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22 October 1976; revised 17 December 1976

Theta-Sensitive Cell and Erythropoiesis: Identification of a Defect in *W/W^v* Anemic Mice

Abstract. *Nonirradiated mice of the* W/W^v *genotype were injected with normal (+/+) bone marrow cells that had been treated with antiserum to Thy 1.2 and complement (C'). Such bone marrow cells had no effect on the number of macroscopic colonies formed in the spleens of these mice, but did not cure the anemia. The addition of +/+ thymocytes to these bone marrow cells restored their ability to cure the anemia in* W/W^v *mice. These data suggest that a theta-sensitive cell is required in the promotion of differentiation of murine hematopoietic stem cells into erythrocytes, and that there is a deficiency of such a cell in the* W/W^v *mouse.*

Macrocytic anemia in *W/W^v* mice is considered to be an experimental model of hypoplastic anemia in man (*1*). At least one of the mechanisms responsible for this genetically determined disorder carried by WBB6F$_1$ mice is a hereditary stem-cell deficiency (*2*). Thus, bone marrow cells from anemic *W/W^v* animals do not form macroscopic colonies in the spleen of irradiated recipients, but microscopic colonies which are reduced both in number and size (*3*). These *W/W^v* anemic animals are a useful model for experimentation, since they have hematologically normal +/+ littermates. Bone marrow cells from +/+ animals form macroscopic colonies in nonirradiated *W/W^v* recipients (*4*), and the final success of the bone marrow graft may be easily determined by testing the red blood cell values of *W/W^v* recipients (*5*). Anemia in *W/W^v* animals, therefore, provides an experimental system particularly suitable for studies concerning the mechanisms which regulate hematopoiesis.

Several studies indicate that the thymus gland exerts a stimulatory effect on erythropoiesis. The addition of 10^6 or more thymocytes to bone marrow cells from transfused into irradiated recipients increased the number of macroscopic colonies (*6*). This cannot be explained by the presence in the thymus of cells forming colonies, since there is only one such cell present in about 100×10^6 thymus cells (*7*). On the other hand, thymectomy of neonatal mice caused anemia and reduced both the total number of bone marrow cells and the number of colony-forming cells present in the bone marrow (*8*). These observations may indicate that while the hematopoietic stem cell, as measured by the assay for colony-forming units in the spleen (CFU-S), is directly responsible for a hematopoietic self-renewal potential, its proliferation, differentiation, and maturation are regulated by lymphoid cells derived from the thymus.

We have performed studies which suggest that the presence of a theta-sensitive cell is required in normal bone marrow, thymus, and spleen for the promotion of normal differentiation of hematopoietic stem cells along the erythroid pathway, and that there is a deficiency of such a cell in the *W/W^v* mouse.

We used WBB6F$_1$ adult male (*W/W^v* and +/+) mice (Jackson Laboratory), that were produced by breeding WB/ReJ-*W*/+ and C57B1/6J-*W^v*/+ parents. Both of these parental strains possess the Thy 1.2 antigen. Bone marrow cells were obtained from the femurs and tibiae of these mice by gentle flushing with medium (RPMI-1640). Thymocytes and spleen cells were obtained by teasing these tissues with a rubber policeman into the medium. Antiserum to Thy 1.2 antigen was made by the method of Reif and Allen (*9*) by immunizing AKR mice with CBA/J thymocytes.

Bone marrow cells were treated with antiserum to Thy 1.2 as follows. The bone marrow cells (10^7) were incubated with 1 ml of a 1 : 30 dilution of antiserum in medium for 20 minutes at 4°C. The unbound antiserum to Thy 1.2 was removed by washing with medium, and the cells were resuspended in 1 ml of a 1 : 8 dilution of rabbit complement (C'), and the suspension was incubated at 37°C for 30 minutes. The cells were washed twice with medium and counted. In control experiments, the bone marrow or spleen cells were treated with normal serum from AKR mice (NMS) and C'. Cell viability was determined by the trypan-blue dye exclusion technique. Viability of the bone marrow cells after treatment with either antiserum to Thy 1.2 plus C' or NMS plus C' was between 90 and 95 percent (*10*). Viability of the spleen cells was about 60 percent after treatment with antiserum to Thy 1.2 plus C' compared to 90 percent after treatment with NMS and C'. Dead cells were eliminated by the Ficoll-Hypaque centrifugation technique, and cell suspensions used for injections consisted of 95 percent viable cells.

