Stable Clonal Expansion of T Cells Induced by Bone Marrow Transplantation


The immune mechanisms of T cells regeneration after bone marrow transplantation (BMT) and the factors maintaining allogeneic marrow graft in the host are still unknown. To pursue this issue, we analyzed T-cell clonality of peripheral blood lymphocytes (PBLs) in BMT recipients, using reverse transcription polymerase chain reaction with T-cell receptor (TCR) Vβ gene segment-specific primers and single-strand conformation polymorphism. PBLs from patients and donors showed a heterogeneous T-cell population with oligoclonal accumulations of CD8+ T cells. When PBLs were cultured in HLA-matched mixed lymphocytes reaction in vitro, no distinct clonal expansion was observed. However, after BMT, oligoclonal expansions were induced in the recipients in vivo, without a restriction of TCR Vβ gene usage. Although part of the expansion was transient, the majority was repeatedly detected even several months later. Our results suggested that certain in vivo mechanisms maintain a stable clonal expansion of distinct T cells in marrow recipients. We also found in a single patient with graft-versus-host disease a replacement of expanded clones by other clones during follow-up. Diminishing numbers of accumulation clones were found in long-term marrow recipients, indicating a general tendency for clonal expansion to subside progressively. Considered together, our data suggest the involvement of clonally expanded T cells in lymphoid regeneration and in acute and chronic immune responses after BMT.

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MATERIALS AND METHODS

Patients. We studied 16 patients who had undergone allogeneic BMT at Tokai University Medical School Hospital from January 1987 through July 1992 (Tables 1 and 2). They were treated with total body irradiation (TBI) or thoracoabdominal irradiation (TAI) and/or immunosuppressive agents, eg, melphalan (L-PAM) or VP-16, just before BMT. All patients received cyclosporin-A (CsA); in some cases, this was followed by prednisolone after BMT as a prophylaxis against GVHD. Supportive treatment in the form of antibiotics or granulocyte colony-stimulating factor was provided on an individual basis when necessary.

Preparation of mononuclear cells. Heparinized blood samples were obtained from patients and healthy donors with informed consent before and after BMT. Peripheral blood mononuclear cells (PBMCs) were prepared by standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation. A proportion of PBMCs was immediately used for RNA isolation, while the rest was cultured as described below. In some experiments, cells were separated into CD4+ or CD8+ subsets by positive selection using antibody-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway) according to the instructions provided by the manufacturer.

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Peripheral T-cell clonality in BMT recipients and donors was analyzed with the PCR-SSCP method. In this system, heterogeneous T-cell populations with diverse CDR3 sequences exhibit a smear-like pattern, whereas a single T-cell clone forms a single band. Thus, a distinct clonotype accumulating within a heterogeneous background can be distinguished as a band on a smear. Based on a DNA sequencing study, we also confirmed that bands migrating to the same position on the same SSCP gel had sequences identical to the TCR CDR3 region, indicating that they represent identical clones.

The PBLs of healthy donors consisted of heterogeneous T cells with several accumulation clones (representative examples are shown in Fig 1A, lower panel). This finding confirmed our previous report on healthy individuals. On the other hand, PBLs of patients with hematologic disorders showed two clonal patterns. The majority of patients, eg, patients KI and DN, had a smear or diverse pattern similar to that observed in healthy individuals. However, a small group of patients, eg, patient HM, showed two clonal accumulations (Fig 1A, upper panel). In either case, most of the accumulation clones were found to be CD8+ T-cell subset.

In vitro stimulation. Mononuclear cells were suspended in RPMI containing 20% human pool serum and stimulated for 7 days by antigens of candida (at 0.05%; Torii Pharmaceutical Co Ltd, Tokyo, Japan), Varicella zoster (at 1:40 dilution), and cytomegalovirus (1:40; a kind gift from Prof K. Shiraki, Toyama Medical and Pharmaceutical University, Toyama, Japan).

