PHENOTYPE OF RECOVERING LYMPHOID CELL
POPULATIONS AFTER MARROW TRANSPLANTATION

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There is little question that the study of recovering lymphoid cell populations following bone marrow transplantation is important to the understanding of the biology of transplantation. With a full understanding of the types of cells, their sequence of appearance, and their relationship to such clinical situations as graft-vs.-host disease (GVHD) and graft rejection, it is likely that it will be possible to significantly improve the therapeutic efficacy of marrow transplantation. In addition, identifying the recovering cells is the first step to establishing their functional role in the recovering immune system.

The availability of monoclonal antibodies (mAb) and flow cytometry has made it possible to easily and rapidly determine the phenotype of blood cells. However, it has become clear that studies of single surface antigens are extremely limited in their ability to define cell types. This study was undertaken to develop as complete a description as possible of the recovering lymphoid cell types after transplantation, using a large panel of mAb and multiparameter flow cytometry.

We summarize here results of over 400 dual-fluorescence analyses done on four marrow transplant patients during the early stages of marrow recovery. The data for the first 90 d after transplantation is presented. Three of the patients received T cell-depleted marrow from an HLA-matched donor. The fourth received untreated marrow from an identical twin. The comparison of these four patients allows us to study the possible effects of marrow T cell depletion on the early recovering immune system.

Our results indicate that the pattern of recovery of lymphoid cells is similar in each of the patients. Six major cell types account for >90% of the lymphoid cells in these patients; the major differences between patients occur in the relative numbers of the cells and their time of appearance. In addition to the six major

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This work was supported in part by grant AI 18060 from the National Institutes of Health, Bethesda, MD. K. A. Ault is recipient of N. I. H. Career Development Award CA 00605. J. H. Antin and D. Ginsburg are recipients of American Cancer Society Junior Faculty Clinical Fellowships, and D. Ginsburg is recipient of an N.I.H. National Research Scientist Award. B. R. Smith is a Fellow of the Leukemia Society of America. Address correspondence to Kenneth Ault, Analytical Cytology Laboratory, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

Abbreviations used in this paper: FACS, fluorescence-activated cell sorter; FL, fluorescein; GVHD, graft-vs.-host disease; mAb, monoclonal antibody; NK, natural killer; PE, phycoerythrin; Th, T helper; Ts, T suppressor.

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J. Exp. Med. © The Rockefeller University Press • 0022-1007/85/6/1483/20 $1.00

Volume 161 June 1985 1483-1502
cell types, a number of subsets can be identified that are much more variable in their appearance and numbers, and a few rare cell types can be identified that are present in some patients in small numbers.

Methods

Patients. All four patients were transplanted for acute leukemia: patient 151, a 22-yr-old female, for acute myelogenous leukemia in remission; patient 152, a 13-yr-old male, for acute myelogenous leukemia in relapse; patient 153, a 34-yr-old female, for acute lymphoblastic leukemia in remission; and patient 155, a 3-yr-old female, for acute lymphoblastic leukemia in remission. All patients received pretransplant conditioning consisting of cytosine arabinoside 500 mg/m²/day by continuous intravenous infusion for 7 d, followed by total body irradiation either with 1,040 rad (151) or 1,200 rad (152, 153, and 155) midline dose in six fractions over three consecutive days at 5 rad/min, and 60 mg/kg/day cyclophosphamide for 2 d. Donors were all HLA- and MLC-compatible siblings. Patient 155 received marrow from her identical twin.

Marrow was harvested by standard methods. Marrow transplanted to patients 151, 152, and 153 was treated with mAb (anti-Leu-1) and complement to remove donor T cells. Briefly, marrow leukocytes were isolated on an IBM 2991 cell washer, and mononuclear cells were harvested on a Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) cushion. These cells were incubated with a saturating concentration of anti-Leu-1 mAb for 30 min at 4°C, followed by incubation with baby rabbit complement (Pel-Freez, Rogers, AR) for 45 min at room temperature. This treatment was repeated three times. Total residual T cells, defined as the number of Leu-4⁺ cells, determined on the fluorescence-activated cell sorter (FACS), ranged from <0.1% to 0.9% of marrow cells. The total dose of treated marrow cells infused in the patients ranged from 0.75 × 10⁸ to 1.5 × 10⁸ nucleated cells/kg.

Patients 151, 152, and 153 received posttransplant intravenous methotrexate 10 mg/m² on days +1, +3, +6, and +11, for GVHD prophylaxis. Patients 151, 153, and 155 showed no evidence of GVHD. Patient 152 had grade II acute GVHD developing at day +48. He received an intravenous infusion of anti-Leu-1 mAb (Becton-Dickinson, Mountain View, CA), in doses sufficient to saturate all peripheral-blood T cell-binding sites, for 5 d with resolution of the GVHD by day +55, and no subsequent recurrence of GVHD. Patients 151 and 155 are alive and well at 6 and 4 mo posttransplant, respectively. Patient 152 died of idiopathic interstitial pneumonitis on day +95. Patient 153 died of recurrent leukemia and sepsis on day +143.

