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Strain Specific Production of a Negative Regulator of IL-3 (NIL-3): Difference in the Negative Feedback Mechanism of Hemopoiesis among Mouse Strains

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ABSTRACT. **The producing cells of the negative regulator of interleukin-3 (NIL-3) were investigated. The 5-fluorouracil-treated bone marrow cells did not produce NIL-3. The bone marrow cells of stem cell-depleted W/W^V mouse did not produce the NIL-3, either.**

The production of NIL-3 was different among mouse strains. Mice of C3H/HeN, A/J and ICR strains produced NIL-3, but the C57BL/6 mice did not produce NIL-3. These results indicate that the negative feedback mechanism of hemopoiesis is different among mouse strains.

In the present study, we could not definitely identify the NIL-3 producing cells, although the present results are suggestive that the stem cells in cycle are a NIL-3 producer. Instead, we found that hemopoietic regulatory mechanisms might be different among mouse strains, especially in C57BL/6 mice.

We have previously reported a production of negative regulator of interleukin-3 from the bone marrow cells of C3H/HeN mice in response to the stimulation of interleukin-3 (IL-3).

A number of negative regulators have been reported; macrophage inflammatory protein-1α (MIP-1α) (6, 11, 22), transforming growth factor-β (TGF-β) (3, 7, 21), tetra-peptide (AcSDKP) (5, 10), hemoregulaory peptide (HP) (9, 13), negative regulatory protein (NRP) (1, 4) etc. But all of them are tissue extracts and may not be physiological negative regulators which should be produced only in response to an excess stimuli with stimulator(s). NIL-3 is produced from the bone marrow cells only in response to IL-3 stimulation, indicating that NIL-3 is in fact a physiological regulator of hemopoiesis.

Production of NIL-3 was dependent on the concentration of IL-3 in the culture medium, and NIL-3 was not produced without IL-3 stimulation. Heat-treatment of the sample at 70°C for 30 minutes inactivated the NIL-3 completely, indicating that NIL-3 is heat-labile (18). Fractionation of bone marrow cells by density gradient centrifugation revealed that NIL-3 was produced from stem cell-enriched fraction ($1.061 \leq \rho < 1.074$), but not from the granulocyte-macrophage-enriched fraction, lymphocyte-enriched fraction or erythrocyte-enriched fraction (18). Five-fluorouracil treatment of the stem cell-enriched fraction suppressed the NIL-3 production completely.

To elucidate whether or not the NIL-3 producing cells were stem cells, production of NIL-3 from bone marrow cells of stem cell-depleted W/W^V mouse was studied. Neither the low density fraction ($1.061 \leq \rho < 1.074$), nor the unfractionated total bone marrow cells of W/W^V mouse produced NIL-3.

To confirm whether the lack of NIL-3 production in W/W^V mouse is due to the depletion of stem cells or due to the strain of mice used, we tested NIL-3 production from the bone marrow cells of C57BL/6 as normal control mice as well as other strains of mice.

Mice of C57BL/6, +/+ and DBA/2 strain scarcely produced NIL-3, whereas mice of C3H/HeN, A/J and ICR strain produced a substantial level of NIL-3, indicating the strain dependent difference in the regulatory mechanism of hemopoiesis.

MATERIALS AND METHODS

Cells. IL-3 producing T cell line (STIL-3 C5) cells were maintained in RPMI-1640 medium supplemented with 5% (v/v) FCS. The conditioned medium was filtered and stored at −70°C (15).

DA-1 cells, an IL-3 dependent cell line (8), were maintained in RPMI-1640 medium supplemented with 10% (v/v) FCS and 10% (v/v) STIL-3 C5 conditioned medium as a source of IL-3.

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Abbreviations: IL-3, interleukin-3; NIL-3, negative regulator of interleukin-3; FCS, fetal calf serum; HS, horse serum; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte/macrophage colony stimulating factor.

Mice. Mice of C3H/HeN (female), DBA/2 (female), A/J (female), C57BL/6 (female), W/W^V (WB-$W/+ \times$C57BL/6-$W^V/+$, F1) (female), $+/+$ (female) and ICR (female) strains were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan), housed under specific pathogen free conditions and used at 8 to 12 weeks of age.

Fractionation of bone marrow cells. Fractionation of bone marrow cells was carried out by Percoll discontinuous density centrifugation method. The details were described in reference (19). Non-adherent low density fraction (Fr.2: $1.061 \leq \rho < 1.074$) of bone marrow cells was used as stem cell-enriched population for the assay of NIL-3 production. The treatment enriches stem cells by a factor of 20, without enrichment of other cell populations (19).

