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Sandy: a new mouse model for platelet storage pool deficiency

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Summary

Sandy (*sdy*) is a mouse mutant with diluted pigmentation which recently arose in the DBA/2J strain. Genetic tests indicate it is caused by an autosomal recessive mutation on mouse Chromosome 13 near the *cr* and *Xt* genetic loci. This mutation is different genetically and hematologically from previously described mouse pigment mutations with storage pool deficiency (SPD). The sandy mutant has diluted pigmentation in both eyes and fur, is fully viable and has prolonged bleeding times. Platelet serotonin levels are extremely low although ATP dependent acidification activity of platelet organelles appears normal. Also, platelet dense granules are extremely reduced in number when analysed by electron microscopy of unfixed platelets. Platelets have abnormal uptake and flashing of the fluorescent dye mepacrine. Secretion of lysosomal enzymes from kidney and from thrombin-stimulated platelets is depressed 2- and 3-fold, and ceroid pigment is present in kidney. Sandy platelets have a reduced rate of aggregation induced by collagen. The sandy mutant has an unusually severe dense granule defect and thus may be an appropriate model for cases of human Hermansky–Pudlak syndrome with similarly extreme types of SPD. It represents the tenth example of a mouse mutant with simultaneous defects in melanosomes, lysosomes and/or platelet dense granules.

1. Introduction

Patients with platelet storage pool deficiency (SPD) have reduced levels of platelet dense granule components such as serotonin, adenine nucleotides and calcium with an associated bleeding tendency (Lages, 1987; Rao & Holmsen, 1986; McEver & Majerus, 1989). SPD was thought to be a relatively rare syndrome. Recently, however, Nieuwenhuis *et al.* (1987) have presented evidence that it is more common than previously suspected. Also, Witkop *et al.* (1989) have reported an incidence of Hermansky–Pudlak Syndrome (HPS), a specific type of SPD, as high as 1/2000 in Puerto Rico. The two most studied types of SPD are Chediak–Higashi Syndrome (CHS) and HPS. CHS patients and animal models for CHS have among many phenotypes (Witkop *et al.* 1989; Barak & Nir, 1987; Blume & Wolff, 1972; Brandt *et al.* 1981) giant lysosomes, a deficiency of natural killer cell activity and cytotoxic lymphocyte activity (Baca *et al.* 1989), diluted pigmentation and decreased neutrophil elastase and cathepsin G activity (Takeuchi & Swank, 1989; Ganz *et al.* 1988). Human patients usually die by their teenage years from repeated infections. HPS is usually not fatal although excessive bleeding after tooth extraction, tonsillectomy, and other surgical procedures is common, and in a few cases massive fatal bleeding has occurred (Witkop *et al.* 1989). HPS is further characterized by diluted pigmentation and by accumulation of ceroid deposits in lysosomes (Witkop *et al.* 1989; Depinho & Kaplan, 1985). Several additional subtypes of human SPD have been described including those with dense granule defects only and those with both dense granule and α granule defects (Weiss *et al.* 1979). SPD has also been described in pigs (Daniels *et al.* 1986) and rats (Raymond & Dodds, 1975) and in several animals with CHS including mice (Brandt *et al.* 1981), cats (Prieur & Meyers, 1981), cows (Meyers *et al.* 1979), mink (Meyers *et al.* 1979), fox (Nes *et al.* 1983), and rats (Nishimura *et al.* 1989).

In our previous studies (Novak *et al.* 1988, 1985), a high correlation has been noted between diluted pigmentation and SPD among mouse mutants. Eight of 26 pigment mutants tested had SPD. The symptoms of SPD, including the prolonged bleeding times, have

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been successfully corrected in the mouse mutants by bone marrow transplantation (Novak *et al.* 1985; McGarry *et al.* 1986) indicating that the mutant defect is intrinsic in platelet progenitor cells. The majority of the mouse pigment mutations also produce lysosomal defects. These results, therefore, indicate the genetic relatedness of melanosomes, platelet dense granules and lysosomes. For each mouse mutant the primary gene product, which is missing or abnormal in each mutant, is likely required for the normal synthesis or function of these organelles.

The above mutations provide useful animal models for human SPD. It is likely that several animal models will be required for investigations aimed at increasing our understanding of human SPD since available evidence suggests that human SPD is quite heterogenous (Lages, 1987; Witkop *et al.* 1989; Weiss *et al.* 1979). We describe a new mouse model for SPD, sandy, which recently arose in the DBA/2J inbred mouse strain at The Jackson Laboratory. The sandy model has an unusually severe dense granule defect. The mutation maps to mouse Chromosome (Chr) 13.

