \times 10^4$ T cells were stimulated with $1 \times 10^4$ irradiated, single, allogeneic non-T cells in 200 μL for 1 to 4 days, and the amount of IL-2 secreted was measured. The median response curve obtained when normal T cells and single normal allogeneic non-T cells were cultured together (— line) is shown; the curves obtained when normal T cells were cultured with patients’ (between 20 to 152 days after ABMT) non-T cells (— line), when patients’ T cells were cultured with single, normal non-T cells (— line), or with other patients’ non-T cells (— line) are also displayed. T cells or irradiated non-T cells alone did not secrete detectable amounts of IL-2 (not shown). Results are expressed as U/mL IL-2 secreted by $1 \times 10^4$ T cells/mL at days 1 through 4 following stimulation.
accessory cells can improve the responsiveness of T cells to non-T cells derived from one different, normal individual (black columns). When the stimulating non-T cells were mixed with equal numbers of non-T cells from another normal individual, either an increased or an identical proliferative response occurred (striped column).

Furthermore, when the stimulating, normal non-T cells were mixed with equal numbers of non-T cells derived from various patients (white columns), only in 3 of 18 cases one can observe a suppressive effect on the MLR. In the 15 other cases, no significant change in proliferation occurred. Therefore the defective stimulating capacity of patients' non-T cells cannot be directly accounted for by an active, suppressive effect of the patients' non-T-cell population on normal T-cell proliferation.

**DISCUSSION**

The group of patients studied here underwent an ABMT using bone marrow treated in vitro with the cyclophosphamide derivative ASTA Z 7557 prior to grafting. In our previous study we demonstrated that the T-cell population (derived from the same group of patients) generated during this second round of ontogeny taking place after ABMT consisted of an excess of CD8* cells and decreased CD4* cells. However, normal membrane expression of the CD2, CD3, and T-cell receptor (TCR) antigens and of the IL-2 receptor defined by CD25 was present. Defective IL-2 synthesis after stimulation with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) was observed in these T cells; furthermore both CD4* and CD8* subsets failed to show either lectin-, CD2-, or CD3-mediated proliferation even in the presence of exogeneous IL-2. It was postulated that the underlying defects could be an intrinsic block in IL-2 synthesis and/or a control at the level of IL-2 receptors function.

To our knowledge, no studies on MLR early after ABMT using ASTA Z 7557-treated bone marrows are available. Earlier studies with patients following allogeneic BMT have described allogeneic MLR impairment of unfractionated cells in patients less than 5 months post-BMT and in those with chronic graft-versus-host disease (GVHD) in short-term patients the responding T cells were defective, but the non-T cells showed only marginally decreased or normal stimulatory capacity.

Accessory cells serve two major functions in the generation of T-cell–mediated immune responses: one is the presentation of antigen, and the other is a sensitization function that triggers a series of events ending in T cells secreting and responding to lymphokines. As shown in the present study, accessory cells from patients post-ABMT failed to provide the necessary signals to activate normal T cells and to induce IL-2 synthesis (Fig 3) and thus to lead to proliferation (Fig 5). From our previous studies and here we see that the number of B cells is low post-ABMT. Whether a low percentage of dendritic cells within the patients' non-T-cell population or whether a lack of B cells is responsible for the defective stimulatory function remains to be investigated.

Amplification of accessory cell function by cytokines such as IFN-γ, IL-1, and GM-CSF has been reported. It is possible that early accessory cells post-ABMT were unable...
were incubated in triplicate for 5 days and 

H-Thymidine added during the last 16 hours. Mixing experiments using normal T+ normal T+ patients non-T cells from a second normal individual (n1+n2) were stimulated 16 hours before harvesting. With T cells alone or irradiated non-T cells plus IL-2, no proliferation was obtained (not shown). Results are expressed as median cpm.

It is known that functioning monocytes are critically required for IL-2 production but not for IL-2 receptor expression. Here we observed that the patients' T cells did not produce IL-2 when cultured with normal allogeneic non-T cells (Fig 3). This implies that there is an inability to secrete IL-2 that is independent from accessory cell failure. In addition, our patient T cells possessed normal levels of CD25+ IL-2 receptors. However, CD25 measures only the expression. Here we observed that the patients' T cells possessed normal levels of IL-2 receptors. However, CD25 measures only the expression.

After a given time interval post-ABMT, the length of which varies from patient to patient, all patient T cells recover their functional ability and the allogeneic MLR returns to normal. Here we have shown the MLR between T cells derived from patients and normal non-T cells at a time.
when defective proliferative responses could be observed (Fig 4). We note that the addition of IL-2 improved the abnormal proliferative responses of nonresponder T cells to normal non-T cells (Fig 4). Similarly, of interest was the improvement of reaction observed with the addition of exogenous IL-2 in the mixed lymphocyte cultures between two patients (Fig 5). This is in marked contrast to stimuli triggering the CD2 or CD3 pathway of T-cell activation, that did not render the IL-2 receptors functional. Here cell-to-cell contact between T cells and either functioning accessory cells (derived from normal individuals) or defective accessory cells (from patients) resulted in the induction of responsiveness of the IL-2 receptors to exogenous IL-2.

We have here a unique model of early T cell ontogeny in an autologous situation. It is possible to use this clinical model for the study of signals required for the expression and function of the IL-2 receptor system. In contrast to CD3 or CD2 stimuli, non-T cells can induce IL-2 responsiveness; thus one has to postulate that additional signals regulate IL-2 receptor expression and function. Non-T cells in a MLR probably activate T cells by a number of different pathways (eg, CD2, CD3, CD4, CD8, CD28, CD44, CD45) which all have a synergistic activating effect and therefore provide a very strong signal, whereas direct activation of T cells via the CD2 or the CD3 pathway provides only one signal. This single signal may not be strong enough to activate T cells regenerated early after ABMT and to render them responsive to exogenous IL-2. Finally, this clinical situation allows the analysis of the nature of monocyte-derived signals or mediators that lead to IL-2 production, a point that is not entirely known at present.

REFERENCES

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Allogeneic mixed lymphocyte reactions during a second round of ontogeny: normal accessory cells did not restore defective interleukin-2 (IL-2) synthesis in T cells but induced responsiveness to exogeneous IL-2

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