Correction of Symptoms of Platelet Storage Pool Deficiency in Animal Models for Chediak-Higashi Syndrome and Hermansky-Pudlak Syndrome

By Edward K. Novak, Michael P. McGarry, and Richard T. Swank

Two human diseases of platelet storage pool deficiency (SPD), Hermansky-Pudlak syndrome and Chediak-Higashi syndrome, are recessively inherited disorders characterized by hypopigmentation, prolonged bleeding, and normal platelet counts accompanied by a reduction in dense granule number. We have recently described seven independent recessive mutations in the mouse regulated by separate genes which are likely animal models for human SPD. Reciprocal bone marrow transplants were carried out between normal C57BL/6J mice and two of these mutants, beige and pallid, in order to test whether the platelet defects are due to a defect in platelet progenitor cells or to humoral factors. Normal and congenic mutant mice were transplanted with marrow after 950 rad whole body radiation. The long bleeding times and low serotonin concentrations of the two mutants were converted to normal values after transplantation with normal marrow. Likewise, normal mice displayed symptoms of SPD when transplanted with mutant marrow. These studies demonstrate that with each of the two mutations, platelet SPD results from a defect in bone marrow precursor cells. Also, the studies suggest that in severe cases, platelet SPD may be successfully treated by bone marrow transplantation.

SEVERAL autosomal recessive human bleeding disorders accompanied by a deficiency in platelet dense granule function have been described.1-3 The most extensively studied of these is platelet storage pool deficiency (SPD), which includes two separate diseases, the Chediak-Higashi syndrome (CHS)7-9 and Hermansky-Pudlak Syndrome (HPS),10 in which the symptoms of SPD are accompanied by a dilution in pigmentation.

SPD is characterized by prolonged bleeding time accompanied by a normal platelet count, decreased platelet dense granule contents and function, and a reduction in the number of platelet dense granules. In addition, patients with CHS and HPS have an abnormal structure and/or function of other subcellular organelles including melanosomes.10,11 and lysosomes.9,10

We, and others, have described a series of seven mouse pigment mutants, mapping at different chromosomal locations, that have characteristics similar to patients with CHS and HPS.12,14 These mice, when compared to normal mice, had a prolonged bleeding time after experimental injury. Platelet counts were similar to those of normal mice, but platelet dense granule components serotonin, adenosine triphosphate, adenosine diphosphate, and morphologically identifiable dense granules were markedly reduced in the mutants. Thus, all seven mouse pigment mutants had symptoms consistent with SPD. The beige mutant, on the basis of the absence of giant lysosomes in many tissues, has been proposed as an animal model for CHS.11 The other mutants, lacking the giant lysosomes characteristic of beige but having lysosomal function defects, have been proposed as animal models for HPS.13,14

The defects in platelet granule formation in these diseases may be due to an intrinsic defect in cellular precursors of platelets or to humoral factors, unrelated to platelet precursors, which can regulate the formation of, or cause an asynchrony in, distribution of platelet dense granules. As a result of reciprocal bone marrow transplantation, we report the cellular nature of the defect in two animal models for SPD: beige (mapping at chromosome 13),11 an animal model for CHS, and pallid (mapping at chromosome 2), an animal model for HPS.14 Furthermore, our results suggest that bone marrow transplantation may be useful as therapy in severe cases of human SPD.

MATERIALS AND METHODS

Animals. C57BL/6J normal (+/+), beige (bg/bg), and pallid (pa/pa) animals were obtained from the Jackson Laboratory, Bar Harbor, Me, and later bred at the animal facilities of Roswell Park Memorial Institute. C57BL/6J mice with a Gus-s structural allele of β-glucuronidase, which is electrophoretically different from the Gus-s structural allele of β-glucuronidase present normally in C57BL/6J and in beige and pallid mice, was developed by Dr V. Chapman (Roswell Park Memorial Institute). The animals were either co-isogenic (beige) or congenic (pallid) to C57BL/6J. Thus, any abnormality found may, to a very high probability, be assigned to the pigment gene rather than differing background genes. Also, the mice were isogenic for histocompatibility genes.