2. Materials and methods

Animals. Sandy, *sdy*, an autosomal recessive coat colour mutation occurred spontaneously in the inbred DBA/2J strain in 1983 at The Jackson Laboratory. The primary breeding colony was maintained in the Mouse Mutant Resource at The Jackson Laboratory and all genetic and allelism crosses were performed at The Jackson Laboratory. Mice for physiological studies were maintained at Roswell Park Cancer Institute.

To facilitate linkage testing of *sdy* with other recessive coat colour genes, the mutation was transferred to the C57BL/6J background. A *sdy/sdy* male from the inbred DBA/2J-*sdy* strain, genotype *a/a* (non-agouti), *b/b* (brown), *d/d* (dilute), was outcrossed to a B6C3Fe-*a/a* (C57BL/6J × C3HeB/FeJLe-*a*) hybrid female. The resulting F1 progeny, genotype a/a +/b +/d +/*sdy*, were intercrossed. An F2 female of the genotype a/a ?/+ ?/+ *sdy/sdy*, was backcrossed to her F1 sire. Several *sdy/sdy* progeny from this backcross mating were tested for the presence of brown and dilute. One homozygous *sdy/sdy* male no. 2685 produced from this mating was shown by test matings to be homozygous wild type ($+^b$, $+^d$) at the brown and dilute loci. When mated to a female homozygous at the brown locus and to a female homozygous at the dilute locus, each from an unrelated strain, male no. 2685 produced no brown progeny among 14 offspring and no dilute progeny among 23 offspring, respectively. Male no. 2685 was then mated to a C57BL/6J female and a backcross-intercross breeding system has been used to establish a C57BL/6J-*sdy* colony. *Sdy/sdy* mice on the C57BL/6J background were used in mapping *sdy* to Chr 13. All other experiments were performed with the *sdy/sdy* and *sdy/+* mice on the DBA/2J background.

Bleeding time. Bleeding time was determined by tail bleeding as described (Novak *et al.* 1988; Dejana *et al.* 1979).

Platelet collections and counts. Platelets were collected for serotonin determinations and thrombin-stimulated secretion studies by the method of Holland (Holland, 1976). For all other studies, platelet-rich plasma was collected from citrated, undiluted blood by centrifugation at 150 ***g*** for 10 min. Platelets were counted according to Brecher & Cronkite (1950).

Platelet serotonin assay. Platelets were lysed in 1 ml distilled water and assayed fluorometrically for serotonin according to Crosti & Luchelli (1962).

Mepacrine uptake. Platelets were incubated with mepacrine (Sigma Chemical Co., St Louis, MO) and analysed with a Leitz MPV-2 fluorescent microscope with photomultiplier attachment as previously described (Reddington *et al.* 1987).

Electron microscopy. Unfixed and unstained platelets were rapidly air-dried on carbon-coated grids according to Hui & Costa (1979). Grids were examined using a Hitachi H-600 transmission electron microscope at 50 kV accelerating voltage and platelets were photographed at 12000-fold magnification. The number of dense granules larger than 50 nm were counted in individual platelets.

Platelet aggregation. Aggregation of platelets was examined by whole blood aggregometry since this method allows the analyses of small blood volumes. Blood was collected as previously described (Novak *et al.* 1981). All analyses were performed at 1–3 h after collection of blood. Aggregation tests were performed essentially as described in the Chrono-Log Manual. 100 μl citrated whole blood was diluted with 800 μl 0·85% NaCl and 100 μl Chronolum (containing buffered firefly extract) was added. Final concentrations of platelet aggregating agents used were 1 and 4 μg/ml collagen and 1·2 and 1·5 mg/ml ristocetin (Chronolog Corp., Havertown, PA). Platelet aggregation was measured at 37 °C in stirred whole blood by the electrical impedence method using the Chrono-Log Whole Blood Aggregometer (Model 500) coupled to a dual channel linear strip chart recorder and quantified by a standard internal resistance. Adenosine triphosphate (ATP) secretion was determined simultaneously with platelet aggregation by coupling with luciferin-luciferase in the firefly extract and measuring the light emitted, in comparison to that produced using known concentrations of ATP.

Thrombin-stimulated platelet secretion. Collected

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platelets were washed 2 times in phosphate-buffered saline containing 2% BSA. Platelets, preincubated with [^{3}H]serotonin (Nishimura *et al.* 1989), were treated with thrombin (Sigma Chemical Co., St Louis, MO) for 3 min with constant shaking. The reaction was stopped with 2·5 nmol/ml of Thromstop (American Diagnostics Inc., Greenwich, CT), a synthetic competitive inhibitor of thrombin (Svendsen *et al.* 1984).

