

Treatment of Hypoplastic Anemia in Mice With Placental Transplants

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A genetic mutation in mice (W/W^y) causes an autosomal recessive disease characterized by hypoplastic anemia which lasts throughout life. Double-dominant W/W^y anemic mice were sublethally irradiated to facilitate repopulation of marrow with transplanted cells and were injected intravenously with suspensions of 5–10 million placental cells of 15 days gestation derived from normal, isogenic donors. Red cell counts fell promptly after irradiation and then rose progressively over a period of weeks, reaching normal levels for the nonmutant. Mean corpuscular volume and hemoglobin electrophoresis patterns of red cells in recipient W/W^y mice resembled those of normal donor animals. The therapeutic effect lasted for the duration of the observation period, in some instances over 9 mo. W/W^y mice that were administered Hanks' solution or fetal blood, instead of placental transplants, remained anemic. Late gestation placentas (18 days) were also ineffective.

PREVIOUS STUDIES from this laboratory have indicated the presence in mouse placenta of cells with hemopoietic capability.¹⁻³ Under appropriate acute experimental conditions, the cells developed the morphologic and functional characteristics of cells of the erythrocytic, lymphocytic, and granulocytic series. The present studies were designed to investigate the potential of placental cells in a "clinical" situation.

Mice bearing the W/W^y genetic mutation are born with congenital hypoplastic anemia which lasts throughout life. Intravenous injection of fetal liver from normal donor animals as a source of erythropoietic cells cures the anemia, demonstrating that the deficiency is in primordial cellular elements rather than environmental developmental factors in the host.⁴ The effect of intravenous injections of suspensions of placental cells on the hypoplastic anemia of W/W^y mice has been investigated in the present study.

MATERIALS AND METHODS

Animals

Male $WBB6F_1-W/W^y$ mice were purchased from the Jackson Laboratory. The mice are produced by mating $WB/ReJ-W/+$ mice with $C57BL/6J-W^y/+$ mice. One-fourth of the offspring are of the W/W^y genotype and are immunologically compatible with $C57BL/6J$ mice.^{4,5} $C57BL/6J$ mice were also obtained from Jackson Laboratory and a mating colony was established. Placentas were obtained from timed pregnancies of $C57BL/6J$ mice.

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Preparation of Cell Suspensions

Suspensions of placental cells were prepared as previously described.¹ Placentas were pressed through a stainless steel screen using a cytosieve. The resulting suspension was cleared of debris by settling and repeated washing with Hanks' balanced salt solution. This step in the procedure was extremely important to remove unidentified materials that cause death of the recipient animal within minutes. Cells were counted in a hemocytometer.

In experiments with 11-12-day gestation fetuses, cell suspensions were prepared from the total animal in a similar fashion.

Transplantation Technique

Recipient W/W^v mice were sublethally irradiated with 200 rads of whole-body irradiation delivered with a Maxitron 300 unit at 300 kV peak with filtration of 2 mm Cu. Target-to-specimen distance was 50 cm and dose rate was 305 rads/min. Placental cells were injected into the cavernous sinus of the penis within 2 hr of irradiation in a volume of 0.5 ml.

In the experiments with 11-12-day gestation placentas and fetuses, isogenic (C57BL/6J) recipient animals were lethally irradiated (800 rads) prior to the injection of cell suspensions. Surviving animals were sacrificed 10 days later and the spleens were examined grossly for nodules and microscopically by making imprints on slides and staining with Giemsa solution.

Blood Counts

For blood analyses, mice were bled from the retroorbital sinus using a heparinized 44.7 lambda pipette. The blood was added to 10 ml of Eagle's diluent and cells were counted in a Coulter Counter, model S.

Pathologic Studies

Pathologic studies were performed on hematoxylin-cosin stained sections after formaldehyde fixation.

Hemoglobin Electrophoresis

Electrophoresis was performed in cellulose acetate at 350 V (47 V/cm) at pH 8.4 in borate buffer. Ponceau-S stain was used.