To kill the cells in cycle, 5-fluorouracil-treatment was carried out (2). Briefly, C3H/HeN mice were injected intravenously with 150 mg/kg body weight of 5-fluorouracil. The mice were sacrificed 36 hours later, and bone marrow cells were re-treated with 2 μg/ml of 5-fluorouracil for 1 hour at 37°C. After the treatment, cells were washed with serum supplemented medium, and used for the NIL-3-production assay.

Culture of total or fractionated bone marrow cells with native IL-3. For the induction of the NIL-3 production, IL-3 was added to the culture medium (18). Briefly, total or fractionated bone marrow cells were cultured at the density of 100 cells/ml in RPMI-1640 medium supplemented with 50% (v/v) STIL-3 C5 conditioned medium and 5% (v/v) FCS. IL-3 activity of the STIL-3 C5 conditioned medium was about 800 U/ml (14).

Assay for NIL-3 activity. An aliquot of the samples was heated at 70°C for 30 minutes before the assay of the NIL-3 activity. NIL-3 activity was completely inactivated by the treatment, but the IL-3 activity was resistant to the heat treatment (18).

DA-1 cells were washed three times with RPMI-1640 medium containing 0.3% (v/v) FCS to remove IL-3, and cultivated at 4×10^4 cells/ml in RPMI-1640 medium supplemented with 10% (v/v) FCS with or without normal (untreated) or heat-treated test samples. Cultures (100 μl) were performed in quadruplicate in 96-well microtiter plates (Falcon 3072) and incubated for 96 hours at 37°C and 5% CO_2 in a fully humidified atmosphere. Proliferation of DA-1 cells was monitored by the 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT Chemicon Int. Inc.) assay.

In this report, NIL-3 activity was calculated by the following formula, because the samples of this step contain high levels of IL-3. Inhibition (%) $= 100 \times$ [1-(Growth of DA-1 cells with native sample)/(Growth of DA-1 cells with heat-treated sample)].

RESULTS

Production of NIL-3 from bone marrow cells in cycle. Bone marrow cells in cell cycle were removed by 5-FU treatment. The remaining bone marrow cells in resting state did not produce NIL-3, suggesting that cells in cycle produce NIL-3 (Fig. 1). As described previously (18), no NIL-3 production was observed without IL-3 stimulation from the total bone marrow cells, either (data not shown).

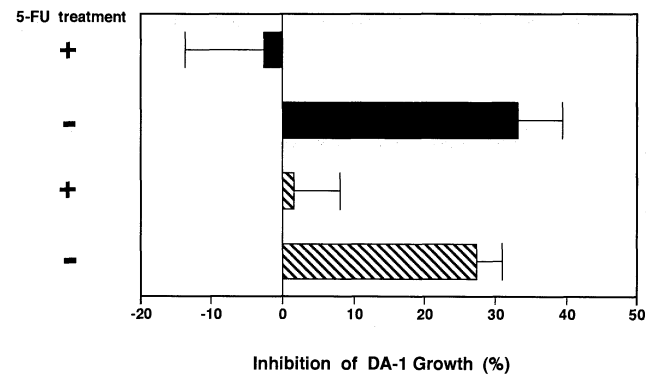

Fig. 1. Effect of 5-FU treatment on NIL-3 production. IL-3 activity of the conditioned medium was measured by the growth of IL-3-dependent DA-1 cells. NIL-3 activity was calculated by the formula indicated in materials and methods. Solid column represents NIL-3 activity produced from fractionated bone marrow cells (Fr.2: $1.061 \leq \rho < 1.074$), and shaded column represents the NIL-3 activity produced from total bone marrow cells. +: treated with 5-FU, −: not-treated. Data represents Mean±S.E.

Lack of NIL-3 production from the W/W^V mice bone marrow cells. To elucidate whether or not NIL-3 producing cells were stem cells, bone marrow cells of stem cell-depleted W/W^V mice were used.

