Identification of the c-kit Ligand: End of the Road for Understanding Aplastic Anemia in Steel Mutant Mice?

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We here report the initiation of hematopoietic recovery in congenitally hypoplastic S1/S1\(^{+}\) mice by the cytototoxic ablation of cells bearing the natural killer (NK) phenotype (NK 1.1\(^{+}\)). The most striking finding was the early several-fold increase in the cycling fraction of stem and progenitor cells (with the exception of progenitors committed to megakaryocytogenesis) in the anti-NK 1.1\(^{+}\) antibody-treated group. This increase resulted in an early, complete restoration of total marrow cellularity to the normal (+/+) littermate level. Our data suggest that NK 1.1\(^{+}\) cells exert functions critical to the negative control of hematopoietic cell proliferation in S1/S1\(^{+}\) mice.

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EXPERIMENTAL PROCEDURES

Mice. Male S1/S1\(^{+}\) mice and their +/+ littermates were purchased from Jackson Laboratories (Bar Harbor, ME) and used at an age of 6 to 8 months as bone marrow donors. Female B6D2F1 mice (DBA/2\(^{n}\) × C57BL/6\(^{n}\)) from our own breeding facility (derived originally from Jackson stock) were used at an age of 3 months as appropriate marrow recipients for the spleen colony-forming unit (CFU-S) assay. Male BALB/c mice from the National Cancer Institute Frederick Cancer Research Facility (Frederick, MD) were used at an age of 6 weeks as hybridoma recipients in the production of anti-NK 1.1 MoAb as an ascites. Mice were maintained in a facility registered by the American Association for Accreditation of Laboratory Animal Care (Washington, DC), provided with acidified water (pH 2.5) and mouse chow ad libitum, and kept in cages covered by filter hoods.

Preparation and use of the anti-NK 1.1 and control ascites. The PK 136 hybridoma producing the anti-NK 1.1 MoAb (Dr. Joseph Kaplan, Children's Hospital, Detroit, MI) was obtained originally from the same hybridoma described by Koo and Peppard.\(^{12}\) The concentration of MoAb in the ascites as well as its specific activity\(^{12}\) was the same as that described by Koo et al\(^{11}\) in that both detected about 6% of spleen cells as NK 1.1\(^{+}\) cells.

NK 1.1\(^{+}\) cells were ablated in situ as described previously.\(^{1,11}\) Briefly, male S1/S1\(^{+}\) mice and their normal (+/+ ) littermates received 2.0 mL intraperitoneal injections of ascites containing 2.0 mg anti-NK 1.1 MoAb at 3 and 2 days before the evaluation of their stem and progenitor cells.

According to our previous work,\(^{11,11}\) the control group was treated similarly but received "control" ascites (CA). Briefly, CA was induced by injection into BALB/c mice of a mouse myeloma cell (P3X63Ag8.653)\(^{12}\) similar to that of the fusion partner (Sp 2/0) of the PK 136 hybridoma.\(^{12}\) The amount of protein (about 10 mg/mL) was similar in both the MoAb-containing ascites and CA. A second control group received no treatment. Because results in the CA-treated group were equivalent to results obtained in untreated mice, only the former data are shown.

Number of marrow stem and progenitor cells. Mice were killed by cervical dislocation and a monodispersed cell suspension of bone marrow was obtained by flushing both femurs with L-15 medium (GIBCO, Grand Island, NY) at room temperature. The marrow was pooled from groups of three donor mice. Total nucleated cell (TNC) counts were obtained by hemacytometer (in duplicate) and

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fractions of the resulting cell suspension were used for assaying (1) "stem cells" committed to myelo-erythroid differentiation (day 10, CFU-S) using the spleen colony assay and (2) progenitor cells committed to granulocyte-macrophage (CFU-granulocyte-macrophage [CFU-GM]), erythroid (burst-forming unit-erythroid [BFU-E] and CFU-E) and megakaryocyte (CFU-megakaryocyte [CFU-Meg]) differentiation using a modified plasma culture technique and incubation at 5% oxygen.

Fraction of CFU in DNA synthesis (S-phase). Mice receiving CA or anti-NK 1.1 ascites were injected via the tail vein with 50 mg/mouse hydroxyurea (HU; Sigma, St Louis, MO). This dose induces the maximum amount of cell kill available for HU. One hour later, their femoral marrow was pooled and assayed for stem and progenitor cell content as described above. The fraction of these cells in S-phase was obtained as the difference between the corresponding values in the respective mouse strain.

Statistical analysis. The Student's t-test was used to test for significance of the differences between the mean values of the femoral contents and cycling (S-phase) fractions of the CFUs measured, comparing the CA- and anti-NK 1.1-treated groups, with significance denoted by P values less than .05.

RESULTS

Marrow cellularity. Table 1 summarizes the changes in marrow cellularity obtained from both +/+ and S1/S1 mice 3 days after the injection of ascites containing anti-NK 1.1 MoAb. For +/+ mice, the femoral content of TNC, CFU-S, CFU-GM, BFU-E, CFU-E, and CFU-Meg did not differ significantly from the corresponding values in CA-treated mice (Table 1A). In contrast, injecting either anti-NK 1.1 MoAb or CA into S1/S1 mice resulted in considerable differences between these two treatments (Table 1B). The CA-treatment had no effect on the hypoplasia in S1/S1 mice, with both stem and progenitor cell values remaining within the reported range (between 20% and 40% of the respective values observed in +/+ mice, Table 1A). However, anti-NK 1.1 treatment resulted in a twofold to fivefold increase in the femoral content of TNC as well as CFU-S, CFU-GM, BFU-E, and CFU-E over those values observed in the CA group (Table 1B). The increase reflected an increase in both total cellularity and concentration of the respective CFUs. Thus, the ablation of NK 1.1+ cells restored marrow cellularity in S1/S1 mice to the values obtained in their +/+ littermates (Table 1A). However, the increase was reflected in a corresponding increase in the respective CA-treated controls.