RESULTS

Sublethal whole-body irradiation, 200 rads, was administered to W/W^v mice to facilitate the repopulation of bone marrow by placental cells. The effect of this procedure and of weekly blood sampling was investigated in a control population of W/W^v mice that received only 0.5 ml Hanks' solution (Fig. 1). The RBC concentration fell during the first 2 wk postirradiation, and then during the next 2 wk gradually returned toward but never reached preirradiation levels. The mean corpuscular volume (MCV) did not change significantly during the observation period. Of the 16 mice originally in the study, 11 survived at 84

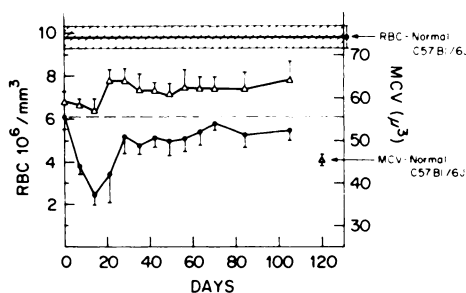


Fig. 1. W/W^v mice received sublethal whole-body irradiation (200 rads) and then were injected i.v. with 0.5 ml Hanks' solution. Broken line, mean RBC concentration at the beginning of study. Δ , MCV; \bullet , RBC. Vertical lines 1 SD. Normal RBC and MCV were derived from twelve C57BL/6J mice.

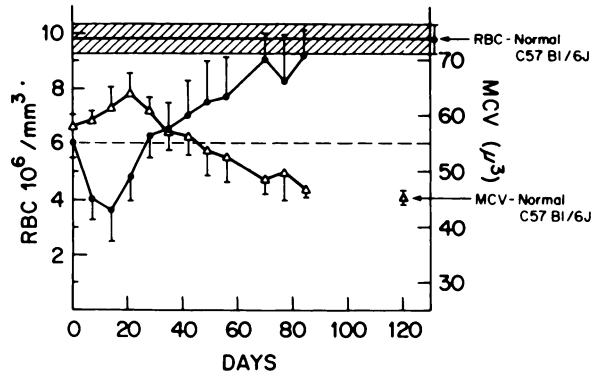


Fig. 2. Same conditions as Fig. 1, but with injection of suspension of 5 million placenta cells from pregnant C57BL/6J mice, 15 days gestation.

days and 7 at 105 days. Irradiation, repeated blood sampling, and possibly other unrecognized factors associated with care in the colony caused a significant mortality.

Eighteen W/W^v mice were exposed to 200 rads whole-body irradiation and then received a suspension of 5 million placental cells intravenously (i.v.) from pregnant C57BL/6J mice at 15 days gestation (Fig. 2). Preirradiation RBC levels were restored by 4 wk. By 70 days the RBC mean concentration reached levels just below normal for C57BL/6J mice. This improvement was maintained for the duration of the observation period, 135 days. Coincidentally, MCV fell, also approaching but not quite achieving the normal range. Despite the improvement in the RBC count, mortality remained high, with seven mice surviving at 84 days. Two of these had relapsed (RBC concentration greater than 2 SD below the mean) and were excluded from the graph.

Nine W/W^v mice received 10 million 15-day gestation placental cells i.v. (Fig. 3). The postirradiation recovery in RBC concentration was accelerated, reaching preirradiation levels in 3 wk and normal levels at 65 days. MCV was also restored to normal. Three mice survived for 9 mo, retaining a normal RBC and MCV before being sacrificed. There was one treatment failure among the nine mice.

In the previously described experiments, no attempts had been made to free the placental suspensions of trapped fetal blood. To exclude the possibility that the favorable results were produced by proliferation of circulating fetal blood

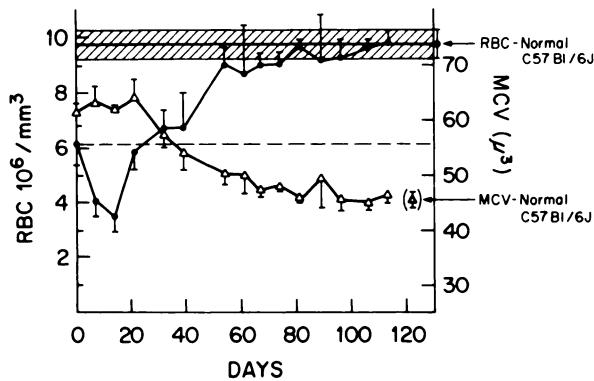


Fig. 3. Same conditions as Fig. 1, but with injection of suspension of 10 million placental cells from pregnant C57BL/6J mice, 15 days gestation.

