We evaluated T-cell-mediated lymphokine-activated killer (LAK) function during the late (greater than 5 months) reconstitution phase after T cell-depleted allogeneic bone marrow transplantation (BMT) for hematologic malignancy. Since LAK cells are sustained by interleukin-2 (IL-2), we also investigated the ability of post-BMT T cells to produce IL-2. These functions were investigated at the clonal level. More than 200 T-cell clones from six long-term BMT recipients were generated and compared with 60 T-cell clones derived from two normal controls. Almost all the CD8+ clonal cultures from BMT recipients expressed cytolytic activity in a lectin-dependent cellular cytotoxicity assay. Interestingly, a higher proportion of BMT recipient-derived cytolytic clones were able to mediate LAK activity in comparison with control clones (28% versus 4%, P < .05). However, T-cell clones from BMT recipients, as opposed to control clones, were largely incapable of producing IL-2. Given the high proportions of post-BMT circulating CD8+ T cells, it appears that, in long-term BMT recipients, the precursors of nonspecific LAK effectors are present at above normal levels. However, their function may be defective in vivo due to poor IL-2 production.

The IMMUNE reconstitution phase that follows allogeneic bone marrow transplantation (BMT) is associated with a persistent deficiency of cellular and humoral immune responses. This results in increased susceptibility to infection and may also determine a defective control of residual leukemic cells that survive the pretransplant conditioning therapy leading to leukemia relapse.

A wave of CD3- cells with granular lymphocyte morphology that are capable of mediating natural killer (NK) and lymphokine-activated killer (LAK) functions has been demonstrated as early as 3 weeks post-grafting. In addition, CD3- clones with anti-leukemia activity have been generated from BMT patients. It has, therefore, been suggested that CD3- cytotoxic cells may control the occurrence of leukemia relapses after BMT. However, the anti-tumor cytotoxicity mediated by T-cell-derived LAK effectors has not been investigated in BMT recipients. T cells are detected in sizeable proportions 2 to 3 months after BMT, but abnormal T-cell phenotypes and functions persist for a prolonged period of time. T-cell abnormalities comprise inverted CD4/CD8 ratios and defects in the proliferative response to mitogens or allogeneic cells. Helper function for B-cell differentiation, cytotoxic T-lymphocyte (CTL) response, and low interleukin-2 (IL-2) production.

We have recently reported that, after T-cell-depleted allogeneic BMT for hematologic malignancy, there is a predominance of T cells expressing NK-related antigens, such as CD57 (Leu 7) and CD11b, within both the T-helper and the T-cytotoxic cell subsets.

In normal blood, the vast majority of cytotoxic cell precursors display a CD8+ phenotype. Among CD8+ cells, only those lymphocytes expressing CD57 and CD11b respond to IL-2 by acquiring nonspecific cytotoxic (LAK) functions against tumor targets. In view of the aforementioned putative role of CD3- cytotoxic cells for the control of leukemia relapse after BMT, we have investigated the cytotoxic function and the production of IL-2 by T cells from six BMT recipients in which co-expression of CD57 and CD11b on the circulating CD8+ T cells was increased to 100%. Mononuclear cell samples were cloned in a high efficiency T-cell cloning system, and the cytolytic functions of more than 200 T-cell clones were subsequently determined. Almost all of the CD8+ clonal cultures from all patients expressed cytolytic activity in a lectin-dependent cellular cytotoxicity (LDCC) assay. Interestingly, a higher proportion of these clones, as compared with control clones, was able to mediate LAK functions. A low production of IL-2 was consistently observed for BMT recipient-derived clones. This suggests that, in spite of the high frequency of LAK precursors with a T-cell phenotype, the cytotoxic function of these cells may be defective in vivo.

**MATERIALS AND METHODS**

**Patients and controls.** Twenty subjects undergoing BMT for hematologic malignancy (4 with acute leukemia, 4 with chronic myelogenous leukemia, 1 with non-Hodgkin’s lymphoma, and 1 with multiple myeloma) were included in this study, after informed consent. Patients were prepared for transplantation using antithymocyte horse immunoglobulin (Merieux, Paris, France) and procarbazine, followed by hyperfractionated total body irradiation and cyclophosphamide. The marrow donors were HLA-identical, mixed lymphocyte culture-compatible adult individuals (age greater than 20 years). Control subjects in this study were either marrow donors or normal individuals age-matched to the donors.