**RNA isolation, cDNA synthesis, and PCR-SSCP analysis.** Total RNA was isolated from cells according to the AGPC method described by Chomczynski et al. RNA was converted to first strand cDNA using reverse transcriptase (Superscript; BRL, Gaithersburg, MD) and random hexamer oligonucleotide priming (100 pmol; BRL) at 42°C for 2 hours. PCR-SSCP using TCR primers was performed as described elsewhere, with minor modifications. Briefly, cDNA was mixed with each primer set (a Vβ sense primer and nonbiotinylated Cβ antisense primer; sequences were as previously described). PCR reaction was performed using dNTP (Takara Shuzo Co, Shiga, Japan) and Taq DNA polymerase (Promega Co, Madison, WI) for 35 cycles (94°C for 1.5 minutes, 60°C for 2 minutes, and 72°C for 3 minutes). Amplified DNA was diluted (1:20), heat-denatured, and then electrophoresed in nondenaturing 4% polyacrylamide gels. In the next step, the DNA was transferred to Immobilon-S (Millipore Intertechn, Bedford, MA). After prehybridization, internal probe (5'-A(A,C)AA(G,C)GTGTCCACCCGAGGCTCGTGTGTT-3') was added to the membrane filter and then hybridized at 42°C for 8 hours, washed, and finally exposed to x-ray films.

**RESULTS**

Peripheral T-cell clonality in patients with hematologic disorders before BMT and in HLA-identical healthy donors.
expansion (Fig 2A and B, upper panel). On the other hand, in vitro stimulation by known viral and microbial antigens induced a marked expansion of a number of T-cell clonotypes (Fig 2A and B, lower panel). Thus, these results indicated that PBLs from both the recipients and donors responded to exogenous antigens and that, at least in HLA-matched cases, the use of MLR in vitro may not provide sufficient stimulation necessary to produce clonal expansion detectable by our RTPCR-SSCP system.

Changes in T-cell clonality caused by allogenic BMT. In the next step, we performed serial analysis of posttransplant PBLs from bone marrow recipients (Table 2) to examine the influence of transplantation on peripheral T-cell clonality. There was a marked increase in the number of clonal accumulations soon after BMT (representative examples are shown in Fig 3A through C). BMT resulted in an increase in the number of bands on SSCP, ie, the accumulation clones, compared with those before BMT. This finding was observed in almost all tested Vβ families. Electrophoretic analysis on the same SSCP gel showed that the posttransplant bands were different from clones before BMT (Fig 4A and B and data not shown). Thus, BMT produced oligoclonal accumulation of T cells in the periphery.

Because MLR is often used to estimate the immune response occurring after BMT, we compared the results of MLR with those of in vivo responses. As described previously, only few clonal expansions were detected in HLA-matched MLR (eg, patient KI, Fig 2A). Nevertheless, a number of clonal accumulations were found in vivo after BMT that were not identical to the in vitro analysis (Fig 3B). Thus, there was a discrepancy between in vivo and in vitro T-cell responses at a clonal level.

Evidence for BMT-induced stable clonal expansions of T cells. The kinetics of clonotype expansion in BMT recipi-
Fig 1. Peripheral T-cell clonality in patients with hematologic disorders and in healthy donors. (A) PBLs were analyzed for clonality using the RT/PCR-SSCP method. Lanes indicate, from left to right of each block, Vβ 1-20 subfamilies. Results from patients and donors are displayed in lines (top, recipients; bottom, donors). (B) Separation and analysis of recipients PBLs into CD4+ and CD8+ subpopulations.
Fig 2. Antigenic stimulation induced distinct clonal accumulations. (A) PBLs from patient KI were cultured with indicated stimuli and analyzed with RT/PCR-SSCP (see legend in Fig 1). (B) PBLs from a healthy donor (MN, HLA-identical to patient MN) were treated in a manner similar to that described in (A). CMV, cytomegalovirus; VZ, varicella zoster virus; Cad, candida.
Changes in peripheral T-cell clonality in allogeneic BMT recipients. (A, B, and C) PBL samples from three recipients were analyzed sequentially with RT-PCR-SSCP. Pre, samples obtained before BMT. Numbers indicate the date of sampling (refer to Table 2). Donor, donor’s response to the recipient’s cells in MLR. See legend to Fig 1.

