In Vitro Inhibition of Normal Human Hematopoiesis by Marrow CD3+, CD8+, HLA-DR+, HNK1+ Lymphocytes


We previously demonstrated that after allogeneic bone marrow transplantation (BMT) a subset of CD8+, HNK+, and DR-positive T lymphocytes are able to inhibit CFU-GM and BFU-E growth with an HLA-DR restriction. In this study we investigated whether these cells, present in normal marrow in low concentration (~1%), play the same role. HNK+-positive sorted marrow cells forming rosettes (E/C) were able to inhibit BFU-E and CFU-GM growth when added back to the marrow E/C at a ratio of 1:10 (HNK+, E/C/E/C) in a range from 40% to 60%. This inhibitory effect was also detected for a cellular ratio of 1:100, which is the normal marrow value for this subset of T cell. HNK+, DR+-sorted E/C after double-immunofluorescent labeling also showed the same inhibitory activity as the HNK+, E/C, whereas the negative fraction including all the other E/C had no detectable inhibitory activity. CD3 and CD8 antigens were also present on the membrane of these cells, as demonstrated in two cases by double-immunofluorescent labeling performed with anti-CD3 or anti-CD8 monoclonal antibodies (MoAbs) and HNK, MoAb, respectively, and subsequent cell sorting. Blocking experiments, performed by adding in culture anti-CD4 and anti-CD8 MoAbs to HNK+, T cells showed that only the last MoAb was able to prevent inhibition of hematopoietic colony growth. These results confirmed that one subset of CD3+, CD8+, DR+, and DR+ T cells was responsible for in vitro inhibition of normal hematopoiesis. In addition, this inhibition was genetically restricted to HLA-class II antigens, since in three co-culture experiments with unrelated bone marrow cells inhibition occurred only when cells with one haploidentical HLA-DR antigen was added back to the culture. Indeed, this effect was really HLA-DR restricted, since in blocking experiments with different anti-HLA class II MoAbs (anti-DR, anti-DP, and anti-DQ MoAbs) only an anti-HLA-DR MoAb was able to prevent the colony growth inhibition by CD3+ HNK+, or CD8+ HNK+, E/C. In conclusion, the CD3+, HNK+, CD8+, DR+ cells may be the T-cell subset able to inhibit normal hematopoiesis with an HLA-DR restriction.

The same subset of T cells is also present in normal marrow but at low concentration (~1%). In this study, we investigated whether this subset of T cells plays a role in the in vitro inhibition of hematopoiesis and showed that these marrow T cells negatively regulate normal in vitro hematopoiesis.

MATERIALS AND METHODS

Bone marrow samples were obtained from BMT donors. Informed consent was obtained from all BMT donors according to institutional review board guidelines.

HLA typing. All normal subjects underwent serotyping of cells for HLA A, B antigens by the standard technique of microcytotoxicity on peripheral blood cells. Typing for DR antigens was performed with a microcytotoxicity test with nylon column-purified B lymphocytes and test cells.

Cell separation. Bone marrow was collected on preservative-free heparin (10 U/mL, Laboratory Roche, Neuilly/Seine, France). Bone marrow light-density cells (LDCs) were separated by density centrifugation over Ficoll-metrizoate (Lymphoprep, Nyegaard, Oslo; density 1.077). The cellular ring at the interface was collected and washed three times in a medium (Eurobio, Paris) at 4°C.

Isolation of marrow E/C. E/C were separated from E/C by rosetting with sheep erythrocytes (E/C) treated with 2-aminoethylisothiouronium bromide (Sigma Chemical, St Louis) followed by Ficoll-metrizoate centrifugation at 4°C. E/C were recovered by lysing sheep erythrocytes with 0.15 mol/L Tris-buffered ammonium chloride. This fraction contained ~85% T cells as determined by an anti-CD3 monoclonal antibody (MoAb). The E/C were recovered from the interphase, washed, and kept for co-culture experiments.

Immunofluorescent labeling. Immunofluorescent labeling was performed on the E/C using HNK, MoAb (Becton Dickinson, Mountain View, CA). In brief, 20 x 10^6 E/C were treated with HNK, antibody at an appropriate dilution (5 x 10^-2 to 1 x 10^-3) in phosphate-buffered saline (PBS), washed, incubated for 30 minutes at 4°C with 2 x 10^-2 diluted fluorescein-conjugated goat IgG directed against mouse μ chain (Nordic, Tullburg, The Netherlands) and washed.