Cell Preparation. Heparinized blood samples were obtained from the patients at various intervals beginning as soon as lymphocytes were identified in the blood. Initially, samples were obtained approximately every 2 wk, the intervals stretching to 1 mo after the first 30–60 d. The blood was mixed with ½ vol of Lymphocyte Separator Reagent (Technicon Instruments Corp., Tarrytown, NY), and incubated at 37°C on a rotating platform for 1 h. This was done to allow the monocytes and polymorphonuclear leukocytes to phagocytize iron particles, improving their depletion during the subsequent density gradient separation step. The blood was then diluted with two vol of Hank’s balanced salt solution and centrifuged over a cushion of Ficoll-Paque (Pharmacia Fine Chemicals). The mononuclear cell interface was harvested, the cells washed, counted, and aliquoted for labeling.

Antibodies. The majority of the antibodies were obtained from Becton Dickinson. They were mAb conjugated either to fluorescein (FL) or phycoerythrin (PE) (1) as indicated in Table 1. In some cases, the antibodies were conjugated to biotin. Other mAb included: biotinylated B1, and unconjugated B2, both from Coulter Immunology (Hialeah, FL).

Conventional antibodies used in these studies included: FL-F(ab′)₂ fraction of rabbit anti–human IgG, IgM, and IgD; these antibodies were obtained from Cappel Laboratories (Cochranville, PA); F(ab′)₂ fraction of rabbit anti–human κ and λ light chains; these were obtained from Accurate Chemical and Scientific Company (Westbury, NY) as intact
immunoglobulin, and the F(ab’)2 fractions prepared in our laboratory by pepsin digestion according to standard methods; FL-goat anti-rabbit IgG, obtained from Cappel Laboratories; FL-goat anti-mouse IgM (Becton Dickinson) was used as a second antibody for the B2 labeling.

The biotinylated antibodies were used in combination with either PE- or FL-conjugated avidin (Becton Dickinson).

Cell Labeling. The labeling protocol followed for each patient is shown in Table II. 25 pairs of mAb were chosen. Additional studies using other antibodies were added in order to further characterize some cell types as described below.

The commercial mAb were used according to manufacturer’s directions, 5 µl antibody solution being used to label 10^6 cells. Double labeling was accomplished in a single step, by addition of both mAb. The cells were incubated on ice for 30 min with the antibodies, washed twice with ice cold phosphate buffered saline, then fixed with 1% paraformaldehyde. During labeling, the cells were carefully protected from light, and subsequently stored at 4°C in the dark before analysis.

Cells labeled with biotinylated antibodies were washed three times, then incubated with PE-avidin (5 µl) or FL-avidin (2.5 µl), washed twice, and fixed. Cells labeled with B2 antibody were incubated with FL-anti-mouse IgM (2.5 µl) as a second step reagent, then washed and fixed.

Conventional antibodies were used at concentrations previously determined to be saturating on normal blood lymphocytes, usually ~50 µg of antibody protein being used to label 10^6 cells.

In the case of the Ig light chain labeling, an indirect method was used in which the cells were first incubated as described above with the anti-light chain antibodies, followed by three washes, a second identical incubation with FL-goat anti-rabbit IgG, three more washes, and fixation.

Conditions for Flow Cytometric Analysis. A Becton Dickinson FACS 440 was used for analysis and sorting of the specimens. The instrument was operated with an Argon ion
laser at 488 nm and 300 mW. Alignment and calibration was performed using glutaraldehyde-fixed chicken erythrocytes. The fluorescence sensitivity of the instrument was checked, and the photomultiplier voltage adjusted to place the chicken erythrocyte standards in the same position for each analysis. 10^4 logarithmic amplification was used for both FL and PE fluorescence. FL and PE fluorescence was separated using a 580 nm long pass dichroic mirror as a beam splitter. FL fluorescence was measured through a 530 ± 30 nm band pass filter. PE fluorescence was measured through a 580 ± 25 nm band pass filter.

The first sample of each run was labeled with FL-HLE antibody, which labels all blood leukocytes, and PE-Leu-M3, which labels monocytes. This sample was used for two purposes. First, a gate was set on the forward and right-angle light scatter parameters, which included all of the brightly HLE-labeled lymphocytes, excluding as much as possible the unlabeled erythrocytes and the Leu-M3-labeled monocytes. Since these samples were monocyte depleted, there was seldom a significant number of monocytes (<3%). Similarly, the few polymorphonuclear leukocytes were identified by lower levels of HLE labeling, and these were easily excluded by gating on right-angle light scatter. Second, this sample was used to adjust the setting of the instrument’s fluorescence-compensation network to eliminate a small amount of spillage of FL fluorescence into the PE channel. When these adjustments were made, a dual-parameter (FL vs. PE) histogram was obtained to confirm the lack of FL fluorescence in the PE channel and to accurately measure the number of erythrocytes that had been included in the light-scatter gates. Although monocytes and polymorphonuclear leukocytes were rigidly excluded from our data using these methods, there were variable numbers of erythrocytes (5–50%) included in our gates. This was intentional, so as not to exclude any small lymphocytes from analysis. In addition, in some cases there were large numbers of nucleated erythrocytes circulating in these patients,

