Natural Killer (NK)-Cell Function and Antileukemic Activity of a Large Population of CD3+/CD8+ T Cells Expressing NK Receptors for Major Histocompatibility Complex Class I After “Three-Loci” HLA-Incompatible Bone Marrow Transplantation

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We have shown that addition of granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to the marrow inoculum allows engraftment of T-cell depleted, “three loci” HLA-incompatible marrow transplants for acute leukemia. The event-free survival of patients at high risk for relapse prompted the present investigation of the antitumor potential of this transplant. Tumor-cell lysis by natural killer (NK) cells is regulated by inhibitory receptors for specific HLA class I alleles. Here, we report the postgrafting emergence of a large, donor-type CD3+/CD8+ T-cell receptor (TcR)-αβ+ cell population, barely detectable in normal subjects, that expresses 58 kD, “p58,” NK receptors for HLA-C locus alleles. Analysis of >900 clones revealed that 40% to 80% of these T cells exhibit NK-like function, ie, they lysed class I targets and were functionally blocked by class I alleles on target cells. Monoclonal antibody-mediated blocking of class I recognition by these cells induced lysis of HLA-protected, autologous targets. The class I-mediated inhibitory signaling through the NK receptors also blocked TcR/CD3-triggered cytotoxicity of these cells, indicating that their antigen-specific responses may be impaired. However, the NK-like function of these cells allows them to discriminate normal cells, protected from lysis, from leukemic cells that were lysed and may be targets for a graft-versus-leukemia effect.

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The applicability of allogeneic bone marrow transplant (BMT) for the treatment of patients with hematologic malignancies is limited by the availability of suitable donors. Because less than 30% of eligible patients have genotypically HLA-identical siblings and only 40% to 50% find an HLA-matched unrelated donor in the international registries of HLA-typed individuals, many patients still fail to find an appropriate donor. However, nearly all patients have a family donor who shares one chromosome 6 and is, therefore, matched for one HLA haplotype but fully, “three loci,” incompatible for the other. To date, transplantation of unmodified BM from two or three loci HLA-incompatible donors has been associated with unsuccessful outcome because of the high incidence (80%) of severe graft-versus-host disease (GVHD). Extensive experience in severe combined immune deficiency patients has shown that GVHD is largely preventable, even in mismatched BMT, when a 3-log T-cell depletion of the donor BM is achieved. However, the use of T-cell-depleted mismatched grafts in patients with leukemia has been hampered by the high incidence (50%) of graft rejection.

We have shown that engraftment of T-cell-depleted allogeneic “three loci” HLA-incompatible BMT for acute leukemia can be successfully accomplished if the transplant stem-cell dose is increased 7- to 10-fold by adding T-cell-depleted granulocyte colony-stimulating factor-mobilized peripheral blood (PB) progenitor cells to the marrow inoculum. As the patients were selected because they had advanced/ resistant leukemia, they were at high risk for postgrafting leukemia relapse. An additional relapse risk factor was the T-cell depletion of the inoculum and the consequent absence of GVHD and antileukemic effect exerted by the alloreactive T cells present in the graft. The event-free survival of many of these patients raised the question of whether the transplant could have exerted a significant and selective graft-versus-leukemia (GVL) effect, distinct from GVHD, and prompted the present investigation of its antitumor potential.

After T-cell-depleted (HLA-matched) marrow transplants, we and others have noted that reconstitution of the immune system is characterized by a short-lived (2 to 4 weeks) natural killer (NK)-cell wave that precedes the emergence of a persistent (from 4 months to 1 year) CD3+/CD8+ lymphocytosis, whereas CD3+/CD4+ cells reach near-normal values only 8 to 12 months postgrafting.