We used *W/W^v* mice as recipients throughout these experiments. The mice were divided into groups, with five mice per group. Each mouse was injected by way of the tail vein with appropriately treated cell preparations. The number of macroscopic colonies formed by transfused bone marrow cells in nonirradiated *W/W^v* recipients was determined on day 7 after the transplantation according to the method of Till and McCulloch (*11*). Mice were bled every 10 days by way of a retro-orbital sinus for hematocrit and red blood cell (RBC) determinations by routine methods.

In the first series of experiments, we depleted the +/+ bone marrow or +/+ spleen cells of theta-sensitive lymphoid cells by treatment with antiserum to Thy 1.2 in the presence of complement, and used such preparations for the correction of *W/W^v* anemia. Injection of 10^7 +/+ bone marrow or spleen cells treated with NMS and C' was equally active in improving the blood values of *W/W^v* recipients (Table 1). In marked contrast, injection of either +/+ bone marrow or +/+ spleen cells treated with antiserum to Thy 1.2 serum and C' failed to produce an increase in the blood values. As expected, control groups receiving either *W/W^v* bone marrow or spleen cells treated either with antiserum to Thy 1.2 and C' or with NMS and C' failed to show an increase in the hematocrit values and RBC counts in *W/W^v* recipients (data not shown). Table 1 shows data of one representative experiment. The whole experiment was repeated six times and gave similar results.

Although antiserum to Thy 1.2 prepared and used for treating cells in vitro as above has been shown previously (*12*) not to affect cells forming macroscopic colonies in the spleen, we argued that the most likely explanation for the observed effect with our preparation of antiserum

to Thy 1.2 could be its cytotoxic effect on stem cells. To rule out this possibility, we injected groups of W/W^r mice with 10^5 bone marrow cells from +/+ or W/W^r mice which had been treated with antiserum to Thy 1.2 and C′; the CFU-S assay was performed. Control mice received bone marrow cells consisting of cells treated with NMS and C′. In parallel experiments, separate groups of W/W^r mice received 10^7 cells treated either with antiserum plus C′ or with NMS plus C′, and the effect of each treatment was evaluated by the determination of the hematocrit value. Table 2 shows that injections of 10^5 +/+ bone marrow cells that had been treated with NMS plus C′ produced after 7 days normal numbers of macroscopic colonies in the spleens of W/W^r recipients, and injections of 10^7 +/+ bone marrow cells significantly increased the hematocrit value in animals of the same genotype. In contrast, injection of 10^7 +/+ bone marrow cells that had been treated with antiserum to Thy 1.2 plus C′ failed to increase the hematocrit values of W/W^r recipients, while an injection of 10^5 bone marrow cells from the same cell suspension in W/W^r mice produced macroscopic colonies which were equal in number to those produced by 10^5 +/+ bone marrow cells treated with NMS plus C′. As expected, 10^5 W/W^r bone marrow cells, both treated with NMS plus C′ and with antiserum to Thy 1.2 plus C′, failed to produce any macroscopic colonies in the spleens of nonirradiated W/W^r recipients and also failed to increase hematocrit values in separate groups of W/W^r recipients.

In a separate experiment, we injected groups of W/W^r mice with 10^7 +/+ or W/W^r thymocytes and evaluated the number of macroscopic colonies in the spleen on day 7 after injection. There was one macroscopic colony present on the surface of spleens of five W/W^r recipients of +/+ thymocytes and no macroscopic colonies on the surface of spleens of recipients of W/W^r thymocytes.

Since depletion of theta-sensitive cells abolished the ability of +/+ bone marrow cells to correct the anemia of W/W^r mice, the next series of experiments was performed to determine if the addition of thymocytes from +/+ mice to bone marrow cells that had been treated with antiserum to Thy 1.2 plus C′ would restore their ability to correct the anemia. Both +/+ thymocytes by themselves, and antiserum to Thy 1.2 plus C′ treated +/+ bone marrow cells alone, failed to produce a significant increase in hematocrits (Table 3). In contrast, injection of 10^7 +/+ bone marrow cells treated with antiserum to Thy 1.2 and C′, together with 10^7 +/+ thymocytes, corrected the anemia in W/W^rmice. All of these effects were seen as early as 10 days after transplantation and persisted for 90 days; that is, for the total duration of this experiment. As expected, injection of W/W^r thymocytes, along with antiserum to Thy 1.2 plus C′ treated +/+ bone marrow into W/W^r recipients, did not lead to the cure of anemia (data not shown).