Proliferative activity. The restoration of marrow cellularity was reflected in a corresponding increase in the respective S-phase fractions of CFU-S and progenitors that was especially evident in the dramatic increase in the S-phase fraction of BFU-E from zero in CA-treated S1/S1 mice to greater than 80% in anti-NK 1.1-treated S1/S1 mice (Table 2). These responses were not observed for megakaryocyte.
oocyte progenitors in which treatment with anti-NK 1.1 MoAb reduced the S-phase fraction of CFU-Meg in +/+ mice with little, if any, effect on these parameters in S1/S1<sup>a</sup> mice.

**DISCUSSION**

Previously, we and others have focused on the physiologic relevance of NK 1.1<sup>+</sup> cells as both immunocompetent cells and negative regulators of murine hematopoiesis using injections of ascites containing anti-NK 1.1 MoAbs to ablate NK 1.1<sup>+</sup> cells in situ.<sup>49</sup> In this study, we have used this approach to gather what we believe are the first data on the physiologic relevance of NK 1.1<sup>+</sup> cells in suppressing hematopoiesis in S1/S1<sup>a</sup> mice. NK 1.1 is not expressed exclusively on CD3-NK cells,<sup>21</sup> although, at present, it represents the most specific antigen for identifying the NK cell phenotype in mice.

Our present results show that the ablation of NK 1.1<sup>+</sup> cells in S1/S1<sup>a</sup> mice led to significant increases in the cycling fraction of CFU-S, CFU-GM, CFU-E, and, especially, BFU-E. This increased proliferation initiated the restoration to normal (+/-) marrow cellularity, both in terms of the femoral content of progenitor cells and TNC count (Tables 1 and 2). We conclude that the proliferation of CFU-S and nonmegakaryocytic progenitors in S1/S1<sup>a</sup> mice was inhibited by the presence of NK 1.1<sup>+</sup> cells, with the most prominent effect observed on early erythroid progenitor cells (BFU-E). Furthermore, we observed a striking reticulocytosis (from 2% to 20%) 11 days after NK 1.1<sup>+</sup> cell ablation in S1/S1<sup>a</sup> mice that was not observed in the +/- littersmates, indicating a specific compensation of the aplastic anemia (data not shown).

Thus, NK-mediated hematopoietic suppression might provide one explanation for both the modernity of the increase in numbers of RBCs after the in vivo treatment with the c-kit ligand and the preferential impairment of RBC production in S1/S1<sup>a</sup> mice.<sup>2</sup> This result occurs despite a microenvironmental defect that primarily affects multipotent stem cells.<sup>1</sup> However, future studies are required to examine whether ligands exist for the c-kit receptor that are capable of directly or indirectly downregulating NK-mediated negative regulation of nonmegakaryocytic hematopoiesis. This would provide a link between impaired NK cell function and a mutation at the steel locus.

Previous investigators failed to demonstrate a significant difference in the lytic activity of splenic NK 1.1<sup>+</sup> cells in S1/S1<sup>a</sup> mice as compared with their +/- littermates.<sup>2</sup> It is possible that lytic activity is not closely related to the hematomodulatory activity of NK 1.1<sup>+</sup> cells, which might explain some of the inconsistency in reports<sup>1</sup> on hematomodulatory effects of NK 1.1<sup>+</sup> cells when using the YAC-cell assay to monitor these cells in marrow. It remains unclear as to whether anti-NK 1.1 MoAb treatment affects the microenvironmental defect in S1/S1<sup>a</sup> mice directly or compensates for the still-existing defect through an increase in the proliferative activity of CFU-S and nonmegakaryocytic progenitor cells.

Our present data are consistent with recent in vitro<sup>3</sup> and in vivo studies<sup>4</sup> of human and murine marrow hematopoiesis that have shown an inhibitory effect of NK 1.1<sup>+</sup> cells on the growth of CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GE(TM)), CFU-GM, BFU-E, and CFU-E and a stimulatory effect on CFU-Meg, respectively. Studies on the mechanisms by which NK 1.1<sup>+</sup> cells may exert a differential effect on hematopoietic stem and progenitor cells are inconclusive,<sup>3</sup> supporting either the release of a humoral factor (interferon-y [IFN-y] or tumor necrosis factor) or direct cell contact. Abnormalities of IFN-y production have been reported in patients with aplastic anemia.<sup>22</sup>

Considering the well-characterized role of NK cells in regulating the immune response in rodents and humans,<sup>21</sup> the present findings provide additional support for the relevance of a regulatory network linking the hematopoietic and immune cell systems. Several reports have shown a close link between the myelosuppression observed in aplastic anemia<sup>21,22</sup> and elevated numbers and/or activities of NK cells. Thus, the present findings may have some relevance for understanding the therapeutic failure of treatment with recombinant growth factors in refractory aplastic anemia.

**ACKNOWLEDGMENT**

We gratefully acknowledge the secretarial assistance of Kay Gounaris. We wish to thank Drs Fred Valeriote, Mark Edelstein, and Stephen Lerman for many helpful discussions and critical review of this manuscript.

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