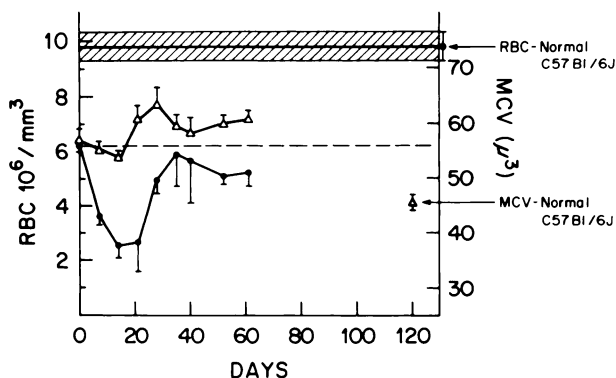


Fig. 4. Same conditions as Fig. 1, but with injection of fetal blood, 0.5 ml, from C57BL/6J fetuses of 15 days gestation.

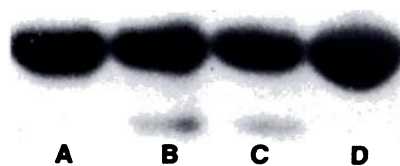
elements, 0.5 ml of blood collected from 15-day gestation fetuses was injected i.v. into 11 irradiated W/W^v mice. It was estimated that at least twice the number of RBC was injected than was trapped in placental cell suspensions. The results were similar to those obtained with Hanks' solution (Fig. 4). Four W/W^v mice were injected with blood from 11-day fetuses and seven with blood from 18-day fetuses with similar results.

The hemoglobin in six W/W^v mice that had received 10 million 15-day gestation placental cells was examined electrophoretically and compared to the normal C57BL/6J mouse and the untreated W/W^v mouse. Three of the reconstituted W/W^v mice were studied 9 mo after placental transplant (Fig. 5). The two bands characteristic of W/W^v mice were replaced by the single band seen in C57BL/6J mice.⁶ Previous analyses of artificial mixtures of blood from W/W^v mice and C57BL/6J mice had demonstrated that the technique was sufficiently sensitive to detect mutant hemoglobin at a concentration of 10% of the total.

Pathologic studies were done on six mice that had received 10 million early gestation placental cells. Three were sacrificed at 4 wk and three at 8 wk after irradiation and the injection of placental cells. Kidneys, liver, thymus, and lymph nodes were not remarkable. At 4 wk, there was a striking increase in splenic weight (194, 122, 214 mg) attributable to a diffuse and focal increase in immature and developed erythroid elements. There was much active mitosis. By 8 wk, spleen weights (88, 76, and 73 mg) and active erythropoiesis had returned to normal levels.

The ability of 18-day placenta to restore the hematologic status of W/W^v mice was investigated with 5 million and 10 million placental cells injected i.v. (10 mice and 7 mice, respectively). There was little difference between the two series. The results of the latter are presented graphically (Fig. 6). There was

Fig. 5. Hemoglobin electrophoresis. (A) W/W^v mouse, 9 mo after placental transplant. (B) W/W^v mouse, 2 mo after receiving fetal blood. (C) W/W^v mouse, untreated. (D) C57BL/6J mouse.



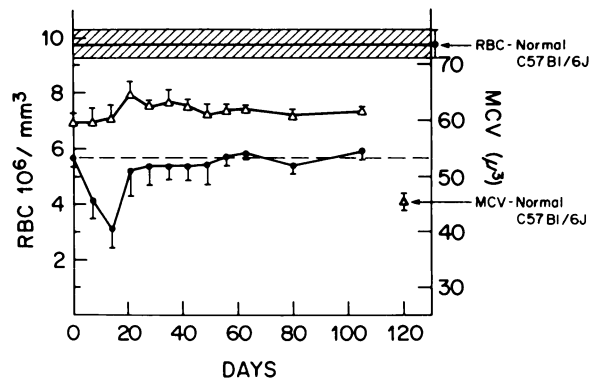


Fig. 6. Same conditions as Fig. 1, but with injection of suspension of 10 million placental cells from pregnant C57BL/6J mice, 18 days gestation.

only slight improvement over the results with fetal blood; RBC concentration reached preirradiation levels. There was no detectable change in MCV.