Bleeding times. Bleeding times14 were determined before bone marrow transplantation and at 30, 60, and 90 days post-marrow transplantation.

Bone marrow transplantation. Prior to transplantation, hosts received 950 rad whole body Co irradiation. Donor marrow was obtained from the long bones of suitable mice, 6 to 8 weeks old. Bone marrow cells (10⁸) from donor femoral marrow were transplanted via lateral tail vein in a volume of 0.5 mL RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY). Mice were routinely maintained on acid water to reduce the possibility of infection.

Test for successful engraftment. Normal mice carried the Gus-s allele of β-glucuronidase while mutant mice carried the Gus-s allele of β-glucuronidase. After final bleeding time measurements, cells were isolated from bone marrow and spleen, washed twice with 0.85% NaCl and resuspended in 0.25 mol/L sucrose, 0.02 mol/L imidazole, pH 7.4, containing 1% Triton X-100. Samples were focused on urea containing isoelectric focusing slab gels according to Lusis and Paigen.15 Glucuronidase activity was visualized using naphthol AS-BI glucuronide as substrate as modified by Swank and Paigen16 and the electrophoretic phenotype of β-glucuronidase was determined. The first experiments yielded identical results with bone marrow and spleen cells. In later experiments, spleen cells alone were used to test for successful engraftment.

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Platelet counts. Platelet samples were diluted in 1% ammonium oxalate and counted according to Brecher and Cronkite. Platelet collection and serotonin assay. Platelets were collected by a modification of the method of Holland. Platelet pellets were lysed in 1 mL of distilled H2O and assayed for serotonin according to Crosti and Lucchelli. Serotonin fluorescence was measured with activation at 295 nm and emission at 540 nm using an Aminco Bowman spectrophotometer (American Instrument, Silver Spring, Md). Fluorescence was quantitated by comparison to fluorescence of standard concentrations of serotonin creatine sulphate.

RESULTS

The normal mice used for this experiment carried the glucuronidase Gus-sb allele while the mutant mice carried the glucuronidase Gus-sb allele. Since the transplanted donor marrow is expected to repopulate the hematopoietic tissues of the recipient, we asked whether bone marrow and spleen cells from the donor and recipient mice could easily be distinguished with respect to this enzyme. Bone marrow and spleen cells from normal mice carrying the Gus-sb glucuronidase allele and from mutant mice carrying the Gus-sb glucuronidase allele were run on urea isoelectric focusing gels and stained for glucuronidase activity as shown in Fig 1. Clearly, both spleen and bone marrow from mice with the acidic Gus-sb allele can be easily distinguished from either bone marrow or spleen with the relatively basic Gus-sb glucuronidase allele. Thus, a successful graft can be distinguished easily from an unsuccessful one by isoelectric focusing for β-glucuronidase phenotype with bone marrow and/or spleen samples.

Reciprocal transplants were accomplished between normal and normal, mutant and mutant, mutant and normal, and normal and mutant mice of both beige and pallid mutant genotypes. The results of the two control transplants and the beige to normal transplant are summarized in Table 1. When normal marrow was engrafted into normal mice, the recipient mice had an expected short bleeding time of about five minutes and the concentration of serotonin was found to be similar to that previously found for normal untransplanted mice. The Gus-sb glucuronidase phenotype was also unchanged, as expected. When beige bone marrow was transplanted into beige mice, the recipient mice retained the Gus-sb glucuronidase phenotype and had prolonged bleeding and abnormally low concentrations of the dense granule marker serotonin. Beige marrow was successfully transplanted into 15 normal mice as determined by bone marrow and spleen cells having the Gus-sb glucuronidase allele typical of beige marrow. Thirteen of these acquired long bleeding times typical of the donor beige phenotype and 13 of 15 mice had serotonin values typical of the beige donor. Two mice had intermediate serotonin values, higher than expected for beige mice but fourfold lower than control values. Although there was some variation in platelet counts, this did not correlate with long bleeding times. When the values of the individual mice were pooled, the results were the same: the normal mice, when transplanted with beige marrow, assumed the characteristics of SPD, suggesting the SPD present in beige mice is due to an intrinsic defect in platelet progenitor cells of the bone marrow.