No NIL-3 production was observed from the bone marrow cells of W/W^V mice regardless of IL-3 stimulation. But, the bone marrow cells of $+/+$ mice studied

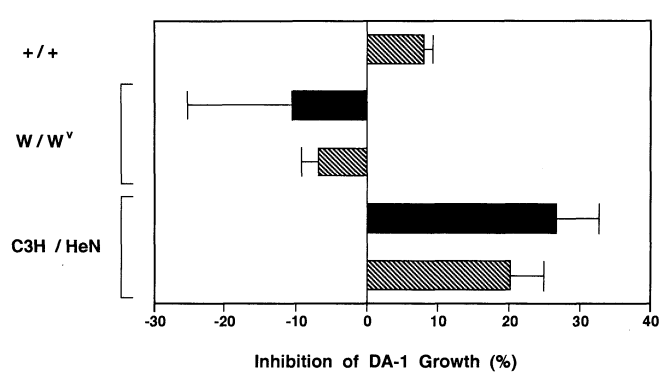

Fig. 2. Lack of NIL-3 production from the W/W^V mouse bone marrow cells. Fractionated or total bone marrow cells of C3H/HeN, W/W^V or $+/+$ mice were cultured in the IL-3-supplemented medium. NIL-3 activity was presented as in Figure 1. Solid column represents NIL-3 activity produced from fractionated bone marrow cells (Fr.2: $1.061 \leq \rho < 1.074$), and shaded column represents NIL-3 activity produced from total bone marrow cells. Data represents Mean± S.E.

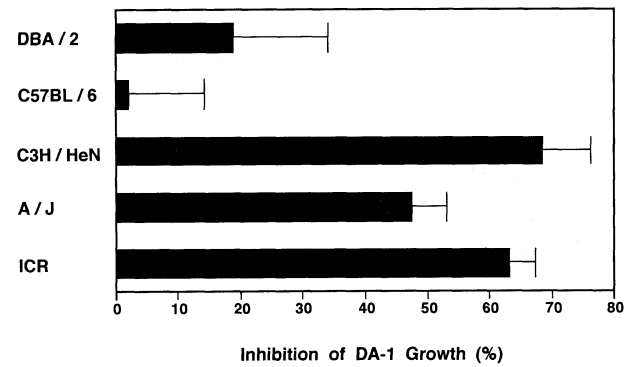

Fig. 3. Strain specific production of NIL-3. Fractionated bone marrow cells from the different strains of mice were cultured in the IL-3-supplemented medium. NIL-3 activity was shown as in Figure 1. Data represents Mean±S.E.

as control did not produce the NIL-3 (Fig. 2), either. We failed to prove the possibility that NIL-3 was produced by the stem cells in cycle. The results suggest instead that the production of NIL-3 is dependent on mouse strain.

Strain specific production of NIL-3. We have reported that response of bone marrow cells to IL-3 stimulation, manifested as the production of G- and GM-CSF, was different among mouse strains (23). Accordingly, we investigated possible strain-dependent differences in the production of NIL-3. This was also confirmed in NIL-3 production.

Mice of C3H/HeN, A/J and ICR strains produced NIL-3. The production of NIL-3 from the bone marrow cells of DBA/2 strain was significantly low compared to the above strains. Bone marrow cells of C57BL/6 mouse scarcely produced NIL-3 (Fig. 3). These results indicate that the negative feedback mechanism against excess IL-3-stimulation is different among mouse strains.

DISCUSSION

Hemopoiesis is regulated by a number of cytokines and cytokine network. We have reported that the production of interleukin-6 (IL-6) was hierarchically regulated by IL-3 (12). Production of granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) was also induced by IL-3 stimulation (20). Thus, IL-3 stimulates and enhances hemopoiesis via the production of other cytokines and interleukins, indicating the presence of a positive feedback mechanism in granulopoiesis.