| FL ANTIBODY | PE-ANTIBODY |
|-------------|-------------|
| 1. HLE      | Leu-M3      |
| 2. Leu-4    | Leu-1       |
| 3. Leu-4    | Leu-5       |
| 4. Leu-9    | Leu-4       |
| 5. Leu-9    | Leu-1       |
| 6. Leu-6    | Leu-5       |
| 7. Leu-2    | Leu-3       |
| 8. Leu-9    | Leu-2       |
| 9. Leu-9    | Leu-3       |
| 10. HLA-DR  | Leu-2       |
| 11. HLA-DR  | Leu-3       |
| 12. Leu-4   | Leu-7       |
| 13. Leu-11  | Leu-7       |
| 14. Leu-4   | Leu-15      |
| 15. Leu-9   | Leu-15      |
| 16. Leu-5   | Leu-15      |
| 17. Leu-7   | Leu-15      |
| 18. Leu-11  | Leu-15      |
| 19. Leu-2   | Leu-15      |
| 20. Leu-7   | Leu-2       |
| 21. Leu-9   | Leu-7       |
| 22. Leu-11  | Leu-2       |
| 23. Leu-11  | Leu-4       |
| 24. Leu-4   | Leu-2       |
| 25. Leu-12  | Leu-1       |
which were, of necessity, included in our gates. As described below, our data was corrected
for the presence of these cells.

All of the samples from a single patient were then analyzed sequentially, collecting
dual-parameter histograms of FL and PE fluorescence data from 20,000–50,000 cells.
These histograms were stored and subsequently analyzed using a Becton Dickinson
Consort 40 computer and software. In some cases, list mode data was taken without
gating, to confirm that all cells of interest were being included in the gates. This was done
primarily when small numbers of cells of particular interest (such as Leu-6+ cells) were
detected. The histograms presented here display FL fluorescence on the abscissa and PE
fluorescence on the ordinate. Contour lines are drawn on the histograms at levels
corresponding to 2, 4, ..., 10, 20, ..., 90% of the highest peak in the histogram.

Data Reduction. The sets of dual-parameter histograms were analyzed by placing
windows around resolved cell types of interest and expressing the number of cells within
the window as a percent of the total cells within the histogram. This percentage was then
normalized to the total percentage of lymphocytes contained within the gates, as deter-
dined by HLE-labeling described above. In this way, unlabeled erythrocytes were ex-
cluded from the analysis. The absolute number of circulating lymphocytes was calculated
from the patient’s total white count and differential count done on a Wright-Giemsa-
stained smear the same day. The absolute number of cells of each type was estimated by
multiplying the percentage obtained above by the absolute lymphocyte count.

Control samples labeled with FL- and PE-conjugated mouse IgG1 mAb confirmed that,
in the regions occupied by labeled cells, there was insignificant nonspecific labeling (<1%).

The placement of windows around cell populations is somewhat subjective. In order to
estimate the accuracy of our data, we made every effort to obtain at least two estimates
of the fraction of each cell type of interest. In most cases, it was possible to identify the
same cell population in at least two different histograms. The mean variation in these
duplicate estimates for 40 different analyses was 11.0 ± 2.1% of the mean.

DNA Polymorphism and Ig Gene Analysis. Before transplantation, blood was obtained
from patients and donors to screen for DNA sequence polymorphisms that could distin-
guish the patient or donor origin of the cells after transplant, as described previously (17).
Briefly, 5–16 μg of DNA from blood leukocytes were digested to completion with the
appropriate restriction endonuclease, fractionated by agarose gel electrophoresis, trans-
ferred to nitrocellulose filters, and then hybridized with the corresponding radiolabeled
probe. Patient 155 was an identical twin, and was not tested. Blood was obtained after
transplantation as soon as peripheral blood leukocyte counts were 500–1,000 cells/mm³,
and again at least one more time when peripheral blood counts had recovered.

Donor, patient (pre- and posttransplant), and posttransplant-sorted Leu-1B cells from
patient 151 were analyzed for clonal somatic rearrangements of the Ig genes, using probes
for the heavy chain switch region (JH), κ chain constant region (κc), and λ chain constant
region (λc), as previously described (18).

Natural Killer (NK) Cell Functional Assay. NK cell function was assayed in a standard
4-h ⁵¹Cr-release assay, using K-562 cells as the target. This assay was done exactly as
described previously (19).

Results

Definition of Cell Types. The following cell types were identified in all four
patients and are discussed in detail below. The manner in which these cells were
identified for purposes of quantitation is described here: Th cells were identified
by the presence of Leu-3 but not Leu-2. Ts cells were identified by the presence
of bright Leu-2 and Leu-4 but not Leu-3 or Leu-11. B cells were identified by
the presence of Leu-12 and absence of Leu-1. Leu-1 B cells were identified by
the presence of Leu-12 and low levels of Leu-1. Their identity was confirmed in
detail as described below. Total NK cells were identified by the presence of Leu-
and Leu-15 but not Leu-4. Leu-2-bearing NK cells were identified by the presence of Leu-11 and low levels of Leu-2 but not Leu-4.