**Marrow graft preparation.** To prevent graft-versus-host disease (GVHD), the marrow transplants were depleted of T cells by sequential soybean agglutination and sheep red blood cell rosetting, according to a procedure described by Reizner et al.

**Posttransplant follow-up.** Engraftment was documented by blood group change or by cytogenetic analysis of sex marker conversion, or disappearance of Ph chromosome. Post-BMT patients selected for this study were all successfully engrafted. No graft failure was observed in the subsequent follow-up of these patients.
Marrow graft recipients received no immunosuppressive therapy for GVHD prophylaxis in the posttransplant period. Only 1 of the 20 patients developed grade I acute GVHD, while none had chronic GVHD.

**Immunofluorescence.** Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation, and partially depleted of monocytes by adherence to plastic dishes. The CD4+ and CD8- cell subsets were enumerated and analyzed for co-expression of CD57 and CD11b antigens by two-color immunofluorescence. Fluorescein-conjugated Leu3 (anti-CD4) or Leu2 (anti-CD8) mouse monoclonal antibodies were used in combination with unconjugated Leu7 (anti-CD57) or Leu15 (anti-CD11b) (Becton Dickinson, Mountain View, CA) developed with rhodamine-conjugated goat anti-mouse IgM or IgG2a antibodies, respectively (Southern Biotechnology Associates, Birmingham, AL). Wet preparations of viable cells were examined by fluorescence microscopy. Co-expression of two markers on the same cell was evaluated by scoring at least 200 cells positive for one marker and determining the percentage of cells also expressing the other marker.

Clonal cultures (see below) were analyzed for their CD3+, CD4+, CD8+, CD56+, and CD16+ phenotypes by indirect immunofluorescence. Leu4 (anti-CD3), Leu19 (anti-CD56) and Leu11a (anti-CD11a) monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA); CK.79 (anti-CD4) was obtained in our laboratories, as previously described, and the B9.4 (anti-CD8) monoclonal antibody was kindly donated by Dr B. Malissen (INSERM, Marseille-Luminy, France). Fluorescein-conjugated goat anti-mouse antibodies (Southern Biotechnology Associates) were used as secondary reagents. Analyses of the expression of CD57 and CD11b antigens were not performed in clonal cultures because our previous studies at the clonal level have shown that both antigens may be lost over a relatively short culture period.

**T-cell cloning.** A preliminary survey of 20 BMT recipients revealed that co-expression of CD57 and CD11b antigens on circulating CD4+ and CD8+ cells ranged from 40% to 85% on CD4+ cells and from 70% to 100% on CD8+ cells (see also reference 16). This is in contrast to co-expression of CD57/CD11b on T cells from normal subjects, which is ≤5% on CD4+ cells and 20% to 40% on CD8+ cells. Six samples (from six individual patients) representative of the observed range of NK-antigen expression were selected for clonal experiments and subsequent functional analyses of the clonal progenies. Mononuclear cells forming rosettes with sheep red blood cells (SRBC) were purified by Ficoll-Hypaque density gradient centrifugation, followed by 0.83% ammonium chloride osmotic lysis of SRBC. E-rosette forming cells were plated under limiting dilution and activated with phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI) for 24 hours before addition of IL-2. This system allows clonal expansion of up to 100% of the peripheral blood T cells. T cells were plated (from 1 to 0.25 cells per well for control cultures and from 0.5 to 0.5 cells per well for BMT recipients' cultures) in groups of 48 microcultures in U-bottom microwells containing 105 irradiated (50 Gy) spleen feeder cells in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FCS) and 0.5% PHA. Recombinant IL-2 (generously provided by the Glaxo Institute for Molecular Biology S.A., Geneva, Switzerland) was added at a final concentration of 25 U/mL after 24 hours. Plates were incubated at 37°C in a humidified atmosphere with 5% CO2. Microcultures were supplemented on day 7 with 105 irradiated feeder cells and IL-2. IL-2 was further added on day 14. Control wells containing irradiated feeder cells, PHA and IL-2 without addition of responder cells. After 14 to 18 days of culture, each microwell was assessed microscopically for cell growth. Immunofluorescence analysis of 50 arbitrarily selected clones revealed that they were all CD3+. Frequencies of proliferating cells were then calculated by the χ2 method from the Poisson distribution relationship between the number of E+ cells plated per culture and the logarithm of the percentage of non-proliferating cultures. In the present experiments, cloning efficiency was ~50% for control cultures and ~10% for patients' cultures. According to the Poisson formulae, if the cloning efficiency is 100%, positive cultures growing from 0.25 (or less) T cells plated per well are highly likely to be clonal. Therefore, growing microcultures were considered clonal when they were derived from 0.5 (or less) T cells plated per well in the case of control cultures and 2 (or less) T cells plated per well in the case of patients' cultures. Only these cultures were used for functional and phenotypic analyses and are referred to as "clones" throughout this article. In addition, those exceptional cultures exhibiting mixed CD4+ /CD8+ cell populations were excluded from the analysis.