The results of these experiments were tested statistically by comparing the RBC count at 50–60 days postirradiation (Table 1). Using Hanks' solution-injected animals as a basis of comparison, a clear beneficial effect was observed with transplants of 15-day gestation placenta ($p < 0.001$) but not of 18-day gestation placenta. There was no significant difference between animals that received 5 million 15-day placental cells and those that received 10 million cells, though the curve (Figs. 2 and 3) suggests improved performance from the latter. The animals that received fetal blood did not differ significantly, as a group, from those that received Hanks' solution. The wide standard deviation with fetal blood at 35 and 40 days (Fig. 4) suggests that some animals may have benefited transiently.

The clear superiority of 15-day placenta over 18-day placenta raised the question as to whether erythropoietic cells were present in placentas of earlier gestation. The number of cells obtainable from 11–12 day placentas was insufficient to permit a series of reconstitution experiments with W/W^v mice. Instead, the technique of Till and McCulloch⁷ was used to determine if hemopoietic cells were present. Five million 11–12-day gestation placental cells from C57BL/6J mice were injected into each of three lethally irradiated isogenic (C57BL/6J) mice. Splenic nodules were grossly visible when the mice were sacrificed at 10

Table 1. RBC in Different Experimental Conditions

Treatment	No. of Animals	RBC $10^6 \pm$ SD	Statistical Significance (p)
Hanks' solution	12	5.40 ± 0.59	—
Fetal blood	7	5.34 ± 0.54	NS
15-day placenta			
5×10^6	11	7.67 ± 1.4	< 0.001
10×10^6	7	8.67 ± 1.7	< 0.001
18–19-day placenta			
5×10^6	6	5.34 ± 0.30	NS
10×10^6	7	5.81 ± 0.16	NS

All W/W^v mice received sublethal irradiation (200 rads) 2 hr before an i.v. injection of Hanks' solution or a cell suspension from compatible C57BL/6J mice. RBC was determined 50–60 days posttreatment. Results of treated animals were compared for statistical significance to Hanks' solution-injected animals. NS: not significant.

days, and examination revealed blastic elements morphologically consistent with the erythroid series. Previous studies done with 18-day gestation placentas had produced splenic nodules with an abundance of leukocytic as well as erythroid elements.² The spleens of irradiated, uninjected animals were small, without nodules, and without erythrocytic cells.

The possibility that the response to very early gestation placenta (11–12 days) reflected a general potential of immature tissues at this stage of gestation was investigated by preparing suspensions of cells from total C57BL/6J embryos and injecting 5 million cells into five lethally irradiated isogenic recipients. Death occurred at 7 days, sooner than would be expected in irradiated, uninjected animals. There was no visible reaction in the spleens. The cause of death was not investigated.

DISCUSSION

Evidence has been previously presented from this laboratory that mouse placenta contains stem hemopoietic cells.¹⁻³ The injection of cells into immunologically defenseless allogenic recipients, either newborn mice or irradiated adults, caused graft-versus-host disease. Appropriate control studies from F1 pregnancies and with fetal blood excluded significant contributions to this phenomenon from either maternal cells or fetal blood elements.¹ The intravenous injection of suspensions of placental cells into lethally irradiated isogenic recipients produced splenic nodules composed of cells at various maturational levels representing the erythrocytic, lymphocytic, and granulocytic series, as well as giant cells resembling megakaryocytes. Experiments with placentas from mice carrying the T6 chromosomal anomaly demonstrated that the nodule cells were derived from the injected placental suspension. In one study, fetectomies were performed at 11–12 days gestation and the retained placentas harvested at 18–19 days gestation. Splenic nodules containing a variety of blood cellular elements were produced by their injection into irradiated isogenic recipients, providing further support of their placental origin.²

In all of these studies, the experimental situations were highly artificial and the observations were of brief duration. The present investigations were undertaken in W/W^v mice, who suffer from a congenital hypoplastic anemia, which resembles clinical conditions seen in man. Prolonged observations of the erythropoietic potential of placentally derived cells were possible.