These results indicate that while +/+ bone marrow cells treated with antiserum to Thy 1.2 plus C′ provide the W/W^r recipient mice with a normal number of hematopoietic stem cells, such bone marrow is depleted by this treatment of some other cells whose presence is critical for the restoration of normal erythropoiesis in W/W^r mice. These cells are sensitive to antiserum to Thy 1.2 and do not derive from cells forming macroscopic colonies in the W/W^r spleen. This suggests the existence of a hypothetical "theta-sensitive regulatory cell" (TSRC) which is not derived from the hematopoietic stem cell and is present in normal adult (+/+) bone marrow, spleen, and in thymus. Therefore, W/W^r mice may be deficient in two cell types which participate in normal erythropoiesis; namely, a hematopoietic stem cell and TSRC, both of which are required for the correction of anemia. Such a deficiency might be due to separate defects of these two cell types, or to the W/W^r mice having a deficiency of a common precursor of these two cell types; for example, a multipotential stem cell. If there were a single defect of a multipotential stem cell, such a cell in the microenvironment of the W/W^r mouse might not be able to differentiate into the hypothetical TSRC. This suggests a subtle selective microenvironmental defect in the W/W^r mouse as an alternative

Table 1. The effect of antiserum to Thy 1.2 on the ability of normal (+/+) bone marrow cells or +/+ spleen cells to correct anemia in W/W^r mice. Tests were conducted 30 days after transplantation. Abbreviations: Hct, hematocrit; RBC, red blood cells; NMS, normal mouse serum; C′, complement. Data are expressed as mean ± standard error (N = 5).

Host	Donor cell type (10^7 cells)	Treatment	Hct (%)	RBC (No. × $10^6/\mu l$)
+/+			47.9 ± 0.7	9.6 ± 0.8
W/W^r			35.8 ± 0.4	5.2 ± 0.1
W/W^r	+/+ Marrow	NMS + C′	47.5 ± 1.1*	9.1 ± 0.2*
W/W^r	+/+ Marrow	Antiserum to Thy 1.2 + C′	35.9 ± 0.8	5.6 ± 0.1
W/W^r	+/+ Spleen	NMS + C′	46.9 ± 0.9*	9.2 ± 0.4*
W/W^r	+/+ Spleen	Antiserum to Thy 1.2 + C′	35.3 ± 0.8	5.2 ± 0.6

*Difference highly significant ($P < .001$) compared to W/W^r recipients treated with other cell preparations.

Table 2. The effect of treatment of normal (+/+) bone marrow cells with antiserum to Thy 1.2 on their ability to form macroscopic colonies in spleen of W/W^r mice and on the ability of these cells to correct the anemia in W/W^r mice. Data are expressed as means ± S.E. (N = 5).

Host	Donor of bone marrow cells	Treatment	Number of colonies formed by 10^5 cells 7 days after transplant	Hct 40 days after transplant of 10^7 cells (%)
W/W^r	W/W^r	NMS + C′	0	36.2 ± 0.7
W/W^r	W/W^r	Antiserum to Thy 1.2 + C′	0	37.5 ± 0.4
W/W^r	+/+	NMS + C′	19.2 ± 3.8	47.4 ± 0.3*
W/W^r	+/+	Antiserum to Thy 1.2 + C′	19.4 ± 3.6	37.0 ± 0.2

*Level of significance ($P < .001$) compared to all other groups.

Table 3. The effect of the addition of +/+ thymocytes on the correction of W/W^r anemia by +/+ bone marrow cells treated with antiserum to Thy 1.2. Data are expressed as means ± S.E. (N = 5).

Host	Treatment of +/+ bone marrow transplant (10^7 cell/recipient)	Transplant of 10^7 +/+ thymocytes	Hct 10 days after transplant (%)	Hct 90 days after transplant (%)
W/W^r	NMS + C′	No	42.8 ± 2.3	45.3 ± 0.3
W/W^r	No bone marrow transplant	Yes	36.5 ± 1.5	35.0 ± 1.0
W/W^r	Antiserum to Thy 1.2 + C′	No	38.0 ± 0.7	37.3 ± 0.5
W/W^r	Antiserum to Thy 1.2 + C′	Yes	41.7 ± 0.5*	44.3 ± 0.5†

*Difference significant ($P < .05$). †Difference highly significant ($P < .001$) compared to groups of W/W^r recipients of antiserum Thy 1.2 plus C′ treated +/+ bone marrow alone or +/+ thymocytes alone; no difference when compared to groups of W/W^r recipients of +/+ bone marrow treated with NMS plus C′.