From INSERM U.91, Service d’Hematologie Hopital Henri Mondor, Creteil; Centre de Cytofluorometrie du C.N.R.S. Villejuif; and Laboratoire Central de Cytofluorometrie de l’Universite Paris XII, Creteil, France.

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Address reprint requests to Giovanna Vinci, MD, INSERM U 91, Hopital Henri Mondor, 94010 Creteil, France.

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Double-membrane labeling was also performed with an HNK, MoAb and either an anti-CD3, or an anti-CD8 (Ortho Diagnostic Systems, Westwood, MA), or an anti-HLA-DR MoAb. Cells were processed as above using incubation by both antibodies, followed by a rhodamine-conjugated goat IgG directed against different subclasses of mouse IgG.

**Purification of hematopoietic progenitors.** Normal progenitors were semipurified using an immune-panning procedure. E-EC were first incubated with a panel of MoAbs recognizing mature differentiation antigens [CD3 and CD5 for the T cell lineage, CD15 and two nonclustered MoAbs for the granulocytic lineage, CD20 for B cells, antilymphocyte protein (GP II) IIIa MoAb for the megakaryocytic lineage, a CD14 for the monocytic lineage, an antiglycophorin A MoAb, and a MoAb against the erythroid-related epitope of leukosialin for the erythroid lineage and an anti-CD36 MoAb]. Incubations were done with an appropriate dilution of each MoAb for 30 minutes at 4°C. Labeled E-EC were separated under sterile conditions into a positive and negative fraction by cell sorting using a FACSSORT. The sorting rate was 1,000 cells/s. Cells were collected in 10-mL sterile plastic tubes containing 2 mL cold fetal calf serum (FCS). The technique of cell sorting was calibrated following the classic procedures.

Assays of CFU-GM and BFU-E. The plasma-clot technique was used as reported previously. The culture medium contained cells, a-medium, 1% deionized bovine serum albumin (BSA, Cohn fraction V, Sigma), 70 μg/mL CaCl2, 10 μg/mL asparagine, 10% human AB serum (Centre Départemental de Transfusion Sanguine du Val de Marne, Créteil, France), and 10% citrated bovine plasma (GIBCO, Paisley, Scotland). Cultures were performed in triplicate in 0.5-mL wells (Falcon 3047, Grenoble, France).

The stimulating factors were either 10% supernatant from the Mo-cell line23 or 5% Mo-medium plus 1.5 IU/mL porcine erythropoietin (25 IU/mg protein; Centre National de Transfusion Sanguine, Paris) for CFU-GM and BFU-E growth, respectively. Cultures were incubated for 12 to 14 days at 37°C in a humidified atmosphere at 5% CO2, and colonies were scored under an inverted microscope. Colonies were grown from bone marrow E-EC. Cultures were obtained by adding the positive fraction obtained by cell sorting from E-EC at different ratios. The cellular concentration was 3 to 5 × 104 E-EC/mL. Results were expressed as the number of colonies/1 × 105 cells.

**Blocking experiment.** Anti-CD8 and anti-CD4 MoAbs were added at a 2 × 10-8 dilution in a coculture experiment between E-EC and HNK, E-EC. Another experiment was performed by adding anti-HLA-DR (K5.2), anti-α2 (B721-1) (Becton Dickinson), and anti-DQ (L3)t) MoAbs at 1 × 10-7, 2 × 10-7, and 2 × 10-8 dilution, respectively, in cocultures between E-EC, CD3* HNK, E-EC, and CD8* HNK, E-EC, respectively. All antibodies used in blocking experiments are IgG1, murine MoAbs.