Acidification assay. Proton pump activity was measured (Colbaugh *et al.* 1989) by the initial rate of acridine orange accumulation within acidic vesicles. Accumulation was measured as the difference between the absorbance at 492 nm and 540 nm in an LKB 4054 Ultrospec Plus spectrophotometer. Collected and washed platelets were resuspended in pH 7·0, 30 mM histidine, 20 mM-KCl, 130 mM-NaCl, 3 mM $MgCl_2$ and 10 μg/ml oligomycin (to inhibit mitochondrial ATPase activity) at 5×10^8 platelets/ml. The combined platelets from three mice were disrupted in a Parr nitrogen bomb at 4 °C by compressing to 1200 lb/in^2 for 15 min followed by decompression. After two compression-decompression cycles, microscopic analysis revealed that greater than 98% of platelets were disrupted. Postnuclear supernatants were prepared by centrifugation at 1000 *g* for 10 min. One ml volume of supernatants (at about 1 mg protein/ml) was made to 2·5 μM in acridine orange. After establishing a baseline reading for 2 min, the reaction was initiated by addition of ATP to 1 mM and accumulation of acridine orange was measured for 7–8 min. Estimates of acidification are expressed in arbitrary optical density units as the initial slope of ATP-induced quenching of acridine orange. In control experiments, no acidification activity was detected in vesicle-free samples prepared by centrifugation of extracts at 100000 *g* for 1 h or in extracts minus ATP. Also, addition of the inhibitor FCCP (carboxyl cyanide *p*-trifluoromethoxyphenylhydrazone) caused rapid reversal of the quenching of acridine orange.

Urine and tissue collection. To amplify lysosomal enzyme concentrations in kidney and urine, mice were treated for 20 days with testosterone as previously described (Brandt *et al.* 1975). At days 20–24, mice were placed in metabolism cages (2–4 per cage) and urine was collected at 24 h intervals. After day 24, mice were killed by anoxia with CO_2 and tissues were homogenized and stored frozen.

Enzyme assays. β-glucuronidase and β-galactosidase were assayed with fluorescent methylumbelliferyl substrates (Brandt *et al.* 1975). Protein was determined using the Bio-Rad protein assay system (Bio-Rad Laboratories).

Table 1. *Bleeding times and platelet counts in heterozygous F_1 control mice and in homozygous* sdy/sdy. *Values represent the mean ± S.E.M. of the number of mice in parentheses. Mice were 6–15 weeks old*

Genotype	Bleeding time (min)	Platelets/ml ($\times 10^{-9}$)
sdy/+	2·00 ± 0·22 (10)	1·44 ± 0·12 (6)
sdy/sdy	> 15 (14)	1·42 ± 0·03 (6)

3. Results

(i) *Abnormal bleeding times in homozygous sandy mutants*

Bleeding times in heterozygous *sdy*/+ mice were within the normal range with a mean of 2 min and a range of from 1·2 to 3·0 min. In contrast, the bleeding times of 14 homozygous *sdy/sdy* mutants were, without exception, greater than 15 min (Table 1). Long bleeding times were obtained with both male and female mice. Sandy is the tenth mouse pigment mutation to produce a prolonged bleeding time (Novak *et al.* 1984, 1988; Ahmed *et al.* 1989).

(ii) *Phenotypic characteristics and genetic analyses of the sandy mutant*

Homozygous sandy (*sdy/sdy*) mice can be distinguished from non mutant mice at birth by lack of pigment in the eye. At 7–8 days of age the fur, ears, feet and tail are considerably lighter in colour than normal littermates. Both juvenile and adult homozygotes are characterized by a reduction in coat colour pigment with light under fur and red eyes. Matings (Table 2) with sandy mice show *sdy* is an autosomal recessive mutation. Homozygous sandy progeny were recovered in the expected numbers after various types of matings and therefore are fully viable (Table 2). Homozygous *sdy/sdy* mice on either the DBA/2J or C57BL/6J inbred strain backgrounds are

Table 2. *Results of matings with* sdy

Pigment				
	Phenotype			
Mating	+/− (normal)	*sdy/sdy* (sandy)	Total	χ^2
+/*sdy* × +/*sdy*	146 (139)	39 (46)	185	1·515
sdy/sdy × +/*sdy*	202 (200)	199 (201)	401	0·0224
+/*sdy* × *sdy/sdy*	236 (227)	217 (226)	453	0·797
sdy/sdy × *sdy/sdy*	— (0)	14 (14)	14	
sdy/sdy × +/+	72 (72)	— (0)	72	

Numbers in parentheses are the numbers of progeny of each phenotype expected assuming that *sdy* is a single autosomal recessive gene.