Table III summarizes the phenotype of each of these cell types. Each of these cell types could be further subdivided on the basis of other studies, as described below. Figs. 1–4 show illustrative examples of dual-parameter histograms taken from patient 151. All of the patients showed similar labeling patterns for each of the cell types described here. The only differences noted between the four patients were quantitative in nature. In particular, patient 155, who had received untreated marrow from an identical twin donor, showed the same pattern of recovering cell types as the other three patients.

**Th Cells.** In each patient, cells having the phenotype of normal blood Th cells appeared between days 10 and 20. These cells are best illustrated in Fig. 1, C and D. Fig. 1 C shows the presence of Leu-3 but not Leu-2 on these cells, and D shows the same cells as Leu-2⁺ and Leu-4⁺. Fig. 1 A illustrates the coordinate expression of both Leu-1 and Leu-4 that was observed in all patients. Additional markers present on these cells included Leu-5 and Leu-9. Fig. 1 B illustrates the expression of Leu-5 on all of the Leu-4⁺ cells. Labeling with Leu-9 was less intense than that seen on the NK cell subsets, as described below (data not shown). Several other markers were variably expressed on these cells, including Leu-15, Leu-7, Leu-8, and HLA-DR. Patient 151 had particularly strong Leu-15 expression on all of her T and NK cells; however, the other patients had variable expression of this antigen. Leu-7, Leu-8, Leu-10, and HLA-DR were even more variable in their expression, even in sequential studies on the same patient.

**Ts Cells.** Each of the patients also began to have cells with the phenotype of normal Ts cells between days 10 and 20. These cells were initially present in numbers comparable to the Th cells. They are illustrated in Fig. 2, C and D. Fig. 1 D shows them as Leu-2-bright cells also expressing Leu-4. In C they are not clearly resolved from the larger population of Leu-2-dim cells discussed below. It is, however, clear from C that they do not express Leu-3. Other markers expressed by these cells included Leu-1, Leu-5, and Leu-9, the latter in quantities less than the NK cells, and comparable to the Th cells. The expression of Leu-15, Leu-7, Leu-8, Leu-10, and HLA-DR was also variable on these cells. They were Leu-11⁻ (Fig. 3, A). Fig. 1, D is of particular importance, since it clearly illustrates that the Leu-2-bright cells are a phenotype distinct from the Leu-2-

### Table III

| Cell type | Leu-4 | Leu-1 | Leu-5 | Leu-2 | Leu-3 | Leu-11 | Leu-12 |
|-----------|-------|-------|-------|-------|-------|--------|--------|
| Ts        | +     | +     | +     | -     | -     | -      | -      |
| Th        | +     | +     | +     | -     | +     | -      | -      |
| Leu-1⁺ B  | -     | dim   | -     | -     | -     | -      | +      |
| B         | -     | -     | -     | -     | -     | -      | +      |
| NK-I      | -     | -     | +     | -     | -     | +      | -      |
| NK-II     | -     | -     | +     | dim   | -     | +      | -      |

Phenotype of Major Lymphoid Cell Populations After Transplantation
dim cells. There is no evidence for any transitional cells between the Leu-2-dim, Leu-4^− cells and the Leu-2-bright, Leu-4^+ cells. In none of the patients were any significant numbers of cells detected bearing both Leu-2 and Leu-3.

B Cells. B cells appeared in three of the patients between days 20 and 30. Patient 152 failed to develop any detectable B cells. In all three of the patients, there were two distinct phenotypes of B cells detectable.

Fig. 2 illustrates data from patient 151 at a time when she clearly had both B cell subtypes. One type of B cells had markers identical with the majority of normal blood B cells. These are illustrated in Fig. 2, A and C. In A, they are seen as Leu-1^−, Leu-12^− cells. In C it can be seen that they are Leu-1^−, B1^+, although the amount of B1 they express is not as great as that of the other B cell subtype.

The population seen in Fig. 2A that expresses both Leu-12 and low levels of Leu-1 is of particular interest. In all three of the patients (151, 153, and 155)
who developed significant numbers of B cells, these were the predominant cell type. We will refer to these as Leu-1 B cells. They are also seen in Fig. 2C, where they are strongly B1+. Fig. 1A also illustrates these cells, where they are seen as Leu-1-dim, Leu-4-. Fig. 2, B and D illustrate the expression of HLA-DR and IgD on both B cell subtypes. The Leu-1 B cells are more brightly staining with HLA-DR, but have amounts of IgD comparable to conventional B cells. Fig. 2B also illustrates the variable expression of HLA-DR on the Leu-1-bright T cells. Studies of heavy chain expression showed that both populations of B cells expressed IgM and IgD but not IgG (data not shown). The Leu-1 B cells stained more brightly with anti-IgM than did the conventional B cells. Both B cell subtypes labeled with both anti-κ and anti-λ in roughly equal numbers (not
FIGURE 3. Analysis of DNA sequence polymorphisms in patient 151. DNA preparations from patient and donor before marrow transplantation, and free fractionated patient cells on days 13, 20, and 64 after transplantation were examined by blot hybridization using probe PAW101. DNA from Leu-1 B cells (prepared on day 64 as described) is shown in the last lane to the right. Patient and donor pretransplant patterns were distinct and easily distinguishable. They share a 16 kilobase (kb) allele, but differ at the second locus. The 14 kb fragment is specific for the patient, and the 19 kb fragment is specific for the donor. All posttransplant cells show exclusively the donor pattern, and thus were of donor origin. Because of its size, the largest fragment (19 kb) is transferred less efficiently in the Southern blot procedure, and is only faintly seen in the last two lanes.