When normal marrow was transplanted into beige mice, 13 mice received successful bone marrow engraftment, as shown by the transfer into either bone marrow or spleen cells of the glucuronidase Gus-sb allele typical of normal control mice (Table 2). When mice were examined for bleeding times, all times were substantially reduced from those of beige control mice and ten of 13 returned to normal bleeding times within 30 days and maintained these times at 60 and 90 days. When serotonin concentrations in recipient mice were determined, all mice had values in the normal range of this dense granule component. In the normal to beige bone marrow transplant, the beige mice lost the characteristics of SPD and assumed the characteristics of normal mice. Thus, the defect in beige mice is cellular in nature, rather than a result of humoral regulatory factor(s) unrelated to platelet progenitor cells.

Similar experiments were performed in the pallid mouse, an animal model for the Hermansky-Pudlak syndrome. The results of the two control transplants between normal to normal (same controls as in Tables 1 and 2) and pallid to pallid mice are shown in Table 3. Normal mice retain the normal glucuronidase Gus-sb allele and bleeding times and serotonin concentrations are similar to values found previously for normal mice. Pallid mice, when transplanted with pallid marrow, retain the characteristics of SPD. When pallid marrow was transplanted into normal mice, 13 mice were shown to have successful transplants by the appearance of the glucuronidase Gus-sb allele in hematopoietic cells (Table 3). Ten of twelve mice were shown to have an increase in bleeding times and each of 13 mice had abnormally low serotonin concentrations. Thus, the characteristics of SPD were transferred from pallid to normal mice by bone marrow transplantation. When normal marrow was transplanted into pallid mice, ten mice received successful transplants, as shown by the appearance of the Gus-sb glucuronidase in hematopoietic cells. In all ten mice, bleeding times returned to normal values within 30 days and were maintained for 90 days as shown in Table 4. Furthermore, serotonin concentration was found to have increased to near normal values in all ten mice. Thus, in the pallid bone marrow transplants, the
Table 1. Transplantation of Beige Marrow Into Normal Mice

<table>
<thead>
<tr>
<th>Donor to Recipient</th>
<th>Platelets/mL ($\times 10^6$)</th>
<th>Bone Marrow Glucuronidase Phenotype</th>
<th>Bleeding Time (min)</th>
<th>Serotonin ($\mu g/10^8$ Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal to normal (n = 9)</td>
<td>6.01 ± 0.46</td>
<td>A</td>
<td>4.96 ± 0.85</td>
<td>5.34 ± 0.65</td>
</tr>
<tr>
<td>Beige to beige (n = 8)</td>
<td>4.80 ± 0.23</td>
<td>B</td>
<td>33.50 ± 4.20*</td>
<td>0.06 ± 0.01*</td>
</tr>
<tr>
<td>Beige to normal (n = 15)</td>
<td>4.80 ± 0.40</td>
<td>B</td>
<td>16.60 ± 2.10*</td>
<td>0.38 ± 0.13*</td>
</tr>
</tbody>
</table>

Bleeding times for individual mice represent an average of bleeding times determined at 30, 60, and 90 days posttransplant. There was no significant difference in bleeding times at these three intervals. All other data represent the means ± SEM for determinations made on recipient mice 100 to 120 days posttransplant. Tests for significance are against normal to normal transplant recipients.

†Data are from individual recipient mice in beige to normal transplants. Fifteen of 17 mice (88%) received successful transplants.