It has recently been shown that human (as well as mouse) NK-cell function, including lysis of tumor cells, is regulated by the expression of multiple, clonotypically distributed, membrane receptors with different specificities for major histocompatibility complex (MHC) class I alleles. Recognition of the specific alleles by their receptors conveys an inhibitory signal that blocks NK-cell lytic activity. In particular, two members of a 58 kD molecular family (“p58”), recognized by the GL183 and EB6 monoclonal antibody (MoAb), function as specific receptors for two different groups of HLA-C alleles, which include all known alleles of the C locus, ie, Cw1, 3, 7, 8, and Cw2, 4, 5, 6, respectively. In normal subjects, these receptors are expressed by NK cells and by a very small (~1%) subset of T-cell receptor (TcR)-αβ T cells, predominantly of the CD8+ phenotype. Because of the relevance of the p58 recep-

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Submitted September 14, 1995; accepted December 20, 1995.

Supported by grants awarded by Associazione Italiana per la Ricerca sul Cancro, and by Consiglio Nazionale delle Ricerche, target project Applicazioni Cliniche della Ricerca Oncologica (ACRO), and Grant No. 94.02936.CIT04.

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Blood, Vol 87, No 9 (May 1), 1996: pp 3933-4000

3993
tors in NK cell-mediated tumor-cell lysis, we used GL183 and EB6 anti-p58 MoAb to analyze their expression in HLA-incompatible BMT recipients. We unexpectedly observed the emergence of a large population of p58−/CD3+/CD8+ TcR-αβ T cells that, like NK cells, lyse HLA class I targets and are functionally blocked by class I alleles on target cells. Furthermore, the NK receptor-mediated negative signaling predominated over the stimulatory signaling through the TcR. Finally, these T cells were able to discriminate normal cells, which are protected from lysis (ie, from GVHD), from leukemic cells, which are lysed and may, therefore, be the targets for a selective GVL effect.

MATERIALS AND METHODS

Patients, conditioning regimen, collection, and T-cell depletion of BM and G-CSF–mobilized PB mononuclear cells (PBMC) have been described. No postgrafting immunosuppressive treatment was administered. A full-donor chimerism of PB and BM was documented in all the recipients by restriction fragment length polymorphism analysis. Patients gave informed consent for phlebotomy for the present study.

Identification of PB lymphocyte subsets. Enumeration of CD3+/CD4+, CD3+/CD4−, CD3−/CD8+ lymphocyte subsets and expression of the p58 receptors GL183 and EB6 by CD16+, CD3−, and CD8− cells were determined by two-color immunofluorescence and flow cytometry using anti-CD3, anti-CD4, anti-CD8, anti-CD16 MoAb, and MoAb to the GL183 and EB6 p58 receptors for HLA-C* and CD8− cells were of the CD8+ phenotype. The CD3+/CD8+ cells and EB6 p58 receptor MoAb were a generous gift from Dr A. Moretta (University of Brescia, Italy). Other MoAbs used were: anti-CD3 Leu 4 (IgG1) and OKT3 (IgG2a); anti-CD4 Leu 3 (IgG1) and OKT4 (IgG2a); anti-CD8 Leu 2 (IgG1) and OKT8 (IgG2b); anti-CD16 Leu 11a (IgG1) and Leu 11b (IgM) (Leu series and an anti-Tcr-αβ MoAb were from Becton Dickinson, San Jose, CA, and OKT series MoAb were from Ortho, Raritan, NJ). Controls were isotype-matched nonreactive MoAb pairs (ie, IOM34 anti-CD34, IgG1, and IOT6 anti-CD1a, IgG2a, from Immunotech, Luminy-Marseille, France) plus the secondary antibodies.

