Interleukin 3 Perfusion in W/W^v Mice Allows the Development of Macroscopic Hematopoietic Spleen Colonies and Restores Cutaneous Mast Cell Number

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Summary

The genetically anemic W/W^v mice are characterized by the inability of their bone marrow cells to form macroscopic pluripotent hematopoietic colonies in the spleen of irradiated recipients upon transfer (colony-forming units). Furthermore, they almost totally lack mast cells, notably in the skin. In the present study, we have tested the effect of recombinant murine interleukin 3 (rmIL-3) on W/W^v mice hematopoiesis. Transfer of W/W^v bone marrow cells into lethally irradiated recipients perfused with rmIL-3 is followed by the appearance of macroscopic spleen colonies. Moreover, perfusion of rmIL-3 in W/W^v mice: (a) restores almost normal total numbers of hematopoietic precursors (colony-forming cells), but without modification of anemia; and (b) leads to the appearance of a normal number of mastocytes in the skin.

The W^v mutation at the W locus is characterized among other features by a macrocytic anemia, a total absence of mature subcutaneous mast cells, a low resistance to irradiation, and the inability of bone marrow cells to form macroscopic hematopoietic colonies in the spleen of lethally irradiated recipients (CFUs). That all these features result from the existence of a hematopoietic cell defect is shown by the fact that bone marrow transplantation from histocompatible $^+/+$ mice results in a complete and permanent cure of these hematopoietic alterations (for review, see reference 1).

These and other abnormalities present in these mice (deficiency in the production of germ cells and melanoblasts resulting, respectively, in sterility and absence of coat pigmentation) appear to result from a defect in the c-kit proto-oncogene, the product of which is a membrane receptor with a structure very close to that of the macrophage CSF (M-CSF) (2, 3), which acts on macrophages and their precursors.

Since we have previously observed that perfusion of recombinant murine IL-3 (rmIL-3) increases hematopoiesis in all myeloid lineages, as well as mast cells (4), we explored the effect of rmIL-3 perfusion on W/W^v mice.

Materials and Methods

Cell Suspensions and Culture. Spleen and bone marrow cells were obtained as previously described (4). Nonparenchymatous hepatic cells were obtained according to Wiltroot's technique (5). The colony-forming cell assay (CFC) was performed in 35-mm diameter petri dishes (Greiner, Nürtingen, FRG) using 0.8% methylcellulose (Methocel Fluka AG, Buchs, Switzerland), according to Eliason et al. (6), with a 10% FCS concentration (Seromed Fakola AG,

Basel Switzerland) instead of purified BSA. rmIL-3 (4) was used at a supraoptimal concentration of 450 U/ml (unless otherwise stated); erythropoietin step II (EPO) (Caunaught Laboratories, Vancouver, Canada) was added at a final concentration of 3 U/ml. Plating concentrations were 2.5 × 10^4 for bone marrow and 10^5 cells/ml for the spleen and liver. CFC were identified and scored with a grid under phase contrast using an inverted microscope with a 6.3 or 10× objective.

Mice and Perfusion. WBB6F$_1$-W/W^v mice (W/W^v), W^v/+ heterozygotes, and normal WBB6F$_1$-$^+/+$ ($^+/+$) littermates were obtained from The Jackson Laboratory (Bar Harbor, ME). Adult males and females, 12–36 wk old, were used. Alzet mini-osmotic pumps (2002; Alza Corp., Palo Alto, CA) were placed intraperitoneally. The pumps contained either purified rmIL-3 at concentrations ranging from 112 to 220 µg/liter) or control solution consisting of the same solvent (10% glycerol in PBS) with 1–5 ng LPS/ml, as previously described (4). Animals were killed after 13–19 d. In the case of the 28-d perfusion, a second pump was placed subcutaneously after 14 d.

