Wheat germ agglutinin-positive cells in a stem cell-enriched fraction of mouse bone marrow have potent natural suppressor activity

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ABSTRACT In the present study we have characterized natural suppressor (NS) cells, which nonspecifically suppress mitogen responses and mixed-lymphocyte reaction. The strongest NS activity was found in a fraction of relatively low-density cells (1.063 < p < 1.075) obtained by equilibrium density centrifugation. Further purification and characterization of these NS cells by using a fluorescence-activated cell sorter indicated that wheat germ agglutinin (WGA)-positive cells have potent NS activity, whereas WGA-negative cells have no NS activity. Spleen colony-forming unit (CFU-S) assays demonstrated a significant correlation between the number of CFU-S cells and the NS activity in the bone marrow. However, WGA-positive cells obtained from the bone marrow of animals treated with 5-fluorouracil, which cells in turn were exposed to 5-fluorouracil, showed a marked reduction in NS activity. These results suggest that the pluripotent stem cells have NS activity when the cells are in the cycling phase but not when the cells are in the G0 phase. It seems possible that apparently primitive cells play an important role in down-regulation of immune responses.

Natural suppressor (NS) activity is defined as the ability of unprimed cells to suppress various immunological functions (1). NS cells have been found in the neonatal spleen (2, 3), in adult bone marrow (4–7), and in the splenic tissue of adult mice that have received total lymphoid irradiation (8). NS cells have been considered to be members of a natural non-specific suppressor cell family that includes natural killer (NK) and natural cytotoxic (NC) cells, which have non-T-cell and non-B-cell (null cell) phenotypes. Recently, additional data have revealed that NS cells may play a crucial role in the induction of self-tolerance and the regulation of immune responses, including prevention of lethal graft-versus-host reactions (9). However, the precise lineage and the characteristic markers of NS cells remains unclear.

Recently, a method has been established to purify pluripotent hematopoietic stem cells by using a fluorescence-activated cell sorter (FACS) (10). With this technique, we have studied the wheat germ agglutinin (WGA)-positive cells in the stem cell-enriched fraction of mouse bone marrow and have shown these cells to have potent NS activity. In addition, by measuring differences in sensitivity to 5-fluorouracil, we have found that the pluripotent stem cells exert NS activity when the stem cells are in a cycling phase but not when these cells are in the G0 phase.

MATERIALS AND METHODS

Preparation of Cells. To remove T cells, B cells, and macrophages, bone marrow cells from C57BL/6N (B6) mice were treated with anti-Thy-1.2 antibody (clone F7D5, Olac, Bicester, U.K.) and complement and were passed through both Sephadex G-10 (Pharmacia) and anti-mouse Ig-coupled Sepharose 4B (Pharmacia) columns. The cells then were fractionated by equilibrium density centrifugation on a discontinuous Percoll (Pharmacia) gradient as described by Lord and Spooner (11). For the density separation, the Percoll solution was prepared in densities of 1.090, 1.075, and 1.063 g/ml. The pH and osmolality (osM) of these solutions were adjusted to pH 7.0 and 300 mosM/kg. After centrifugation at 1400 x g for 30 min, cells were collected in fractions of various densities: p < 1.063 (fraction 1); 1.063 < p < 1.075 (fraction 2), 1.075 < p < 1.090 (fraction 3), and 1.090 < p (fraction 4).

Cell Sorting. Cells in fraction 2 were labeled with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA-FITC) at 0.4 µg/ml and sorted on a fluorescence-activated cell sorter (FACS; FACStar, Becton Dickinson). Electronic windows were set to select highly WGA-FITC-labeled cells with moderate-forward and low-perpendicular backscatter as described by Spooner et al. (10).

5-Fluorouracil Treatment. 5-Fluorouracil (Kyowa Hakko, Tokyo) was injected intravenously into mice (150 mg/kg of body weight). The mice were sacrificed 36 hr later, and bone marrow cells were collected (5 x 10^6 cells per ml) and then treated again in vitro with 5-fluorouracil (5 µg/ml) for 2 hr at 37°C.

Assay of NS Activity. Bone marrow cells were assayed for NS activity in mixed lymphocyte reaction (MLR) or mitogen responses.