RESULTS

Emergence of a large population of CD3−/CD8+ cells expressing NK receptors for HLA-C alleles. After a brisk but transient NK-cell wave, the lymphocyte subset, which predominated during the long-term reconstitution phase after HLA-mismatched BMT, exhibited a CD3−/CD8+ phenotype (Fig 1A). Two members of the p58 molecular family that function as NK receptors for HLA-C alleles were identified by the GL183 and EB6 MoAb, respectively. P58 molecules were not expressed by cells of the post-BMT NK-cell wave, but were, instead, expressed on a large proportion of the recovering CD3+ cells (Fig 1B). Moreover, most of the p58− cells were of the CD8+ phenotype. The CD3−/CD8+ cells that expressed GL183 consistently outnumbered those that displayed EB6. Additional two-color analyses (not included in Fig 1) showed that only few CD4+ cells coexpressed p58, and that virtually all CD3+ cells, including those that expressed p58 receptors, were TcR-αβ+. Postgrafting time-course of the expression of the GL183 receptor on CD3+ cells in six BMT recipients are shown in Fig 1C. Expression increased from ~1% donor values up to ~80% by 3 to 5
Figure 1. A large population of CD3+/CD8+ cells expressing the p58 NK receptors for HLA-C is observed after HLA-incompatible BMT. (A) Absolute counts of T-helper, T-cytotoxic, and NK cell subsets. (B) Expression of the p58 receptors GL183 and EB6 by CD16+, CD3+, and CD8+ cells in a representative BMT recipient at 4 months post-BMT. (C) Proportion of CD3+ cells expressing GL183 as a function of time post-BMT.

Lysis is regulated by HLA class I molecules expressed on target cells. Because a fraction of p58+ cytolytic T-cell clones lysed the HLA class I target DAUDI, we tested the ability of these cells to lyse six EBV-transformed lymphoblastoid cell lines that display different homozygous HLA haplotypes. Some of the clones exhibited a sizeable degree of cytotoxicity also against the EBV-transformed cell lines. In addition, different clones exhibited heterogeneous patterns of lysis, ie, they lysed lines with different HLA haplotypes (Fig 3A). Clones 1 and 2 (GL183-/EB6-) lysed the homozygous cell line 8.1 (A1, B8, Cw7) but not the homozygous line 57.1 (A1, B57, Cw6). Clones 3 and 4 (GL183+/EB6+)...
Fig 3. The lytic function of p58'/CD3'/CD8' cells is regulated by HLA class I molecules expressed by target cells. (A) Similar to NK cells, a given HLA haplotype confers protection in a dominant fashion and susceptibility to lysis is inherited in a recessive manner. P58'/CD3'/CD8' clones with lytic activity against DAUDI were tested for lysis of EBV-transformed lymphoblastoid cell lines homozygous for given HLA haplotypes (lines 8.1, 57.1, 13.1, 46.1, 44.1, and 7.1) and of lines exhibiting heterozygous HLA haplotypes derived from the combination of two distinct homozygous haplotypes (lines 8.1/57.1, 13.1/46.1, and 44.1/7.1). Four representative clones are shown (see Results for HLA typing of cell lines and for the p58 phenotype of clones). Clones that lysed only one of two homozygous cell lines also failed to lyse the heterozygous cells (E:T = 10:1). (B) Receptors specific for HLA-C alleles are responsible for inhibition of cytotoxicity. GL183' T-cell clones with lytic activity against the P815 murine cell line fail to lyse a P815 transfectant expressing the HLA-Cw3 allele (E:T = 10:1).
Fig 5. Engagement of the NK receptors mediates inhibition of CD3/TCR-triggered cytotoxicity. (A) p58⁺ T-cell clones were analyzed by hybrid anti-CD3/anti-HLA class II MoAb-redirected killing of the HLA class I⁺ (and class II⁺) U937 cell line. In the group of clones with NK-like function against DAUDI (no. 1 to 5), class I inhibits CD3/TCR-triggered cytotoxicity, because (1) the hybrid MoAb does not significantly trigger CD3/TCR-mediated lysis and (2) masking of class I by addition of the 3A3 anticlass I MoAb restores CD3/TCR-triggered cytotoxicity. In contrast, clones without NK-like function (no. 6 to 10) mediate efficient lysis that is unaffected by addition of the anticlass I MoAb (E:T = 5:1). (B) Anti-CD3 MoAb-redirected killing of the FcγR⁺ murine P815 cells mediated by the former group of clones is also inhibited by mimicking human HLA class I on target cells with target Fcγ receptor-immobilized anti-p58 GL183 or EB6 MoAb (E:T = 5:1).