Recipient mice assumed the characteristics of the donor. Characteristics of SPD were transferred by pallid marrow into normal mice. Normal mouse marrow was able to correct the symptoms of SPD in pallid mice. Thus, the defect in pallid mice is due to cellular platelet progenitors rather than regulatory humoral factors unrelated to platelet production.

All transplanted mice appeared to be healthy throughout the >100 days of the experiments and no external signs of graft-v-host rejection were observed. When the mice were killed, only two of 51 mice transplanted had a slightly enlarged (~20%) spleen. No other tests of graft rejection were done.

Table 2. Transplantation of Normal Marrow Into Beige Mice

<table>
<thead>
<tr>
<th>Donor to Recipient</th>
<th>Platelets/mL ($\times 10^6$)</th>
<th>Bone Marrow Glucuronidase Phenotype</th>
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<td>0.06 ± 0.01*</td>
</tr>
<tr>
<td>Normal to beige (n = 13)</td>
<td>5.05 ± 0.59</td>
<td>A</td>
<td>6.73 ± 1.1</td>
<td>8.30 ± 1.00</td>
</tr>
</tbody>
</table>

Bleeding times for individual mice represent an average of bleeding times determined at 30, 60, and 90 days posttransplant. There was no significant difference in bleeding times at these three intervals. All other data represent the means ± SEM for determinations made on recipient mice 100 to 120 days posttransplant. Tests for significance are against normal to normal transplant recipients.

*P < .001.
†Data are from individual recipient mice in normal to beige transplants. Each of 13 mice (100%) received successful transplants.
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Table 3. Transplantation of Pallid Marrow Into Normal Mice

<table>
<thead>
<tr>
<th>Donor to Recipient</th>
<th>Platelets/mL (x 10^9)</th>
<th>Bone Marrow Glucuronidase Phenotype</th>
<th>Bleeding Time (min)</th>
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<td>4.96 ± 0.85</td>
<td>5.34 ± 0.65</td>
<td></td>
</tr>
<tr>
<td>Pallid to pallid (n = 8)</td>
<td>4.59 ± 0.32 B</td>
<td>30.90 ± 2.90*</td>
<td>0.14 ± 0.04*</td>
<td></td>
</tr>
<tr>
<td>Pallid to normal (n = 13)</td>
<td>6.15 ± 0.44 B</td>
<td>16.20 ± 2.10†</td>
<td>0.21 ± 0.06*</td>
<td></td>
</tr>
</tbody>
</table>

Mouse No. †

1 7.8 B 20.9 0.28
2 3.5 B 16.1 0.84
3 4.5 B 13.2 0.26
4 4.0 B 22.6 0.28
5 8.1 B 3.5 0.05
6 7.2 B 15.0 0.32
7 6.1 B 14.2 <0.05
8 7.3 B 16.5 <0.05
9 5.3 B 19.0 <0.05
10 5.6 B 28.8 0.13
11 5.1 B 21.8 0.11
12 8.2 B — 0.28
13 7.3 B 3.2 0.05

Bleeding times for individual mice represent an average of bleeding times determined at 30, 60, and 90 days posttransplant. There was no significant difference in bleeding times at these three intervals. All other data represent the means ± SEM for determinations made on recipient mice 100 to 120 days posttransplant. Tests for significance are against normal to normal transplant recipients.