Our results showed an additional change of clonality between 50 and 113 days after transplantation, ie, around the occurrence of GVHD. In this case, the previously expanded clones disappeared, whereas different clones started to emerge. These clones were found to remain stable afterwards.
Peripheral T-cell clonality tends to be heterogeneous in long-term marrow recipients. To further explore the fate of accumulating T-cell clonotypes, we analyzed PBLs from 9 recipients who had undergone BMT approximately 1.5 to 6.0 years before the present study (the exact times of sampling for individual recipients are shown in Table 2). The majority of long-term recipients exhibited a heterogeneous T-cell population with various degrees of clonal accumulations of CD8+ T cells (representative results are shown in Fig 5). When serial samples from the same recipient were compared, the majority of accumulated clones were persistently seen in the same recipient at different sampling time points (data not shown).

One patient with chronic GVHD (patient SK, Tables 1 and 2) showed some clonal accumulations (data not shown). However, the total number of distinct accumulation clones in this patient was less than that of the early-phase recipients. For instance, whereas the sum of distinct bands of all Vβ subfamilies was 57 in patient K1 on day 20 (Fig 3B), it was 33 only in patient SK at 19 months (data not shown).

DISCUSSION

The involvement of T cells in antigen-specific immune responses has been widely investigated in several fields, especially through the analysis of TCR gene usage. It has been suggested that the usage of TCR component may be restricted in antigen-specific responses. In this regard, T-cell specificity has been also explored in BMT, because modulation of allospecific T cells may accelerate the restoration of impaired immune function after BMT and/or prevent GVHD. However, until today, the specificity of T cells had been estimated only through in vitro studies, because no appropriate technique had been so far available to detect clonalities in vivo. To overcome this problem, we established an RT-PCR and SSCP system with TCR Vβ gene family-specific primers that allowed the detection of accumulated T-cell clonotypes in diverse populations at a frequency of one to several thousands. Using this system, we reported recently that peripheral T cells from healthy individuals exhibit heterogeneity with several stable accumulations of CD8+ clones. Moreover, we showed that stimulation by exogenous antigens, such as PPD in vitro or an infection in vivo, produced distinct clonal expansions within the heterogeneous T-cell population. We applied the same technique in the present study to investigate T-cell clonality in BMT recipients.

Because BMT is considered as a reconstitution of the host hematopoietic system (for a review, see Atkinson), it is important to understand how and what host T-cell clonotypes would grow from the transplanted donor marrow. Using Vα 5-transcripts, Rencher et al reported that the new population of T cells was highly diverse. On the other hand, Yamanaka et al analyzed CDR3 sequences of sequential samples from a BMT recipient and found a restricted oligoclonality in one specimen among serial samples. Thus, a controversy exists regarding in vivo T-cell clonality of BMT recipients.

In the present study, T-cell clonality of the recipients was analyzed directly using the RT/PCR-SSCP method. We focused on 22 subfamilies of the Vβ gene segment. Our results showed that multiple clonotypic accumulations occurred in...
the PBLs of recipients after HLA-identical transplantation. This phenomenon may be considered as an acute-phase expansion of host-reactive donor T cells that may be responding to distinct minor histocompatibility antigens. In addition, the initially expanding clones may consist of several subpopulations, each of which is either an allospecific or nonspecific (secondary) responder. In this regard, our previous studies suggested that removal of the stimulating factor resulted in the disappearance of clonal accumulations. Therefore, the disappearing clones may initially be induced by transient stimulation after BMT, e.g., an acute infection.