A proportion of CD3⁺/CD8⁺ cells that emerged after HLA-incompatible BMT, thus, coexpress two receptors for HLA-class I with opposing functions, ie, an inhibitory NK receptor (such as pS8) and the stimulatory, class I-restricted TcR. To probe the functional outcome of coengagement of the NK receptors for class I and of the TcR on these clones, we used the HLA-class I⁺ human cell line U937 in a hybrid anti-CD3/anti-HLA class II MoAb-redirected killing assay, in which HLA-class I recognition was blocked by addition of the 3A3 antihuman class I MoAb. Figure 5A shows representative clones belonging to the two pS8⁺/CD8⁺ T-cell populations, ie, that with and that without NK-like function. The ability of the hybrid MoAb to redirect CD3/TCR-triggered killing of U937 targets was preliminarily indicated by the efficient lysis mediated by a set of BMT recipient-derived pS8⁺/CD8⁺ T-cells (not shown). In contrast, in the group of pS8⁺/CD3⁺/CD8⁺ clones with NK-like activity (clones no. 1 to 5 in Fig 5A), the hybrid MoAb did not trigger significant CD3/TCR-mediated lysis of the U937 target. As expected, addition of anticlass I MoAb restored efficient NK-like lysis. The observation that CD3/TCR stimulation promoted limited, but consistent, additional increases in anticlass I-redirected lysis, suggests that physiological engagement of functional NK receptors (such as pS8) by the natural ligand, HLA-class I, may mediate inhibition of CD3/TCR-triggered effector functions. Unexpectedly, in p58⁺/CD3⁺/CD8⁺ clones that did not exhibit NK-like function (clones no. 6 to 10), physiological engagement of the NK receptors (p58) by class I did not block CD3/TCR-triggered cytotoxicity, as shown by the facts that hybrid MoAb-redirected lysis was efficient and it did not increase on addition of the anticlass I MoAb. Further evidence that p58 receptor engagement mediates inhibition of the CD3/TCR-triggered cytotoxic function of these clones was obtained by the use of an anti-CD3 MoAb-redirected killing assay of the FcγR⁺ murine P815 cells mediated by the former group of clones is also inhibited by mimicking human HLA class I on target cells with target Fcγ receptor-immobilized anti-p58 GL183 or EB6 MoAb (E:T = 5:1).
CD3+/CD8+ lymphocytes that express p58 (GL183 and/or P58) of these cells is associated with the capacity to discriminate normal cells from the same patients.

In this study we describe the emergence of a subset of CD3+/CD8+ lymphocytes that express p58 (GL183 and EB6) NK-cell receptors for the HLA-C locus alleles, during the reconstitution phase after three loci HLA-incompatible (T-cell depleted) marrow grafting for acute leukemia. This subset, barely detectable in normal subjects, undergoes an impressive and long-lasting expansion in HLA-incompatible BMT recipients. Furthermore, we find that this subset can be subdivided into two distinct populations. In one population, the expression of NK receptors for HLA-class I is associated with the capacity to discriminate normal cells, which are protected from lysis (ie, from GVHD), from leukemic cells which are not protected and may, therefore, be targets for a selective GVL effect.

**DISCUSSION**

In this study we describe the emergence of a subset of CD3+/CD8+ lymphocytes that express p58 (GL183 and EB6) NK-cell receptors for the HLA-C locus alleles, during the reconstitution phase after three loci HLA-incompatible (T-cell depleted) marrow grafting for acute leukemia. This subset, barely detectable in normal subjects, undergoes an impressive and long-lasting expansion in HLA-incompatible BMT recipients. Furthermore, we find that this subset can be subdivided into two distinct populations. In one population, the expression of NK receptors for HLA-class I is associated with the capacity to discriminate normal cells, which are protected from lysis (ie, from GVHD), from leukemic cells which are not protected and may, therefore, be targets for a selective GVL effect.