Table 4. Transplantation of Normal Marrow Into Pallid Mice

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<td>Pallid to pallid (n = 8)</td>
<td>4.59 ± 0.32 B</td>
<td>30.90 ± 2.90*</td>
<td>0.14 ± 0.04*</td>
<td></td>
</tr>
<tr>
<td>Normal to pallid (n = 10)</td>
<td>6.02 ± 0.72 A</td>
<td>5.85 ± 0.86</td>
<td>6.16 ± 0.97</td>
<td></td>
</tr>
</tbody>
</table>

Mouse No. †

1 3.0 A 7.0 6.14
2 7.6 A 4.3 4.03
3 4.9 A 8.6 9.60
4 3.1 A 7.9 11.90
5 5.1 A 6.4 7.70
6 3.9 A 8.3 7.40
7 8.1 A 1.5 2.60
8 10.8 A 8.2 3.10
9 5.5 A 1.5 6.20
10 7.0 A 4.8 3.00

Bleeding times for individual mice represent an average of bleeding times determined at 30, 60, and 90 days posttransplant. There was no significant difference in bleeding times at these three intervals. All other data represent the means ± SEM for determinations made on recipient mice 100 to 120 days posttransplant. Tests for significance are against normal to normal transplant recipients.

DISCUSSION

We have established the cellular nature of SPD in two distinct mouse models of this disease. When marrow from SPD mice was transplanted into normal recipients, the recipient mice assumed the characteristics of SPD. When marrow from normal mice was transplanted into mutant mice, the characteristics of SPD were corrected and the mutant mice exhibited normal bleeding times and normal concentrations of the dense granule marker serotonin. The simplest interpretation of these results is that the defect in each of these distinct animal models is due to a cellular platelet progenitor rather than to systemic regulatory factors. These mice should be useful in identifying at what stage of progenitor cell development, megakaryocyte maturation, or platelet formation the abnormality in dense granule formation first occurs.

The fact that normal bone marrow transplantation can correct the characteristics of SPD in animal models may
have significance for therapy in severe cases of human SPD. Our model system, in which the mutant mice are congenic to the normal mice, differing by only single genes from normal mice, represents an ideal transplantation system. Caution should be used in extrapolating these results to human SPD because of difficulties in achieving successful marrow transplants. However, bone marrow transplantation has been used in several other stem cell diseases including a platelet disorder, Wiskott-Aldrich syndrome, lysosomal storage diseases, anemias, and leukemias. In fact, some characteristics of human CHS have been corrected by bone marrow transplantation in at least one patient. Twenty months after bone marrow transplant from his HLA-identical sister, a 4-year-old boy had normal leukocyte granules and normal natural killer cell function and was free of infection associated with this disease. These authors did not determine whether SPD was corrected.

There are seven independent mutations in the mouse, mapping at distinct loci, which exhibit the characteristics of SPD. These genes represent seven distinct steps in granule development. It will be interesting to determine whether the remaining five are also cellular in nature or whether any are due to abnormal humoral factors regulating granule development. This, in turn, will be helpful in ascertaining the likelihood that the subclassifications of human SPD can be corrected by bone marrow transplantation. It is not known whether the subclassifications of human SPD as described by Weiss et al are, as in mouse models, due to independent genetic lesions or a single genetic lesion expressed on different genetic backgrounds.

The beige and pallid mutants are the most characterized of the seven mutants that affect the function of several subcellular organelles. The primary gene product defective in either of these models has not yet been identified. However, a large number of physiologic and biochemical abnormalities have been described for these mice and in human CHS and HPS (reviewed). Which, if any, of these is the primary lesion responsible for the granule defects remains to be established. However, identification of the progenitor cell types involved in expressing SPD may bring us closer to identifying the basic genetic defects in these and other SPDs.

ACKNOWLEDGMENT

We thank Ronald Casey, Madonna Reddington, Steven Gates, Andrea Pascarella, Jeff LaDuca, and Charlotte Abraham for able technical assistance and Cindy Bell, Nancy Holdsworth, and Cheryl Mrowczynski for secretarial aid. Dr V. Chapman provided congenic mice containing the sub structural variant of /beta-glucuronidase.

NOTE ADDED IN PROOF

Kazmierowski et al were able to correct the high susceptibility to infection by bone marrow transplantation.

REFERENCES

CORRECTION OF PLATELET STORAGE POOL DISEASE


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