Spleen CFU Assay. 10^5 W/W^v bone marrow cells were injected intravenously in 17-wk-old W/+ mice that had been lethally irradiated (1,000 rad from a ^{137}Cs source). Two groups were injected, one without treatment, and another receiving rmIL-3 subcutaneously for 8 d (27 µg/mouse; Alzet 2001; Alza Corp.). Mice were killed at day 10. Colonies were scored after fixation in Bouin's solution. Histological sections were examined after hematoxylin-eosin staining.

Preparation of Spleen Cell Supernatants. Spleen cells at a concentration of 5 × 10^6 cells/ml were incubated for 24 or 48 h with or without Con A (5 µg/ml) in DME containing 2% FCS. Supernatants were centrifuged and filtered through a filter (0.22-µ pore size) before being tested on an IL-3-dependent cell line for quantification of IL-3 production (7).

Histology. Examination for the presence of mastocytes in paraffin-embedded sections was performed after fixation of the skin in neutral formol. Sections were stained with toluidine blue and alcian blue 8 GX-safranin O (8). Mast cells were scored in 10 different fields (0.04 mm²).

Results

IL-3 Perfusion Stimulates Hematopoiesis in W/W^v Mice but Does Not Improve the Anemia. After 13–19 d of rmIL-3 perfusion in W/W^v and $^+/_+$, the spleen was enlarged, and numerous hematopoietic foci were observed on histological sections of spleen and liver, as already reported for normal mice (4). To quantify this hematopoietic enhancement, CFC assays were performed with bone marrow, spleen, and non-parenchymatous liver cells. As previously observed (4), rmIL-3 perfusion resulted in a shift of hematopoiesis from the bone marrow to the spleen and liver, where the number of CFC was massively augmented (Table 1). However, due to the fact that splenic and hepatic contribution to hematopoiesis is low when compared with that of bone marrow, rmIL-3 induced only a modest calculated enhancement of total hematopoiesis. W/W^v mice, which have about four times less splenic CFC than $^+/_+$ mice (Table 1), also showed a considerable increase in spleen and liver CFC under rmIL-3 perfusion, with a total amount of CFC reaching a value close to that observed in normal mice without IL-3. Using supraoptimal doses of rmIL-3 and EPO for the in vitro assay, the increase in early erythropoietic precursors (BFU-E) per spleen was comparable in $^+/_+$ and W/W^v mice (6 vs. 174 × 10³ and 2 vs. 64 × 10³ per spleen in each strain of mice, respectively). However, total BFU-E enhancement per mouse, calculated as in Table 1, was moderate. Since in previous experiments rmIL-3 perfusion in normal mice increases the total BFU-E rather than the CFU-E content, CFU-E were not determined in the

present experiments. No improvement in the anemia was observed: the number of RBC per ml of blood was 5.0 ± 0.5 × 10⁹ and 5.2 ± 0.2 × 10⁹ in PBS and IL-3 treated W/W^v mice, and 9.0 ± 0.3 × 10⁹ and 8.3 ± 0.4 × 10⁹ in comparable groups of $^+/_+$ mice (mean ± SEM of four experiments with two or three mice in each group). Even when rmIL-3 perfusion was prolonged for 28 d, the RBC number and the hematocrit of the W/W^v did not change (29 ± 2 vs. 30 ± 1% for untreated mice). In contrast, rmIL-3 perfusion had a marked effect on leukocytosis, which increased about six times (23 ± 13 × 10⁶ vs. 38 ± 15 × 10⁶ cells/ml for the W/W^v and $^+/_+$ mice, respectively); this increase consisted in large part of polymorphonuclear cells: 36 ± 10 and 57 ± 3% for the W/W^v and $^+/_+$, respectively (percent of PMN relative to total white blood cells in untreated animals ranged between 11 and 20).