**Cytotoxicity assay.** K 562 cell line was grown as a suspension culture in RPMI 1640 with 10% FBS and used as target in cytotoxicity assay. Before cytotoxicity, target cells were washed and labeled with 51Cr (ORIS Compagnie, Gif sur Yvette, France) at a concentration of 50 μCi/106 cells for 90 minutes. Effector cells were CD8* HNK, E-EC, and used as target in cytotoxicity assay. Before cytotoxicity, target cells were washed and labeled with 51Cr (ORIS Compagnie, Gif sur Yvette, France) at a concentration of 50 μCi/106 cells for 90 minutes. Effector cells were

**RESULTS**

**Effect of HNK, E-EC on colony growth of CFU-GM and BFU-E.** E-EC from two normal marrows were stained with HNK, MoAb by indirect immunofluorescent labeling. HNK, E-EC were obtained by cell sorting. This fraction represents ~3% of the E-EC. CFU-GM and BFU-E growth inhibition was observed when E-EC were cocultured with HNK, E-EC at different ratios from 6:1 to 100:1. The degree of inhibition was higher for CFU-GM than for BFU-E growth and this effect was observed until a ratio of 100:1 was reached (E-EC/HNK, E-EC) in one case (Fig 1). In another experiment, CFU-GM and BFU-E colony growth was inhibited until a ratio of 50:1 was reached (data not shown).

**Effect of HNK, DR*, E-EC on CFU-GM and BFU-E growth.** To demonstrate that the inhibitory HNK, E-EC bear the HLA-DR antigen as previously demonstrated in allogeneic BMT, E-EC from two normal marrows were stained by indirect double-immunofluorescent labeling with both HNK, and anti-HLA-DR MoAbs (Fig 2). The percentage of HNK, E-EC labeled by the anti-HLA-DR MoAb was 66% and 80%, respectively, in Figs 2A and B. The HNK, DR*, E-EC were sorted as the positive fraction; all other E-EC were included in the negative fraction.

Cells from the positive fraction were added back in coculture to the E-EC at a ratio of 12:1 (E-EC/HNK, DR*, E-EC), whereas cells from the negative fraction

**Fig 1.** Effect of marrow HNK, E-EC on colony growth of (A) CFU-GM and (B) BFU-E. E-EC obtained by sheep RBC rosetting were labeled by HNK, MoAb, and cell-sorted HNK, E-EC were subsequently added back to the E-EC at different ratios. Cultures were performed in triplicate in 500-μL vol, and results were expressed as mean ± SD. E-EC ( ) ; E-EC + HNK, E-EC ( ) ; E-EC + HNK, E-EC ( ) ; E-EC + HNK, E-EC ( ) ; E-EC + HNK, E-EC ( ) ; E-EC + HNK, E-EC ( ) ; and E-EC + HNK, E-EC ( ) . All results are different from control A (p < .001).
were added back in one case in a ratio of 1:1. In Figs 2A and B, the positive fraction was able to inhibit colony growth significantly (30% for CFU-GM and from 53% to 33% for BFU-E growth). No inhibition was observed when the negative fraction was added to the Ec (Fig 2).

Effect of HNK1, DR E and HNK1, DR E on colony growth of CFU-GM and BFU-E. E' were stained by an indirect double-immunofluorescent technique with both HNK1, MoAb and anti-DR MoAb. The marrow HNK1, DR E were stained as the positive fraction; all other E' were included in the negative fraction. Cells from the positive or negative fraction were added back in culture to the Ec at a ratio of 12:1 and 1:1, respectively. E' + HNK1, DR E (ratio 12:1 between Ec and HNK1, DR E) (Ο); E' + HNK1, DR E + HNK1, DR 3' cells (ratio 1:1) (■).

Effect of HNK1, CD8 and HNK1, CD8 on colony growth inhibition of CFU-GM and BFU-E. To demonstrate that the same subset of HNK1, DR E also showed CD3 and CD8 antigens, two different experiments were performed (Fig 3A and B). In one case (Fig 3A), the marrow Ec were stained by indirect double-immunofluorescent labeling using an anti-CD3 and HNK1, MoAbs; 66% of the HNK1, cells exhibited the CD3 antigen. In another case (Fig 3B), anti-CD8 and HNK1, MoAbs were used; 75% of the HNK1, cells exhibited the CD8 antigen. After cell sorting, Ec from the marrow donor were cocultured with the positive fraction, CD3' HNK1, E' (A) or CD8' HNK1, E' (B) at ratios of 6:1 or 11:1, respectively. A significant inhibition was observed (from 28% to 38% for CFU-GM and from 51% to 44% for BFU-E in A and B, respectively), whereas the negative fraction had no inhibitory effect. CD8' HNK1, E' also inhibited a purified fraction of hematopoietic progenitors (2.2% CFU-GM, 1.5% BFU-E) in a ratio of 1:40 (38% inhibition of CFU-GM; 37% for BFU-E). In the same experiment, increasing numbers of CD8' HNK1, E' from 1:40 to 1:100 also enhanced inhibition (data not shown).