The injection of compatible placental cells of 15 days gestation from normal donors (C57BL/6J) into sublethally irradiated W/W^v mice corrected the anemia. The RBC concentration increased and the MCV fell. Correction lasted for months, the duration of the observation period (Figs. 2 and 3). Placental cells from 18–19-day pregnancies did not produce a significant therapeutic effect even though cells with hemopoietic potential have been demonstrated in such placentas by other techniques.² It would appear that the 15–16-day placenta had a higher concentration of erythropoietic cells. Erythropoietic cells were demonstrated in 11–12-day placentas by the Till and McCulloch technique,⁷ and it is possible that these very early gestation placentas might be even more effective therapeutically. The placentas were too small to permit a series of constitutive experiments with W/W^v mice.

The therapeutic response in W/W^v mice probably resulted from repopulation of host tissue with normal placental cells from the donor, rather than from a stimulatory effect on host tissue. The presumption is based in part on our previous extensive studies demonstrating that placental cells can repopulate host animals with hemopoietic cells.¹⁻³ The beneficial effect extending over a 9-mo period, observed in the present studies, is also more consistent with the establishment of a new cell line in the host animals. In the reconstituted W/W^v mouse the MCV and the hemoglobin electrophoretic pattern are those of the donor C57BL/6J mice, indicating that the mutant cells were displaced or suppressed.⁶

The origin of the stem cells in the placenta remains uncertain. It may be that a small population of embryonic cells that have pluripotential capacity remains in the placenta, or that trophoblast cells are capable of dedifferentiation. It is also possible that the stem cells originate in the yolk sac and migrate into the placenta in the same manner that other fetal hemopoietic loci are populated. A surprising feature is the apparent density of stem cells in the placenta although there is normally no evidence of hemopoietic activity. Ten million placental cells are sufficient to reconstitute W/W^v mice for an extended period of time, in spite of the burden of frequent blood sampling. Russell et al.⁴ have used 8 million fetal liver cells from 15-day gestation fetuses (at which time the liver is a major hemopoietic organ) to achieve similar results.

Clinical experience suggests that human placenta may also have hemopoietic potential. Severe erythroblastosis may be associated with intense hemopoietic activity in the placenta,⁸ suggesting that the placenta contains primordial cells, as well as those environmental factors necessary for the support of erythropoiesis.

The question arises as to whether placental stem cells serve a significant function in normal pregnancy. As noted above, the placental stem cells can differentiate into lymphoid cells with immunologic function and must be assumed to be equipped with appropriate membrane receptors. Mouse placenta transfers Ig gamma globulin to the fetus, as does human placenta, and both have specific receptors to facilitate the process.⁹ Large numbers of cells with Fc receptors have been demonstrated in mouse placenta¹⁰ and in human placenta.¹¹ The role of placental stem cells may be to provide cells with receptors designed for IgG transport. Transfer of maternal IgG to the fetus is vital to the survival of the newborn.

The present studies were undertaken to determine if placental cells could repopulate a host animal with hemopoietic cells and whether such cells could regenerate and survive over extended periods of time. The answer was clearly in the affirmative. No attempt was made to determine the optimal conditions for achieving such results. Survival could probably be improved by modifying or eliminating irradiation and by altering other aspects of treatment. A more significant question was whether placental cells offer any advantage over customary sources of hemopoietic cells in transplantation. In addition to their availability, placental cells may be less subject to rejection. Human trophoblast is deficient in blood group A antigens¹² and in HLA antigens.¹³ In view of our earlier experiments, the possibility of placental transplants inducing a graft-

versus-host reaction must also be considered.¹ The studies were performed with term placenta and it is possible that early gestation placenta may not have the same disadvantage. In the relatively few experiments undertaken with 11–12-day gestation placentas, cells of the leukocytic series appeared to be absent from the splenic nodules induced by injection into isogeneic recipients.

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