**Assays for the cytolytic activities.** The cytolytic activity of the clones was detected in an LDCC assay as described elsewhere. This assay allows detection of all cytotoxic cells, irrespective of their specificities. Briefly, clonal microcultures were counted and the cell counts were adjusted at 1 x 104/mL. Fifty microliter aliquots (containing 5 x 104 cells) of each growing microculture were incubated in V-bottomed wells of microtiter trays with 5 x 104 [3H]-labeled P815 cells with 1% (vol/vol) PHA. Plates were centrifuged at 100 g for 2 minutes and were incubated for 4 hours at 37°C. Target cell lysis was determined by counting the radioactivity of 0.1 mL supernatants, and the specific lysis was calculated according to the formula:

\[
\% \text{ Specific lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100
\]

Maximum release was obtained by lysing [3H]-labeled cells with 1 N HCl. Spontaneous release from [3H]-labeled target cells was determined by incubating these cells in culture medium in the absence of effector cells. Cultures in which [3H]-release exceeded the mean spontaneous release by more than 3 SD were considered positive for cytolytic activity.

Assessment of LAK function of the clones was performed using [3H]-labeled fresh melanoma cells as target cells in the [3H]-release assay described above.

**Assay for IL-2 production.** Clonal microcultures were washed several times and were resuspended at a cell concentration of 1 x 105/mL in 200 μL culture medium containing 1% (vol/vol) PHA. Supernatants were removed after 24 hours for determination of IL-2 activity.

As an indicator system we used the IL-2-dependent mouse CTLL cell line. Clonal supernatants were added to 5 x 105 indicator cells at a final concentration of 25%. IL-2 activity was assessed by [3H]-thymidine uptake of CTLL cells after a 6-hour pulse at the end of a 24-hour culture period.

Calculation of the IL-2 produced by T-cell clones was based on a standard source of recombinant IL-2 that contained a known amount of international standard units.

**RESULTS**

**Cloning of T cells from BMT recipients.** Circulating CD4+ and CD8+ T cells from 20 long-term (greater than 5 months) BMT recipients were analyzed by two-color immunofluorescence for co-expression of the NK-related antigens, CD57 and CD11b. Six samples representative of the observed range of NK-antigen expression on CD4+ and CD8+ cells were selected for cloning under limiting dilution conditions, and for the determination of cytolytic function and IL-2 production at the clonal level. As shown in Table 1,
or CD11b. Data refer to peak values determined during follow-up (see "Materials and Methods"). The purpose of the subsequent experiments was to determine the effects of such phenotypic imbalances on the CD4:CD8 ratios detected in the original peripheral blood lymphocyte (PBL) preparations used for the cloning experiments; ie, CD4:CD8 = 1.4:1.2 in normal controls. Differences between patients and controls were highly significant (P < .001 by Student’s t test) in all cases.

Freshly isolated T cells from these samples co-expressed CD57 and CD11b in very high, although variable, proportions; ie, 40% to 87% for CD4+ cells and 70% to 98% for CD8+ cells. The purpose of the subsequent experiments was to determine the effects of such phenotypic imbalances on the cytolytic potential of either the CD4+ or CD8+ subset. To this end, mononuclear cells were plated under limiting dilution conditions and activated with PHA for 24 hours before addition of IL-2. Frequencies of proliferating T cells were then calculated.