In addition, both subtypes expressed B2 and Leu-14 in comparable amounts. Both labeled very weakly or not at all with anti-CALLA, and were Leu-11-.

DNA prepared from B cells of patient 151 was studied by DNA sequence polymorphism analysis on days 13, 20, and 64. They were also examined for clonal somatic rearrangement of the Ig genes on day 64. Sorting for the Leu-1 B cells enriched them from 18% to 65% of total lymphocytes. DNA from these enriched cells was analyzed with DNA from unsorted posttransplant mononuclear cells, pretransplant recipient cells, and donor cells. The DNA polymorphism shown).
analysis demonstrated that there was complete engraftment of donor cells, and that the sorted Leu-1 B cells were of donor origin (Fig. 3). This analysis can detect residual host elements when they comprise as few as 1% of the total (17). Thus the Leu-1 B cells did not derive from residual host lymphocytes. Furthermore, examination of Ig gene configuration detected no evidence for clonal somatic rearrangements either before or after transplantation (data not shown). Therefore, it is unlikely that they represent either induction of a lymphoproliferative disorder posttransplantation or transplantation of a preexistent lymphoproliferative disorder from the donor.

**NK Cells.** At least two distinct subtypes of NK cells were identified in all four patients. These cells appeared between days 10 and 20 and, taken together, they were the most numerous lymphoid cells present throughout our studies. Both subtypes expressed Leu-11, which was not expressed on either T cells or B cells. Fig. 4A illustrates that the Leu-11+ cells could be separated into those that were Leu-2- and those that expressed low levels of Leu-2. These two types are not

![Figure 4](image_url)

**Figure 4.** Analysis of NK cells after transplantation. (A) Leu-11 (horizontal) vs. Leu-2 (vertical). The Ts cells that are brightly Leu-2+ are Leu-11-. The population of Leu-11+ NK cells can be divided into those that express low amounts of Leu-2, and those that are Leu-2-. These two populations are not clearly resolved here, but can be clearly seen in other panels. (B) Leu-7 (horizontal) vs. Leu-2 (vertical). There is some expression of Leu-2 on Ts cells. The majority of the Leu-2-dim cells are Leu-7-, whereas the majority of Leu-7+ cells are Leu-2-. (C) Leu-11 (horizontal) vs. Leu-7 (vertical). A minority of the Leu-11-bearing cells also express Leu-7. (D) Leu-11 (horizontal) vs. Leu-15 (vertical). Most of the NK cells are Leu-11+ and Leu-15+. The cells that are Leu-15+ but Leu-11- are primarily T cells.
clearly resolved, and thus may not be totally distinct. The Leu-2-dim cells were
shown above to be clearly Leu-4−, and distinct from Ts. Fig. 4B illustrates that
the expression of Leu-7 was variable on these cells. In general, the Leu-2− cells
seemed to express more Leu-7 than did the Leu-2-dim cells. This panel also
shows some expression of Leu-7 on Leu-2-bright Ts cells. Fig. 4C clearly
illustrates that the majority of the Leu-7 labeling is on the Leu-11-bearing cells,
and D shows that all of the Leu-11-bearing cells were Leu-15+. All of the NK
cells, both Leu-2− and Leu-2-dim, were characterized by the presence of Leu-
11, Leu-15, Leu-5, bright Leu-9, and variable amounts of HLA-DR. They all
lacked Leu-4, Leu-1, and Leu-3.

The relative proportions of Leu-2− and Leu-2-dim NK cells varied widely in
each of our patients. Leu-2-dim NK cells as a percent of total NK cells varied
from 24 to 79%, with a mean of 52% and a standard deviation of 18%.

Although the phenotype of these cells is consistent with that of NK cells
defined in normal blood (see discussion) the designation of these cells as “NK”
cells implies a functional definition. In order to confirm this, we performed
sorting experiments on cells from patient 151 at a time when these cells were
very numerous (days 30–40). In two experiments, we showed that all of the
ability to kill K-562 cells could be recovered in the Leu-11+ population. Sorting
of the cells into those that were Leu-11+ and either Leu-7+ or Leu-7− showed
that both the Leu-7+ and Leu-7− cells had equivalent ability to kill. Similarly,
sorting cells that were Leu-11+ and either Leu-2− or Leu-2-dim showed that
these two subtypes had equivalent killing activity (data not shown). Thus, these
cells were “NK” cells by functional criteria, and there were no apparent functional
distinctions detectable between the subtypes.