W/W^v Bone Marrow Cells Give Rise to Macroscopic Colonies When Injected into Recipients Perfused with IL-3. When irradiated recipients of W/W^v bone marrow cells were perfused with rmIL-3 for 8 d after transfer, macroscopic colonies, undetectable without rmIL-3 perfusion, were observed on the 10th day (Fig. 1). Histologically, these colonies were not distinguishable when compared with those observed in $^+/_+$ irradiated mice injected with $^+/_+$ bone marrow cells, in particular with respect to their content in erythroid cells, all types of hematopoietic cells being observed. However, in this assay, the number of bone marrow–derived CFUs from W/W^v mice was deficient, since 6 ± 2 CFUs per 10⁵ injected W/W^v bone marrow cells were recovered, compared with 54 ± 10 CFUs scored for the same number of $^+/_+$ bone marrow cells injected to unperfused mice.

IL-3 Perfusion Corrects the Cutaneous Mast Cell Depletion of W/W^v Mice. After 28 d of rmIL-3 perfusion, mature cutaneous mast cells, which cannot be detected on skin sections of untreated W/W^v mice, were almost as numerous as in

Table 1. *Effects of IL-3 Perfusion on Hematopoietic Progenitors*

| | IL-3 (450 U/ml) + EPO (3 U/ml) | | | |
| | $^+/_+$ | | W/W^v | |
Organ	PBS-perfused CFC/organ	IL-3-perfused CFC/organ	PBS-perfused CFC/organ	IL-3-perfused CFC/organ
		× 10³		
Femur	61 (4)	22 (2)	43 (6)	32 (4)
Spleen	71 (3)	948 (3)	14 (4)	429 (4)
Liver	0.6 (1)	27 (1)	0.06 (2)	58 (2)
Total per mouse	1,292*	1,415*	874*	1,127*

Numbers in parentheses indicate numbers of experiments. Each experiment consisted of two to three cultures from two to three mice for each condition. Total number of CFC (erythroid + nonerythroid) was scored on day 9–11.
* Based on the assumption that one femur contains 5% of total bone marrow. Total bone marrow, total splenic, and total hepatic CFC were added.

Figure 1. Spleens of lethally irradiated +/+ mice after transfer of W/Wᵛ bone marrow cells (×10). (A) Under rmIL-3 perfusion for 8 d, there is the presence of macroscopic colonies. (B) Without IL-3 (osmotic pumps with solvent alone), no colonies are seen.

the skin of untreated +/+ mice: 10.5 ± 0.7 per microscopic field (0.02 mm²), compared with 14.5 ± 3.5 in +/+ mice and 23 ± 5 in rmIL-3-treated +/+ mice (Fig. 2). These cells stain positively with safranin, indicating that they are indeed of the serosal type.

W/Wᵛ Mice Have No Defect in Responsiveness to, and in Production of, IL-3. In a CFC assay, no difference in dose-response to rmIL-3 was detected between bone marrow cells of W/Wᵛ and +/+ mice over a range of concentrations varying between 2,250 and 0.23 U/ml. The in vitro release of IL-3 by spleen cells of W/Wᵛ or +/+ mice stimulated by Con A for 24 or 48 h was also found very similar (data not shown).

Discussion

Two striking effects of prolonged rmIL-3 perfusion on the hematopoietic defects of W/Wᵛ mice were observed in the present study: (a) the restoration of the capacity of early hematopoietic progenitors to undergo, in the spleen of irradiated recipients perfused with rmIL-3, the proliferative expansion giving rise to macroscopic multilineage hematopoietic colonies; and (b) the restoration of subcutaneous mast cells, the bone marrow origin of which is known (9). In culture, IL-3 allows the growth of mucosal mast cells, but it has been shown by transfer experiments that the mucosal or connective tissue mast cell phenotype is regulated by the tissue microenvironment; thus, it is not surprising that these restored cutaneous mast cells were stained by safranin, a characteristic of connective tissue mast cells (10). A similar observation has been made in the skin of aged W/Wᵛ mice of C57L/6 genetic background when they develop a dermatitis in which mast cells progressively accumulate (11).