Blocking experiments were performed with an anti-CD4 or anti-CD8 MoAb. In this case, HNK1, E' Ec from a normal marrow were added back to the Ec at a constant ratio (1:12). Anti-CD4 and anti-CD8 MoAbs were added in culture in an adequate dilution. The suppressive effect mediated by HNK1, E' was abolished only when an anti-CD8 MoAb was included in culture (Fig 4).

Effect of HNK1, E' from a normal subject on colony growth from normal unrelated subjects. HNK1, Ec from normal marrow donors were obtained by cell sorting. Two normal unrelated subjects who had no HLA class I antigens in common with the T-cell marrow donor were chosen. One of them had a partial HLA-DR homology (HLA-DR7); the other had no HLA-DR homology. HNK1, E' Ec were added in coculture to Ec from the donor himself and from these two normal subjects at different ratios (from 1:16 to 1:100). A significant inhibition (30% to 44% of inhibition for CFU-GM and BFU-E, respectively) of colony growth from the donor and the normal subject with an HLA-DR7 homology was observed. This inhibition was observed up to a ratio of 1:50; in contrast, no inhibition was observed in the normal subject with no common HLA-DR haplotype (Table 1).

In another experiment, HNK1, E' Ec obtained from an HLA-DR 3-5 normal subject were able to inhibit his own colony growth, colony growth of a subject with complete HLA-DR homology (DR 3-5), and of a subject with partial HLA-DR homology (DR5). In contrast, no colony growth inhibition was observed in a subject with HLA-DR3 homology or in another subject without HLA-DR identity (HLA-DR...
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Fig 4. Effect of addition of anti-CD4 and anti-CD8 MoAbs on inhibition of colony growth by HNK₁ E⁺ C. Sorted HNK₁ E⁺ C from normal marrow were added back in culture to the E C at a constant ratio.² Blocking experiments were performed by addition of anti-CD4 and anti-CD8 purified MoAbs in culture at a dilution of 1 x 10⁻². Both antibodies were IgG1 antibody. E C (☐); E C + HNK₁, E⁺ C (ratio 12:1 between E C and HNK₁, E⁺ C) (☐); E C + HNK₁, E⁺ C + anti-CD4 MoAb (▲); and E C + HNK₁, E⁺ C + anti-CD8 MoAb (▼).

Effect of CD8⁺ HNK₁⁺ E cells on lysis of K562 cells. To study the functional activity of CD8⁺ HNK₁⁺ E cells, we analyzed whether these cells showed a natural killer (NK) activity. No significant percentage of lysis was found against the K562 target (at a ratio of effector to target cell of 20:1) (data not shown).

DISCUSSION

We investigated the role of lymphocytes in negative regulation of normal hematopoiesis. The results of our study provided two kinds of information: First, a subset of marrow T cells was able to inhibit in vitro hematopoiesis; second, this inhibitory effect was genetically restricted to the HLA-DR locus. Previously, in normal subjects, some subsets of T cells characterized by HLA-DR antigen on their surface inhibited CFU-GM and BFU-E growth, this suppressive effect was genetically restricted to cell combinations phenotypically identical to at least one HLA-DR locus.⁵ Furthermore, suppressive T cells have been observed in different pathologic conditions: We demonstrated that after allogeneic BMT a subset of T lymphocytes was able to inhibit CFU-GM and BFU-E colony growth⁶ and that these cells express mature T cell antigens, CD3, CD8, and CD2 associated with HNK₁ and HLA-DR antigens. In addition, this inhibition is genetically restricted to the DR antigen in the absence of class I antigen recognition, and only one of the two haplotypes appears to be involved in the genetic restriction.⁷ A similar observation was made in a malignant T-cell proliferation exhibiting a similar phenotype and capable of inhibiting erythropoiesis with a class II antigen restriction.⁸ Our results show that HNK₁⁺ T cells from normal marrows are able to inhibit CFU-GM and BFU-E colony growth when added to cells depleted of T cells; this effect is observed at a very low concentration (1 x 10⁶ cells/1 x 10⁶ E⁻ C/mL). These cells belong to the T-cell lineage, as shown by the presence of CD3 antigen and of a mature T cell antigen, CD8, in sorting experiments. On the other hand, they do not express the CD4 antigen since other sorted T cells (the negative fraction) have no inhibitory activity and an anti-CD4 MoAb does not abolish the inhibitory activity of HNK₁⁺ cells.