MLR. MLR was determined by measuring the incorporation of 0.5 µCi (1 Ci = 37 GBq) of [3H]thymidine (New England Nuclear) in DNA as described (12). Triplicate cultures were set up in 96-well round-bottom microtiter plates (Corning). Each well contained 4.5 x 10^5 responder cells (BALB/c spleen cells), and various concentrations of B6 bone mouse marrow cells (as the source of NS cells) in a total volume of 0.2 ml of RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum and 50 µM 2-mercaptoethanol (Wako Pure Chemical Industries, Tokyo). The cultures were incubated for 96 hr at 37°C in humidified 5% CO2/95% air. [3H]Thymidine was present during the last 4 hr of the culture period. Percent suppression was calculated by the following formula: % suppression = 1 – (cpm with BMC/cpm without BMC) x 100, in which BMC = bone marrow cells.

Mitogen responses. Mitogen responses were determined by measuring incorporation of [3H]thymidine into DNA.

Abbreviations: NS, natural suppressor; NK, natural killer; NC, natural cytotoxic; FACS, fluorescence-activated cell sorter; WGA, wheat germ agglutinin; B6 mice, C57BL/6N mice; MLR, mixed lymphocyte reaction; FCS, fetal calf serum; LPS, lipopolysaccharide; CFU-S, spleen colony-forming unit.

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**RESULTS AND DISCUSSION**

To remove T cells, B cells, and macrophages, bone marrow cells from B6 mice were treated with anti-Thy-1.2 antibody and complement and were passed serially through both Sephadex G-10 and anti-mouse IgG-coupled Sepharose columns. We first confirmed that the cells obtained in this manner have nonspecific suppressive activity in MLR and mitogen responses—features described by Claman and coworkers for NS cells (1). To characterize the NS cells, the cells were further fractionated by equilibrium density centrifugation (11). Cells from a low-density fraction (fraction 2: 1.063 < ρ < 1.075) showed the strongest NS activity against either of the two mitogen responses (concanavalin A and LPS responses) or the responses in MLR (Fig. 1). These findings are largely consistent with those of Dorshkind and Rosse (5). To further purify the NS cells, fraction 2 cells were labeled with WGA-FITC and analyzed with a FACS (FACStar) (10).

WGA− cells in fraction 2 had no significant NS activity (Table 1). In contrast, WGA+ cells in fraction 2 had marked NS activity inhibiting either MLR or LPS responses. It should be noted that 3 × 10^5 WGA+ cells (about 1/100th of responder cells) suppressed LPS response or MLR response by approximately 40%. CFU-S assays revealed that the number of CFU-S on both day 8 (1025) and day 12 (1280) (data not shown in Fig. 2) among the WGA+ cells of fraction 2 was about 50 times greater than the number of CFU-S (20) in the original bone marrow cells (Fig. 2). Thus, there was a significant correlation between the number of CFU-S and the activity in the NS assay. It appears likely, therefore, that WGA+ stem cells exhibit NS activity.

The next step was to investigate whether the stem cells with NS activity are in the cycling or the noncyling (Go) phase. It has been demonstrated that early multipotential stem cells (Go stem cells) are not affected by 5-fluorouracil treatment (14). Therefore, we used the procedure described above to separate bone marrow cells from 5-fluorouracil-treated mice. The WGA+ cells (1 × 10^5) in fraction 2 produced 1380 CFU-S on day 12 (but no CFU-S on day 8), whereas the WGA− cells in fraction 2 produced no CFU-S on days 8 or 12. The WGA+ cells from 5-fluorouracil-treated bone marrow showed a marked reduction in NS activity in MLR and mitogen responses. FACs analysis with acridine orange revealed that both the RNA and DNA content of these WGA+ cells are very low. These results therefore indicate

Table 1. Enrichment of NS cells in WGA+ cells of low-density fraction

<table>
<thead>
<tr>
<th>Assay</th>
<th>BM Cells added</th>
<th>Number of BM cells added, Δcpm ± SD (% suppression)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>MLR*</td>
<td>None</td>
<td>19,519 ± 2605</td>
</tr>
<tr>
<td></td>
<td>Original BM</td>
<td>13,926 ± 6732 (29)</td>
</tr>
<tr>
<td></td>
<td>Fraction 2</td>
<td>11,190 ± 5480 (43)</td>
</tr>
<tr>
<td></td>
<td>Fraction 2 WGA+</td>
<td>20,232 ± 4450 (0)</td>
</tr>
<tr>
<td>LPS†</td>
<td>None</td>
<td>21,423 ± 2060</td>
</tr>
<tr>
<td></td>
<td>Original BM</td>
<td>15,978 ± 608 (25)</td>
</tr>
<tr>
<td></td>
<td>Fraction 2</td>
<td>13,568 ± 2962 (37)</td>
</tr>
<tr>
<td></td>
<td>Fraction 2 WGA+</td>
<td>22,873 ± 1124 (0)</td>
</tr>
</tbody>
</table>