General Observations. In addition to defining the distinct cell types identified
above, our studies allow us to make several general statements concerning
recovering lymphoid cells after transplantation. The first concerns those antigens
whose expression we have found to be “variable”. These include HLA-DR, Leu-
7, Leu-15, Leu-8, and Leu-10. We have found that each of these antigens is
expressed on varying numbers of T cells during the posttransplant period. In
general, the expression of each of these markers is much higher in our transplant
patients than it is in normal volunteers. This is particularly true for Leu-15 and
HLA-DR. Further studies will be required to determine the relationships between
these various markers.

A second comment must be made about the appearance of Leu-6. Two of our
patients (153 and 155) had significant numbers of Leu-6-bearing T cells (2–4%).
Thus, although Leu-6-bearing T cells may be seen in some posttransplant
settings, they are not consistently present. The two patients who had detectable
numbers of Leu-6-bearing cells did not show detectable numbers of cells bearing
both Leu-2 and Leu-3.

Quantitative Analysis of Cell Types. Fig. 5 shows the numbers of each of the
cell types as a function of time after transplant for patients 151 and 155. The
quantitative data for the other two patients are similar. These data have several
features in common. Most striking is the very large numbers of NK cells that
appear very early. In patient 151 at day 27, they accounted for 93% of the
circulating lymphoid cells. In patient 155 at day 26, they accounted for 47% of
Figure 5. Recovery of lymphoid cells after transplantation. The time course of recovery of the major cell types is shown for the first 90 d after transplantation. (A), patient 151; (B), patient 155. (○), Th cells; (□), Ts cells; (●), conventional B cells; (■), Leu-1 B cells; (△), total NK cells. The recovery of all cell types was more rapid in patient 155, who received a transplant from an identical twin, but the general features of the cells were the same. In these figures, the two types of NK cells described in the text have been combined.

the lymphoid cells. This is compared with the level of these cells in normal blood, ranging from 5 to 15% (20, and our unpublished results). In addition, in each patient, Ts and Th cells appear early and in roughly comparable numbers. The identical twin, who received marrow that had not been T cell-depleted, showed higher numbers of T cells earlier than the patients receiving T cell-depleted marrow. In normal samples, Th cells outnumber Ts by ~2:1, but this is not the case in our posttransplant patients. Third, in three of the patients (151, 153, and 155), the appearance of B cells was delayed to near day 30, but they then rose rapidly and became a predominant cell type. Finally, the predominance of the Leu-1 B cell is striking in all three patients. Patient 151 first showed Leu-1 B cells at day 30. Conventional B cells appeared on day 46, but then decreased in numbers, while the Leu-1 B cells persisted. Patient 155 first showed Leu-1 B cells on day 26, and then beginning at day 67 showed a progressive increase of normal B cells.

Also shown on these figures are data from studies done on the normal donors for patients 151 and 155. The donor for patient 151 has normal proportions of NK, Th, and Ts cells. He appears to be somewhat unusual, in that he had equal
numbers of conventional B cells and Leu-1 B cells. The studies on this donor have been repeated twice, with nearly identical results, separated by a period of three months. Thus, these lymphoid cell phenotypes appear to be stable over time in the donor. The donor for patient 155 also had normal proportions of NK, Th, and Ts cells. The proportion of Leu-1 B cells in this donor was similar to what we have observed in other normal volunteers (1-2%).

**Screening for Chimerism.** Studies of DNA sequence polymorphisms in the donors and recipients have been carried out in each of the three cases involving nontwin donors. The results, done at several time points following transplantation, failed to reveal any evidence for lymphoid chimerism in patients 151 and 152. Patient 153 did show evidence for mixed lympho-hematopoietic chimerism at time points before the appearance of leukemic relapse. Since this method will detect residual host cells at levels as low as 1% (17), we can conclude that in two of our patients, all of the cell types we have identified have arisen from donor cells.

**Discussion**

The studies described here are unique in their use of multiparameter dual immunofluorescence analysis and genetic studies to obtain as complete a description as possible of the recovering lymphoid cell populations after transplantation. Previous work on the phenotype of recovering cells began with studies using only sheep erythrocyte rosetting to detect T cells. These studies showed that the absolute number and proportion of T cells was depressed for a period of 1-3 mo (21-26). More recently, several reports (26-31) have appeared showing that the early T cells have a markedly abnormal distribution with regard to “helper” and “suppressor” subsets. These results show that “helper” cells remain depressed, but suggest that “suppressor” cells are markedly increased, especially in patients with GVHD. Our results suggest that this may be due to confusion between Ts cells and Leu-2-bearing NK cells. Previous studies of B cell function have shown marked deficiencies in antibody production for a year or more following transplantation (21-25). Despite this, levels of phenotypic B cells returned to normal within 1-2 mo (21-24, 32). Our studies may shed some light on this issue by showing that a large proportion of the recovering B cells belong to an unusual phenotype that may not make normal antibody responses. Elevated levels of NK activity (33) and cells, as judged by HNK1 (Leu-7) labeling (34) have been reported in some transplant patients, especially those with chronic GVHD.