The T cells we studied shared a phenotype HNK₁⁺, CD8⁺ similar to other cells implicated in negative regulation of hematopoiesis. Since other sorted T cells (the negative fraction) have no inhibitory activity and an anti-CD4 MoAb does not abolish the inhibitory activity of HNK₁⁺ cells.
hematopoiesis both in physiologic and pathologic conditions. Two types of inhibition of hematopoiesis mediated by HNK\textsuperscript{+}, CD8\textsuperscript{+} cells which may correspond to cells with different functions can probably be distinguished. A first type of inhibition is not genetically restricted to the HLA-class II antigens, and generation of the inhibitory effect requires cell–cell contact by preincubation with the target cells. These results were obtained previously\textsuperscript{27-28} by NK cells and again recently by NK clones that suppress hematopoiesis in a heterogeneous but clonally stable manner.\textsuperscript{29} We also showed previously that in some patients with CD8\textsuperscript{+} lymphocytosis and neutropenia, lymphocytes are able to inhibit CFU-GM colony growth after overnight incubation with the target cells as reported for NK clones.\textsuperscript{30} The inhibition mediated by NK cells and these T cells may be related to secretion of lymphokines such as interferon γ\textsuperscript{29} and tumor necrosis factor.\textsuperscript{31}

The second type of inhibition mediated by some CD3\textsuperscript{+}, CD8\textsuperscript{+}, HNK\textsuperscript{+} cells differs by the presence of a constitutive inhibitory activity not requiring preactivation by the target. This inhibitory effect is genetically restricted to the HLA-DR locus. These cells have been essentially described in some pathologic conditions.\textsuperscript{9,12,13} Our results show that normal marrow T cells are able to inhibit hematopoiesis with an HLA-DR restriction. In the present study, we cannot conclude whether this phenomenon of inhibition was restricted to one or both HLA-DR haplotypes. Among the HLA-class II antigens, only the DR locus is implicated in this effect, as demonstrated by blocking experiments with different anti–HLA-class II MoAbs.

We noted, as have other researchers, that target progenitor cells for suppression may be either CFU-GM or BFU-E cells, which also express the HLA-DR antigen on their surfaces.\textsuperscript{32,33} Therefore, the HLA-DR antigen could operate as a locus negatively regulating proliferation of hematopoietic progenitor cells.\textsuperscript{34-36} Our data suggest that a subset of marrow T cells with a CD3\textsuperscript{+}, CD8\textsuperscript{+}, HLA-DR\textsuperscript{+}, HNK\textsuperscript{+} phenotype negatively regulates in vitro hematopoiesis with an HLA-DR genetic restriction. Further investigations, probably at the clonal level, are needed to investigate the mechanisms of this inhibition, especially whether this effect is dependent on cell interactions or secretion of lymphokines.

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**Table 2. Effect of CD8\textsuperscript{+} HNK\textsuperscript{+} E\textsuperscript{+} on CFU-GM (a) and BFU-E (b) Growth in Coculture Experiments**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ratio</th>
<th>HLA-DR 4-6 (Autologous)</th>
<th>HLA-DR 4-8</th>
<th>HLA-DR 3-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^8 E\textsuperscript{+}$</td>
<td>a</td>
<td>562.5 ± 47.5</td>
<td>183.6 ± 4.7</td>
<td>204 ± 30</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>140 ± 7.5</td>
<td>42.5 ± 4</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>$E\textsuperscript{+} + T\textsuperscript{+} HNK\textsuperscript{+} E\textsuperscript{+}$</td>
<td>a</td>
<td>305 ± 14</td>
<td>132.6 ± 6</td>
<td>217 ± 15</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>63.2 ± 13</td>
<td>17 ± 3.5</td>
<td>34 ± 5.9</td>
</tr>
</tbody>
</table>

Sorted CD8\textsuperscript{+} HNK\textsuperscript{+} E\textsuperscript{+} cells from an HLA-DR 4-6 subject were added in a ratio of 1:10 in coculture experiments to E\textsuperscript{+} cells from the bone marrow donor himself and from two normal unrelated subjects—one with an HLA-DR 4 common haplotype and one without common HLA-DR haplotype.
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