In any event, the NK-like function of these cells endows them with the capacity to discriminate normal cells, which are protected from lysis, ie, from GVHD, from leukemic cells, which are lysed and may, therefore, be targets for a GVL effect. It is of note that in HLA-mismatched BMT recipients, these cells are the predominant PB cell subset for an extended period of time postgrafting and may, therefore, be the major effector system of a GVL effect. Because as previously described, NK receptor-mediated recognition of HLA-class I depends on the presence of specific self-peptides complexed with class I, the observed loss of protection of leukemic cells from the NK-like lysis mediated by these clones could be the consequence of a replacement of specific self (protective) peptides with others that may result from the leukemic transformation.

The marked expansion of this cell subset after HLA-incompatible marrow transplants contrasts with the limited increase observed after HLA-matched transplants, indicating that the degree of disparity between donor and host HMC, ie, one entirely mismatched HLA haploptope, may be responsible for this expansion. Because expression of NK receptors can inhibit antigen-specific T-cell responses, these cells may contain a potentially harmful repertoire of alloreactive donor-type T cells, which would not need to undergo clonal deletion because they would be kept in check by the NK receptor-mediated inhibitory signals. However, additional events must be envisaged to propose an explanation that also accounts for the observation that there is another CD8+ T-cell population that expresses p58 receptors but (1) does not exhibit NK-like activity against HLA class I targets or (2) in which physiologic engagement of the NK receptors by HLA class I is apparently inefficient in blocking the TcR-mediated re-
sponsors. The lack of inhibition of the TcR-mediated responses may possibly reflect a still immature functional status of the p58 receptors, which might recognize their MHC class I ligands with lower efficiency or, alternatively, might be unable to transduce efficient inhibitory signals. In addition, the two functionally distinct p58− subsets may differ for the presence, or functional status, of as yet unidentified stimulatory receptor(s) responsible for the triggering of the lytic machinery by HLA class I targets or anticlass I MoAb-masked autologous cells. Circumstances leading to reduced signaling through the TcR, such as anergy induced by the host incompatible MHC antigens, might enable the stimulatory NK receptor(s) to become functional. Expression of a full set of functional NK receptors, ie, the inhibitory receptors for HLA class I, and the (unidentified) stimulatory receptor(s), would in turn rescue the anergic T cells and allow their expansion as operational NK cells. This hypothesis is consistent with our observation that, among the p58+/CD3+/CD8+ clones, the proportion of clones with NK-like activity increases progressively over time (see Fig 2), and also with the already mentioned finding that such a dramatic expansion of p58+/CD3+/CD8+ cells is not seen after HLA-compatible BMT. In addition, the T cells that reconstitute after mismatched (data not shown), as well as matched, BMT are largely CD28− and therefore susceptible to anergy by lack of costimulation through the CD28 receptor. The somewhat limited contribution that CD3/TcR stimulation adds to the lytic ability of these cells (upon masking of HLA class I on target cells) (see Fig 5) would also seem to support this contention.

Because the very small T-cell subset that expresses NK receptors in normal donors has also recently been shown to be functionally inhibited by recognition of specific HLA-class I alleles, the data presented here may define a unique model for a more general involvement of these cells in the regulation of T-cell responses against alloantigens, ie, of autoimmunity.

ACKNOWLEDGMENT

We are particularly grateful to Dr Alessandro Moretta (University of Brescia, Italy) for helpful discussions and critical review of the manuscript, and for the generous gift of the anti-p58 GL183 and EB6, anti-CD3 JT3A, anti-CD16 KD-1, and Z-27 MoAb. We also thank Drs Antonio Lanzavecchia (The Basel Institute for Immunology, Basel, Switzerland) and Miguel Lopez-Botet (Hospital de la Princesa, Madrid, Spain) for the hybrid HB55E3 anti-CD3MLA-MoAb-

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