It is also possible that the predominance of distinct T-cell clonotypes is in part a consequence of altered thymic selection after BMT. Because a different thymic environment is introduced to the donor origin T-cell precursors after BMT, it is likely that T-cell maturation or selection would be highly altered. In addition, certain factors, such as CsA treatment or GVHD, are reported to affect thymic positive and negative selection in the posttransplant period. Thus, the relationship between thymic regeneration and degree of clonal expansion is worth investigating, and studies concerning T-cell surface markers may provide insight into such process.

Accumulation clones in the posttransplant period were observed in CD4+ and CD8+ subsets at a relatively early stage (data not shown). In contrast, analysis of PBLs in long-term recipients showed that CD8+ T-cell clones were the main subset at that stage (Fig. 5). We have recently observed an early and transient expansion of CD4+ T cells after in vivo antigenic stimulation. Thus, a proportion of CD4+ T-cell clones may represent the quick responders after antigen exposure. On the other hand, clonal expansion of CD8+ T cells has been also observed by several investigators in healthy individuals. Results of a recent study on BMT recipients also suggested oligoclonal expansion of CD8+ T cells with inhibitory activity to cytotoxic function. However, the specificity of these CD8+ clones has not been fully investigated. Results from our laboratory showed that a sustained antigenic stimulation would maintain clonal accumulation of T cells. Thus, it is possible that the expansion of CD8+ T-cell clones in BMT recipients is maintained by active and chronic host immune responses.

Our results also showed that MLR could not predict the responding T-cell clones. This may be due to the sensitivity of the method used in the present study. In HLA-matched MLR, the response of minor histocompatibility antigen-specific clones in vitro may be relatively low so that it would not be shown as a band in SSCP. In addition, it is possible that apparent T-cell responses are induced by certain antigens or conditions that exist exclusively in vivo.

From a clinical point of view, it is desirable to know the relationship between the appearance (or disappearance) of distinct T-cell clonotypes and the occurrence of GVHD. Die-
trich et al. showed that most Va and Vβ subfamilies were expressed in PBLs and dermal GVHD, suggesting a diversified TCR gene segment expression in allogeneic BMT. However, because the antigen specificity of T cells is mainly determined by the CDR3 region, a more direct analysis of this region may also be necessary. In this regard, our RT/PCR-SSCP study showed that the occurrence of GVHD seems to alter the expanding population of peripheral T cells. The TCR usage of these clones was not restricted to certain Vβ subfamilies. In addition, even after recovery from GVHD, a number of these clones was still found to accumulate. Thus, the remaining clones may be present as long-term remnants that would preserve T-cell memory of GVHD. Naturally, comparison of accumulating T-cell clonotypes between PBLs and GVHD lesions may be helpful to our understanding of the function of each T-cell clone.

Gorski et al. have recently analyzed T-cell repertoire complexity in BMT recipients using CDR3 size spectratyping. The method used in their study is similar to RT/PCR-SSCP, although it focuses on CDR3 length of the TCR, rather than the sequence. These workers suggested that the state of immune function or T-cell competency of marrow recipients correlated with the T-cell repertoire complexity.

Finally, this study showed for the first time that the overall T-cell clonality of BMT recipients had a tendency to become heterogeneous in the long-term. This observation may represent an active immunologic mechanism maintaining homeostasis of T-cell clonality that was skewed by BMT.

In conclusion, we showed a profound change in T-cell clonality after BMT and provided evidence for a stable clonal expansion in PBLs of BMT recipients. Our results suggest the involvement of in vivo distinct T-cell clones in acute and chronic immune responses occurring in BMT recipients.

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Fig 5. Long-term observations of T-cell clonality after BMT. PBLs from 9 BMT recipients at chronic, stable phase were analyzed and representative data of 3 patients are shown with initials and the post-BMT sampling date in months (refer to Table 2). Top, whole population; middle, CD4+ population; bottom, CD8+ population.

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