The major conclusions we have reached from these studies on four patients are that (a) for the six major lymphoid cell types identified, identical phenotypes of recovering cells were seen in each patient; (b) the patients differed only in the relative numbers of the various cell types as a function of time, with the identical twin transplant recipient making the fastest recovery to near normal proportions of cells; (c) the three patients receiving T cell–depleted marrow differed quantitatively but not qualitatively in the types and numbers of recovering T cells, when compared to the identical twin, who received untreated marrow; (d) T cells of the helper and suppressor phenotypes appear in nearly equal numbers after transplant; (e) NK cells are present in strikingly elevated numbers during the first 90 d after transplantation; (f) an unusual phenotype of B cell (the Leu-
1 B cell) was identified as a major cell type in all three of our patients who developed B cells.

The two patients who were most informative were 151 and 155. Both of these patients had uncomplicated posttransplant courses with no evidence for GVHD or overt infection. Thus, we have emphasized the data from these two patients. Our other two patients were more complicated in that one (152) developed GVHD, received intravenous anti-T cell mAb therapy, and subsequently died of interstitial pneumonitis; the other (153) died of recurrent leukemia. Nevertheless, these two patients are instructive because there were no striking differences in recovering lymphoid cell types observed in these patients, with the exception of the absence of B cells in patient 152. Thus, despite the small number of patients studied, we feel it is likely that the pattern of lymphoid recovery described here is relatively characteristic. Much more extensive studies will be necessary to determine whether quantitative variations in the phenotype of recovering lymphocytes offer insight into posttransplant complications. Deviations from the "normal" pattern of reconstitution described here may indicate early or subclinical GVHD or viral infection. In addition, failure to observe a normal pattern of lymphoid reconstitution may be indicative of partial failure of lymphoid engraftment, or of abnormalities of lymphocyte differentiation in some recipients.

Several of the cell types we have observed deserve special comment. All of the T cells in our patients, both Th and Ts, displayed varying amounts of HLA-DR, a surface structure thought to represent activation of T cells (35). In addition, they displayed variable amounts of several other antigens, including Leu-7, Leu-8, Leu-10, and Leu-15. The significance of this fluctuating surface display is unknown. It will be important in the future to establish the correlations between these various antigens, which we have not done here. They may all reflect various states of activation or stages of differentiation of the same cells, or some of them may define unique functional subsets, as has been proposed for Leu-15 (12). It is clear that in this setting, Leu-7, which in normal individuals is largely confined to non-T NK cells, can be variably expressed on conventional T cells in transplant recipients.

We failed to detect any significant number of T cells that fit cleanly into the category of thymic cells. Only two of our patients showed significant numbers of Leu-6-bearing cells, and none had detectable numbers of cells bearing both Leu-2 and Leu-3. There have been previous reports (36) confirming the presence of cells expressing T6 (similar to Leu-6); however, the absence of both Leu-2 and Leu-3 on these cells raises a question as to whether they truly conform to the phenotype of early thymocytes. Thus, it seems that if the T cell population is recapitulating thymic differentiation posttransplantation, the characteristic thymic cells are not circulating.

The two major types of NK cells we have identified here, distinguished by their expression of Leu-2, are also seen in varying numbers in normal volunteers. It may be possible to further subdivide NK cells according to their expression of Leu-7, but, since the expression of Leu-7 appeared to be variable on the T cells, we chose not to use it to define further subtypes of NK cells. The only marker we have used that appears to be unique to NK cells is Leu-11, in that we did not
observe Leu-11 on T cells at any time. The elegant three-marker studies of Lanier and Loken (20), using Leu-11, Leu-2, and Leu-7, defined several subsets of NK cells in normal blood. A review of their data shows that, in three normal individuals, the mean percent of Leu-11+ NK cells was 9.0%. Of these, only 0.5% also expressed dim Leu-2. Thus, each of our patients not only had markedly elevated numbers of total NK cells, but also had a marked disproportionate increase in Leu-2-dim NK cells. Since the Leu-2 molecule can be released in soluble form (37) it remains to be established whether these cells are synthesizing their surface Leu-2 or have passively acquired it. Preliminary data in our laboratory suggests that, when cultured in the absence of Leu-2-bearing T cells, these cells continue to express low levels of Leu-2 for at least 3 d, suggesting that they are indeed synthesizing the molecule.

Previous single-parameter studies of post-marrow transplant patients have noted a very large number of circulating Leu-2- or T8-bearing cells, which were identified as Ts cells (26-31, 38). Our results suggest that the inclusion of the Leu-2-dim population, which is clearly distinct from Ts cells, may account for these reports. The inclusion of variable numbers of these Leu-2-dim cells may also significantly alter T4/T8 ratios calculated from single-parameter studies. Interestingly, one report (39) of patients with decreased numbers of "suppressor" T cells was carried out with a different marker (TH2). This discrepancy may be explained if the TH2 antibody does not label the Leu-2-bearing NK cells (see also 31).