On the other hand, the hemopoietic system produces

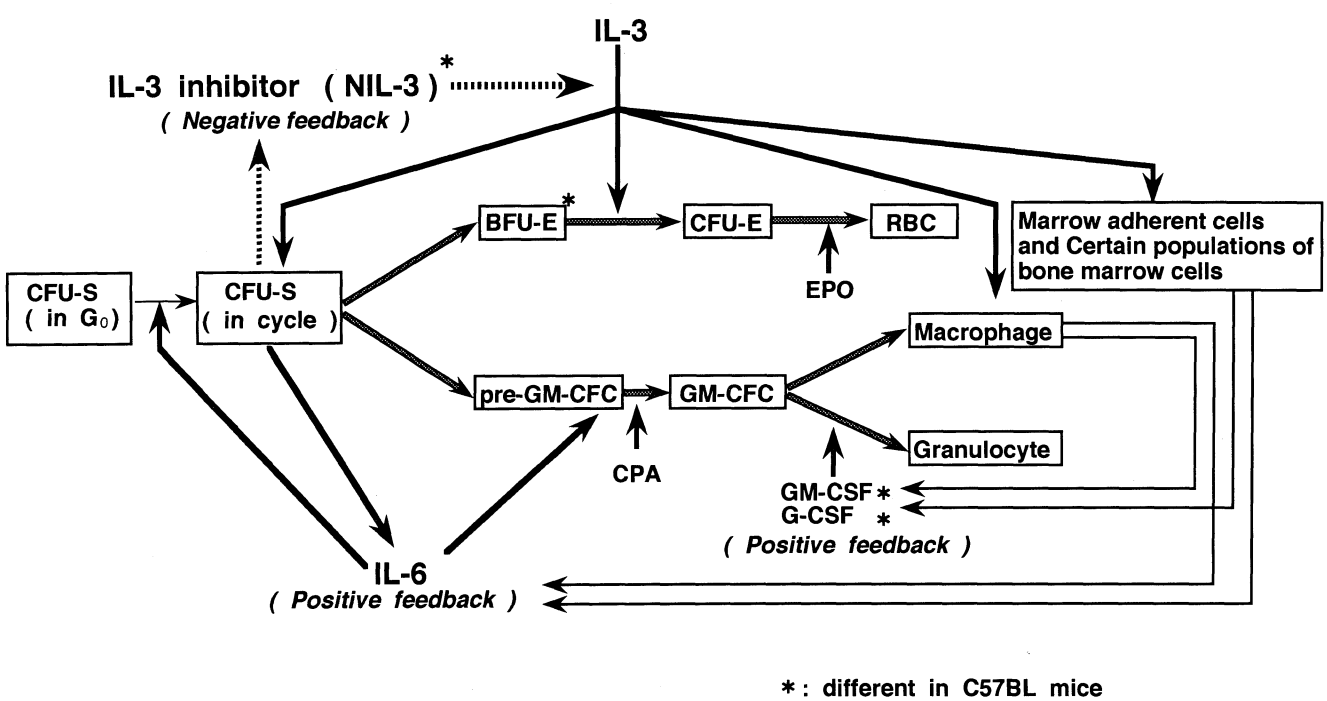

Fig. 4. Possible feedback network of cytokine production in granulopoiesis. Certain populations of hemopoietic system produce IL-3. IL-3 thus produced stimulates bone marrow adherent cells, macrophages and stromal cells to produce IL-6, GM-CSF and G-CSF, resulting in an accelerated granulopoiesis. On the other hand, excess stimulation of bone marrow cells in cell cycle with IL-3 resulted in the production of NIL-3, which would prevent mice from the exhaustion of hemopoietic stem cells.

a negative regulator antagonistic to IL-3 perhaps to compensate for excess stimulation of IL-3. This would protect mice from exhaustion of hemopoietic stem cells. NIL-3 is one such negative regulators.

Present studies have revealed that NIL-3 producing cells are IL-3-responsive bone marrow cells in cycle. Although lack of FACS in our university or nearby institution prevented us from performing a cell sorter analysis of the NIL-3 producing cells, the NIL-3-producing cells appear in the stem cells in cycle, because neither the 5-fluorouracil treated bone marrow cells nor bone marrow cells of stem cell-depleted W/W^V mouse produced NIL-3. In the presence of NIL-3, granulopoiesis might be suppressed. In fact, by the excess stimulation of IL-3 in STIL-3 bearing mice, the features of granulocytes showed two phases. At first the number of granulocytes increased, and then the number decreased gradually (16, 17). Production of NIL-3 was observed in the latter phase (unpublished observations). This represents a negative feedback mechanism of hemopoiesis. These positive and negative feedback mechanisms appear to play a key role in the regulation of granulopoiesis (Fig. 4).

We found also in the present study that some strains of mice did not produce NIL-3. We have previously reported strain-dependent differences in the production of G-, and GM-CSF from the bone marrow adherent cells and macrophages in response to IL-3 (23). The observations indicated that positive feedback regulatory mechanism of hemopoiesis was different among mouse strains. We have shown herein that the negative feedback regulatory mechanism differs also among mouse strains. These differences in the positive and negative feedback mechanisms indicate that there are delicate differences in the hemopoietic regulatory mechanisms among mouse strains.

As described previously, semi-purification of NIL-3 was carried out with hydrophobic interaction chromatography, ion-exchange chromatography and gel filtration chromatography (18). The semi-purified NIL-3 was a protein with a relative molecular mass (*Mr*) of 57,000 (gel filtration) (unpublished data). Further determination of amino acids sequence is in progress.

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