The frequency of post-grafting T cells undergoing clonal expansion in this system was lower than that of control T cells (1 in 10 versus 1 in 2, as calculated by the Poisson formula). A total of 209 clones from six BMT recipients and 62 clones from two control subjects were examined (see "Materials and Methods" for selection procedure and definition of clonality). Immunofluorescence analyses performed on separate aliquots of each growing microculture (between days 18 and 21) demonstrated that 29% of the BMT recipient-derived clones were CD4+ and 71% were CD8+. Of the 62 control clones examined, 56.3% were CD4+ and 43.5% were CD8+. This distribution closely reflected the CD4/CD8 ratios detected in the original peripheral blood lymphocyte (PBL) preparations used for the cloning experiments; ie, CD4:CD8 = 1.4 to 1.2 in normal control subjects and CD4:CD8 = 0.5 to 0.3 in BMT recipients. This indicates that the relatively low cloning efficiency of BMT recipients' T cells, as opposed to that of control cells, was not a consequence of a selective inability of a given T-cell subset to give rise to clonal progenies in our system. This is also suggested by the identical cloning efficiency of CD8+ cells from the one sample in which the virtual totality of CD8+ cells co-expressed CD57 and CD11b compared with that of other BMT recipient-derived CD4+ or CD8+ cell populations not co-expressing NK-related antigens at the same rate (see "Discussion").

**LDCC of BMT recipient-derived T-cell clones.** One aliquot (50 μL) of each clonal microculture (5 x 10⁶ cells) was removed on day 18 of culture to test the cytolytic activity in an LDCC.

As shown in Table 2, the cytolytic potential of BMT recipient-derived clones was largely a property of CD8+ clonal cultures, whereas approximately 1/3 of the CD4+ clones exhibited cytolytic activity. Ninety-six percent of the CD8+ cells derived from either control CD8+ cells (therefore largely NK antigen-negative) or patients' CD8+ cells (predominantly CD57+/CD11b+) exhibited a marked cytolytic activity in the LDCC assay.

**LAK function of BMT recipient-derived T-cell clones.** Of BMT recipient-derived clones, 87 of 209, and 45 of 62 control clones, yielded enough cells to allow further evaluation of their LAK activity. These clones were tested against [¹¹Cr]-labeled fresh melanoma cells. In Fig 1 the LAK function of BMT recipient-derived clones (abscissa) is compared with the LDCC activity (ordinate). Interestingly, as many as 28% of post-BMT cytolytic clones also exhibited LAK activity as opposed to the exceptional occurrence of clones with this function among those generated from normal donors (Fig 1 and Table 2).

**IL-2 production after BMT.** In order to evaluate their ability to produce IL-2, clonal microcultures (containing 4 x 10⁴ cells) were stimulated with PHA for 24 hours. The culture supernatants were then harvested and tested for IL-2 production. Of control CD4+ and CD8+ clones, 63% and 41% produced detectable amounts of IL-2. In contrast, 15% and 7% of BMT recipient-derived CD4+ and CD8+ clones, respectively, produced IL-2 (Fig 2). These results indicate that clonable T cells in the late reconstitution phase after BMT contain fewer cells than normal that are capable of giving rise to IL-2 producing clones.

### DISCUSSION

This study was aimed at the definition of cytolytic functions of clonable T cells from T-cell-depleted BMT recipients in a high efficiency cloning system. A lower cloning efficiency was observed in BMT recipients as compared with normal controls. Phenotypic analyses of the clones closely reflected the inverted CD4+CD8+ ratios detected after BMT. In contrast to control clones, the proportions of IL-2–producing clones were low, irrespective of their CD4+ or CD8+ phenotypes. On the other hand, virtually 100% of the CD8+ clones were cytolytic when tested in an LDCC assay. Similar analyses were performed previously by Daley et al, who have shown an overall reduction of proliferating T-cell

<table>
<thead>
<tr>
<th>Table 1. Phenotypic Analyses of BMT Recipient-Derived CD4+ and CD8+ Cells Used in the Cloning Experiments</th>
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<tbody>
<tr>
<td>Cell Phenotypes*</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>CD4+ co-expressing</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>CD4+ co-expressing†</td>
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Phenotypes determined by two-color immunofluorescence (described in "Materials and Methods").

*Range of percentages of CD4+ or CD8+ cells also expressing CD57 or CD11b. Data refer to peak values determined during follow-up (see "Results"). The CD4:CD8 ratio in these patients was 0.5:0.3 versus 1.4:1.2 in normal controls.

†Less than 1% of these cells also expressed CD16 or CD56.

‡Differences between patients and controls were highly significant (P < .001 by Student’s t test) in all cases.