The presence of large numbers of NK cells in the posttransplant setting raises the important question of their possible effect on hematopoiesis. There is evidence in both mice and man that NK cells interact with hematopoietic progenitor cells, and thus may have a regulatory role. Hansson et al. (40) have reported that density gradient-enriched human large granular lymphocytes, which contain NK activity, are capable of inhibiting granulocyte colony-forming units, but have no effect on the development of erythroid burst-forming activity. A similar result was recently reported (41) showing an interaction with erythroid colony-forming units. We have recently reported (42) an inhibitory interaction between murine NK cells and pluripotent marrow progenitors. A recent report (43) of NK cells bearing both Leu-2 and Leu-15 and mediating suppression of B cell differentiation adds the possibility that these cells may be responsible for some of the B cell defects described above. Our patients have had no evidence for defective marrow engraftment, despite the large numbers of circulating NK cells. Thus, if there is such a regulatory interaction, it is clearly a more complicated issue, requiring further study.

The final striking finding in these studies was the presence of large numbers of dim Leu-1-bearing B cells in all three of our patients who developed B cells. These cells were characterized in our patients by the presence of relatively large amounts of surface IgM and lower amounts of surface IgD. This is in agreement with an earlier report (52) of B cells with large amounts of IgM posttransplant. This phenotype of Leu-1-bearing B cells is also found in chronic lymphocytic leukemia; however, examination of these cells from one of our patients failed to detect evidence for clonal somatic Ig gene rearrangements, and suggests that these cells in our patients are polyclonal in origin.
There are several reports of similar cells in mice and man. In mice, the analogous cell type is the Lyt-1 B cell (44, 45). These cells have been described in some murine lymphomas, in NZB mice, and in small numbers in normal murine spleens. There is data implicating these cells in the regulation of Ig production by other B cells, and in the production of autoantibodies. They have been shown to express relatively large amounts of surface IgM, and to be responsible for the high levels of spontaneous IgM secretion seen in the autoimmune NZB mice. In man, the Leu-1 B cell has been described in small numbers in normal individuals (46), as well as being the characteristic phenotype of chronic lymphocytic leukemia. We have recently observed large numbers of these cells in human fetal spleens (J. H. Antin and K. A. Ault, unpublished observations). The role of these cells in the human immune system is still unknown, and their significance in the setting of marrow transplantation will be of considerable interest in future investigations.

Summary

Four patients who received bone marrow transplants were studied sequentially during the posttransplant period to define the pattern of recovering lymphoid cell types. Three patients received T cell-depleted, HLA-matched marrow, and one received untreated marrow from an identical twin. Blood lymphoid cells were labeled with 25 different pairs of monoclonal antibodies. In each sample, one antibody was conjugated to fluorescein and one to phycoerythrin, thus allowing simultaneous assessment of the expression of the two markers using the fluorescence activated cell sorter. A total of 14 antibodies were used, routinely including HLE, Leu-M3, Leu-4, Leu-1, Leu-5, Leu-6, Leu-2, Leu-3, HLA-DR, Leu-7, Leu-11, Leu-15, and Leu-12. Other antibodies were used to further define some populations.

This study has allowed us to define six distinct cell types that have appeared in all four patients by day 90 posttransplantation, and which account for 90–100% of all circulating lymphoid cells. These cell types are (a) T helper cells expressing Leu-1, Leu-4, Leu-9, Leu-5, Leu-3, and variable amounts of HLA-DR; (b) T suppressor cells expressing Leu-1, Leu-4, Leu-9, Leu-5, Leu-2, and variable amounts of HLA-DR; (c) B cells expressing Leu-12, B1, HLA-DR, IgD, and IgM, but none of the T cell antigens; (d) an unusual B cell phenotype (Leu-1 B) expressing all of the B cell markers, and also having low amounts of Leu-1, but none of the other T cell antigens; (e) natural killer (NK) cells expressing Leu-11, Leu-15, Leu-5 but none of the other T cell or B cell markers; (f) NK cells expressing Leu-11, Leu-15, Leu-5, and low levels of Leu-2. Both NK types also express Leu-7 on some, but not all cells.

The relative frequencies of these cell types varied among the patients and with time, but the striking findings were the presence of relatively few mature T cells, large numbers of NK cells, and the preponderance of the unusual Leu-1 B cell over conventional B cells in all three patients who developed B cells. Sorting experiments confirmed the NK activity of the major NK cell phenotypes, and DNA analysis confirmed that all of the cells studied were of donor origin. In addition, analysis of Ig genes in one patient showed that the Leu-1 B cells were not clonally rearranged.
These dual-parameter immunofluorescence analyses clearly define the pattern of lymphoid reconstitution in these patients, and reveal previously unsuspected cell types. They also suggest that depletion of T cells from donor marrow does not drastically alter the pattern of recovery of the immune system.

We thank the Becton Dickinson Monoclonal Antibody Center, and particularly Dr. Noel Warner for contributions of mAb and many helpful discussions. Dr. P. Leder provided the Ig gene probes. We also acknowledge the help of the following persons involved in our marrow transplant program: Dr. David G. Nathan, Karen Baker, R. N., Dr. Steven J. Burakoff, Dr. Robert Handin, Dr. Fred S. Rosen, Dr. Jeffery Lipton, and the house staff and nurses of the Brigham and Women's Hospital and Children's Hospital Medical Center.

Received for publication 24 September 1984

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