explanation for these results. The inability of bone marrow cells treated with antiserum to Thy 1.2 plus C′ to give rise to TSRC may indicate that at least certain thymic lymphoid cells are not derived from the hematopoietic stem cell. On the other hand, Wu *et al.* (*13*) infused W/W^v mice with irradiated +/+ bone marrow cells and found that a proportion of cells from the bone marrow, spleen, thymus, and lymph nodes of the recipient mice all showed the same radiation-induced chromosomal abnormalities. Based on these observations, they reasoned that hematopoietic colony-forming cells, erythroblasts, granulocytes, thymic cells, and cells of the lymph nodes may all constitute the same clone. Further experiments are necessary to resolve these differences.

Our data also indicate some of the characteristics of the TSRC. These cells are sensitive to treatment with antiserum to Thy 1.2 serum, are present in 10^7 of +/+ bone marrow cells, and may constitute less than 1 percent of the total bone marrow, since it has been reported that the frequency of cells sensitive to Thy 1 in mouse bone marrow is below 1 percent (*14*). Also, there must be a sufficient number of such cells present in 10^7 thymocytes and in 10^7 spleen cells.

One of the critical questions in the present study is the use of antiserum to Thy 1.2 prepared by immunizing AKR mice with CBA/J thymocytes, which also may contain another specificity, namely, antiserum to Ly 3.2 (*15*). The Ly 3.2 antigen is present in C57B1/6 parents and, therefore, this specificity may be responsible for the observed effect. It must be mentioned, however, that both Ly 3 and Thy 1 antigens are carried exclusively by T lymphocyte cells. It is also possible that this serum contains other specificities directed against unknown surface antigens expressed on CBA/J but not on AKR thymocytes.

It has been reported (*14*) that mouse bone marrow does not contain mature T cells; therefore, it seems that the TSRC is possibly a T-lymphoid precursor cell type. Moreover, Harrison and Cherry (*16*) and Harrison and Astle (*17*) recently have found, using a T6 chromosome marker, that the thymus in W/W^v anemic mice injected (60 to 90 days after transplantation) with +/+ bone marrow cells is completely repopulated by cells of the donor type. This suggests that the T-cell precursor of the W/W^v mouse is unable to compete with normal T-cell precursors from the +/+ mouse.

Our evidence on the role of TSRC in the correction of the anemia in W/W^v mice prompted us to examine the hematological data available concerning athymic *nu/nu* mice. Values for Hct and RBC in the *nu/nu* mice were reduced but not statistically different from their +/*nu* littermate controls (*18*). However, there is evidence for a reduced number of nucleated cells in the bone marrow, a reduced number of hematopoietic stem cells in the bone marrow, and a reduced life-sparing capacity of the bone marrow in irradiated recipients (*19*). This, together with numerous reports of the stimulatory effect of thymocytes on erythropoiesis (*6–8*), suggests that collaboration of a hematopoietic stem cell (as determined by the CFU-S assay) with the TSRC may be a normal event, at least in mice, and for the erythropoiesis, not only in the W/W^v mice but also in normal strains.

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30 September 1976; revised 26 November 1976

Neoplastic and Possibly Related Skin Lesions in Neotenic Tiger Salamanders from a Sewage Lagoon

Abstract. *Tiger salamanders (*Ambystoma tigrinum*) inhabiting a sewage sedimentation lagoon become neotenic, and approximately one-third develop neoplastic skin lesions including cancer. Circumstances suggest a chemical etiology for the neoplasms.*

An estimated 28,000 (*1*) neotenic tiger salamanders, *Ambystoma tigrinum*, are the only vertebrates inhabiting a small isolated lagoon which is heavily polluted with secondary treated domestic sewage. On an annual basis 30 to 50 percent of this population have had skin lesions, 84 percent of which are neoplasms. The neoplasms are of epidermal, fibrocytic, and melanocytic origin; while most are well differentiated and superficial, some are poorly differentiated and invasive. In contrast, tiger salamanders from uncontaminated lagoons in the same general vicinity metamorphose normally (*1*), and no neoplasms were discovered among 12,600 larvae sampled from 16 proximal nonsewage lagoons over the past 6 years.

The contaminated lagoon, a natural playa centrally located in a grass field on Reese Air Force Base, Hurlwood, Lubbock County, Texas, covers 13 ha at an average depth of 2 m. It was chosen in 1970 for an in-depth ecological study because of its many desirable features: its size, depth, and the density of the salamander population facilitated seining; its