*NS activity in MLR was determined by culturing 4.5 × 10^5 responder cells (BALB/c spleen cells), 7.5 × 10^5 irradiated (20 Gy) stimulator cells (B6 spleen cells), and various concentrations of B6 BM cells (as NS cells).

†NS activity in LPS response was determined by culturing 5 × 10^5 cells from B6 spleen with various concentrations of B6 BM cells (as NS cells) in the presence of 25 μg of LPS per ml.

**Fig. 1.** Enrichment of NS cells in the low-density fraction (fraction 2). NS activity was determined by assays for mitogen (Con A and LPS) responses and MLR. Triplicate cultures were set up in wells of flat-bottom microtiter plates (Corning), each containing 5 × 10^5 cells from B6 mouse spleen in 0.2 ml of RPMI 1640 medium containing 5% fetal calf serum. The cells were cocultured with various concentrations of B6 mouse bone marrow cells (as NS cells) in the presence of concanavalin A (Con A; Calbiochem) at 5.0 μg/ml or Escherichia coli lipopolysaccharide (LPS; Difco) at 25 μg/ml. The cultures were incubated for 72 hr at 37°C in humidified 3% CO2/95% air. [3H]Thymidine was present during the last 4 hr of the culture period.

**Spleen Colony-Forming Unit (CFU-S) Assay.** Twenty-four hours before cell transfer, recipient mice were irradiated at 8.0 Gy by a 60Co γ-ray emitter. Appropriate concentrations of cells were injected into the recipient mice to determine the CFU-S content. The mice were sacrificed 8 and 12 days later, and their spleens were removed and fixed in Bouin's solution. Visible surface colonies were counted.

**Cell-Cycle Determination.** The cell-cycle distribution of bone marrow cells was measured by flow cytometry after differential staining of cellular DNA and RNA with the metachromatic dye acridine orange as described by Darzynkiewicz et al. (13). Approximately 5 × 10^5 cells were suspended in 0.2 ml of phosphate-buffered saline and mixed with 0.5 ml of 0.1% Triton X-100 (Sigma)/0.2 M sucrose/0.1 mM EDTA/20 mM citrated phosphate buffer, pH 3.0. One minute later, the cells were stained by addition of 1 ml of 0.002% acridine orange (Polyscience, Warrington, PA)/0.1 M NaCl/20 mM citrated phosphate buffer, pH 3.8. After 5 min of equilibration at room temperature, the fluorescence intensity was measured on a flow cytometer (Ortho Spectrum III, Ortho Diagnostics).
that WGA + stem cells exert NS activity when in the cycling phase but not when in the G₀ phase.

Further analysis of cell-surface markers revealed that cycling stem cells with NS activity are asialo GM-1−, Ia−, Thy-1−, and surface Ig−. In addition, they were found to kill neither the NC target Meth A nor the NK target YAC-1. However, since it has been reported that NS activity was found in cells that possess markers such as Thy-1, surface Ig, Mac-1, asialo GM1, or Ly-5 (1, 2), it may be that cells that are in the process of differentiation from WGA + stem cells have NS activity. The data in Table 1 reveal that WGA + stem cells show potent NS activity; a single WGA + stem cell in the cycling phase almost completely suppresses the responses of 50 effector cells.

It is noteworthy that NS cells are present in hematopoietic organs such as bone marrow and fetal liver, and that NS activity is augmented during recovery from lymphohemopoietic tissue damage, including damage from total lymphoid irradiation, cyclophosphamide treatment, or chronic graft-versus-host disease after bone marrow transplantation (1).

The biological and clinical significance of NS cells is still speculative, but increasingly, data show that NS cells play a crucial role in induction of self-tolerance and in downregulation of immune responses, including the prevention of lethal graft-versus-host disease (8). Further investigations of these suppressor functions should contribute to elucidation of the real biological role of WGA + stem cells in the cycling phase.

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