### Table 2. Functional Evaluation of BMT Recipient-Derived T-Cell Clones

<table>
<thead>
<tr>
<th>Proportion of Cytolytic Clones</th>
<th>Patients (n = 6)</th>
<th>Controls (n = 2)</th>
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</thead>
<tbody>
<tr>
<td>Defined by LDCC</td>
<td>209</td>
<td>62</td>
</tr>
<tr>
<td>Expressing LAK function</td>
<td>87*</td>
<td>45*</td>
</tr>
</tbody>
</table>

IL-2 producing clones 153* 16 7 62 63† 41†

*Of 209 clones derived from six long-term BMT recipients, 87 clones and 45 of 62 control clones were available for analysis of LAK function. Shown are the proportions of cytolytic clones, as defined by LDCC, also exhibiting LAK function (see also Fig 2). Tested for IL-2 production were 153 clones derived from BMT recipients and 62 control clones. The lower number of clones tested for LAK function and IL-2 production is due to availability of cell numbers suitable for these functional studies.

†P < .05.
Fig 1. Comparison of CTL (ordinate) and LAK functions (abscissa) of control (A) versus BMT recipient-derived T-cell clones (B). CTL activity was measured by LDCC as indicated in "Materials and Methods," and LAK activity was evaluated against fresh melanoma cells. Eighty-four of a total of 209 clones derived from six long-term BMT recipients and 45 of 62 control clones were available in cell numbers suitable for this analysis. Analogous to control clones, the vast majority of CD8+ T-cell clones from long-term BMT recipients exhibited CTL function. A higher proportion of BMT recipient-derived cytolytic clones, as compared with control clones, also mediated LAK activity (28% versus 4%) (P < .05).

Fig 2. Defective IL-2 production by BMT recipient-derived T-cell clones. Of a total of 209, 153 clones derived from six long-term BMT recipients (B) and 62 of 62 control clones (A) were available in cell numbers suitable for this analysis. Clonal microcultures (containing 4 x 10^6 cells) were stimulated with PHA for 24 hours. The culture supernatants were then harvested and tested for IL-2 production. Of control CD4+ (○) and CD8+ (●) clones, 83% and 41%, respectively, produced detectable amounts of IL-2. In contrast, 15% and 7% of BMT recipient-derived CD4+ (○) and CD8+ (●) clones, respectively, produced IL-2 (P < .05).
level as long as the first post-grafting year. The anti-tumor cytolytic function of these cells in BMT recipients has not been investigated thus far. Moreover, in view of this persistant CD8+/CD11b- lymphocytosis as opposed to the transient elevation of CD3- cells, it is conceivable that CD3+/CD8+ cells could account for a major part of the overall LAK precursors in the post-BMT phase.

In this study we show that increased proportions of BMT recipient-derived cytolytic T-cell clones, as compared with control clones, expressed LAK function in an IL-2-dependent in vitro system and that all of them had the CD8+ phenotype. Since post-grafting CD8+ T cells co-expressed CD57 and CD11b in very high proportions (70% to 98%), as compared with control CD8+ cells (20% to 30%), it would seem that CD3+ LAK precursors are preferentially contained within the CD57+/CD11b+ (rather than the CD57-/CD11b-) subset of CD8+ T cells also in BMT recipients. Given the relatively low cloning efficiency of post-BMT T cells, it could be argued that the minor T-cell subset not expressing CD57/CD11b could give rise to the bulk of the observed clones, and, consequently, the clonal progenies might not necessarily reflect the original distribution of CD57+/CD11b+ versus CD57-/CD11b- cells within the CD4+ or CD8+ subset. However, the cloning efficiency and the cytolytic pattern observed in the CD8+ cells from a patient in which the virtual totality of CD8+ cells co-expressed CD57 and CD11b were comparable with those observed in the other CD8+ samples, which were greater than 70% CD57+/CD11b+. These findings would indicate that the data were not the result of an outgrowth of a minor (NK antigen-negative) cell population.

We also show that the proportion of clonable T cells capable of producing IL-2 is extremely low in long-term BMT recipients (irrespective of their CD4+/CD8+ phenotype). It follows that LAK-mediated defences against residual leukaemia or relapse may be impaired in long-term BMT recipients, although LAK precursor cells either with CD3--4+ or CD3+ phenotype (present data) are found in normal or even increased numbers.

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Cytolytic function of clonable T cells after human bone marrow transplantation

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