On the other hand, in spite of an enhancement in the number of early erythropoietic precursors (BFU-E), IL-3 perfusion, even prolonged during 4 wk, failed to significantly correct the anemia of W/Wᵛ mice. This indicates that the anemia does not only result from an insufficient expansion of early erythropoietic precursors. It is unlikely, however, to simply reflect a relative lack of erythropoietin required for the final maturation of BFU-E progeny, since W/Wᵛ mice have high erythropoietin levels.

IL-3 does not interact with the c-kit receptor, which is absent or defective in W/Wᵛ mice, since the bone marrow hematopoietic progenitors of W/Wᵛ or +/+ mice display the same response in vitro to a wide range of IL-3 concentration; the gene of the mouse IL-3-R has recently been cloned (12), and it is totally unrelated to the c-kit gene. The ligand of the c-kit receptor is still unknown; it appears to be released or presented, among other sources, by cells of the bone marrow stroma and by spleen fibroblasts (13, 14). It is the lack of
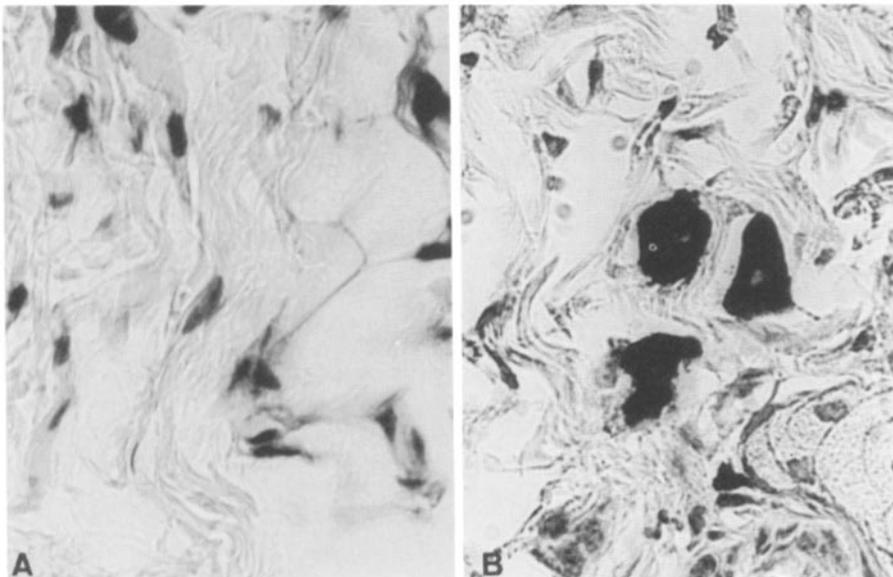

Figure 2. Mast cells are absent from the skin of untreated W/Wᵛ mice. (A), but are present after rmIL-3 perfusion (28 d) (B). (toluidine blue staining; ×200).

this ligand that is likely to characterize the S1/S1^d mutant mice, which are phenotypically identical to W/W^v mice, but which contain early hematopoietic progenitors giving rise to normal macroscopic colonies when injected into irradiated $^+/_+$ mice, or into W/W^v mice, curing their anemia and their mast cell defect (1). Thus, it appears that, in the mouse, at least two hematopoietic growth factors promote the expansion and pluripotent differentiation of early hematopoietic progenitors: IL-3, which may not be involved in basal hematopoiesis since lymphocytes are its major source, and the ligand of the c-kit receptor, a soluble, membrane-bound or matrice-bound growth factor that, in some way, is also necessary for complete RBC maturation.

It has recently been shown that the murine EPO receptor (EPO-R) can display either high or low affinity for EPO, and it has been suggested that this EPO-R might require interaction with another receptor to function as a high affinity receptor (15). If there were some kind of functional cooperation between the EPO-R and the c-kit receptor bound to its ligand, the EPO-R of W/W^v or S1/S1^d mice would be present only in the low affinity state. Such a mechanism could then account for the inability of IL-3 perfusion to cure the anemia of these mice in spite of its effects on hematopoietic progenitors.

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