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Table 3. *Two point crosses showing linkage of* sdy *with* cr, *and* Xt^J

Cross	Mating (female × male)	Phenotype of progeny: ++ (normal)	*sdy*+ (sandy)	+*cr* (crinkled)	*sdy cr* (sandy-crinkled)	Total	Recombination ±S.E.M. (%)
1.	*sdy* +/+ *cr* × *sdy* +/+ *cr*	73	27	36	—	136	(⩽ 29, 95% UCL)
2.	*sdy cr*/+ + × *sdy cr*/+ +	102	9	7	28	146	11·70±2·86
3.	*sdy cr*/*sdy cr* × *sdy* +/+ *cr*	5	25	29	6	65	16·92±4·65
		Xt^J +	+ *sdy*	*Xt^J sdy*	++	Total	Recombination ±S.E.M. (%)
4.	*Xt^J* +/+ *sdy* × + *sdy*/+ *sdy*	41	47	5	11	104	15·38±3·54

indistinguishable from homozygous pallid (*pa*/*pa*) (Green, 1989) on the C57BL/6J background. Homozygous *sdy*/*sdy* mice on a C3H (*A*/*A*) background resemble homozygous muted (*mu*/*mu*) (Green, 1989) in coat colour including light under fur, but differ in eye colour with *sdy* producing the lighter eyes of the two. Eye pigmentation darkens with age in muted homozygotes but not in sandy homozygotes. Tests for allelism between *sdy* and each of the genes, *pa* and *mu*, as well as beige (*bg*) and pearl (*pe*) (Green, 1989) also on Chr 13 were negative.

Crosses that confirmed the Chr 13 location of *sdy* are given in Tables 3 and 4. The mutant gene crinkled (*cr*) (Green, 1989) was selected as a Chr 13 marker that produces an easily scorable phenotype (Table 3). No *cr sdy*/*cr sdy*, recombinants were produced in a repulsion intercross among a total of 136 progeny (cross 1), giving a recombination estimate of ⩽ 29% linkage at the 95% confidence level. To obtain *cr* and *sdy* on the same chromosome, 11 of the *cr* +/*cr*? offspring and 11 of the +*sdy*/?*sdy* offspring were mated together. Four of the 11 *cr* +/*cr*? mutants were found to carry *sdy* and one of the 11 + *sdy*/?*sdy* mutants was found to carry *cr*. These data give a recombination estimate of 12·82±5·69% between the *cr* and *sdy* genes calculated according to the formula given by Deol & Green (1966). Mice homozygous for *cr* and *sdy* were recovered from descendants of these tested F2 individuals and used for crosses 2, 3 and 5 in Tables 3 and 4. A separate two point cross using the dominant Chr 13 marker, extra toes-J (Xt^J) (Green, 1989), gave a recombination estimate of 15·38±3·54% between the *Xt* and *sdy* genes (Table 3, cross 4). Recombination estimates for intercrosses were calculated using a computer program devised by E. L. Green for analysing linkage data (Green, 1985). Using the weighted average method (Mather, 1938) to combine the data from Table 3, crosses 2 and 3 (Table 3), crosses 5 and 6 (Table 4) and the test matings in cross 1 gives a combined recombination estimate of 15·96±1·49 for the *cr*-*sdy* interval. To determine the position of sandy in relation to *cr* and Xt^J, three points backcrosses (crosses 5 and 6) were made. The data show the most likely order is *cr*–Xt^J–*sdy* with the *sdy* gene being about 17 map units from the closely linked *cr* and *Xt* genes. This order is supported by preliminary data from a three-point cross in progress in which a Robertsonian chromosome is used to mark the centromere end of the chromosome. The arrangement of marker genes and genes causing platelet

Table 4. *Results showing linear order of* cr, Xt^J *and* sdy

Cross	Matings (female × male)	Phenotype of progeny: *cr*+*sdy* (crinkled-sandy)	+Xt^J+ (extra toes)	*cr* Xt^J + (crinkled extra toes)	++ *sdy* (sandy)	*cr* ++ (crinkled)	+ Xt^J*sdy* (extra toes sandy)	*cr* Xt^J*sdy* (crinkled extra toes sandy)	+++ (normal)	Total
5.	$\frac{cr+sdy}{+Xt^J+} \times \frac{cr+sdy}{cr+sdy}$	141	144	1	5	34	28	—	6	359
6.	$\frac{cr+sdy}{cr+sdy} \times \frac{cr+sdy}{+Xt^J+}$	25	52	1	—	3	3	—	—	84
	Total	166	196	2	5	37	31	—	6	443

Recombination estimates ±S.E.M.

	Females	Males	Combined
cr–Xt^J	12/359 = 3·34±0·95	1/84 = 1·19±1·18	13/443 = 2·93±0·80
Xt^J–*sdy*	68/359 = 18·94±2·07	6/84 = 7·14±2·81	74/443 = 16·70±1·77
cr–*sdy*	68/359 = 18·94±2·07	7/84 = 8·33±3·01	75/443 = 16·93±1·78

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SPD on Chr 13 together with their intervening cM recombination distances is:
centromere–17–*cr*, *bg*-2-*Xt*^{J}–17–*sdy*–27–*pe*

(iii) *Other physiological analyses*

The long bleeding times of sandy mice were not caused by thrombocytopenia since normal platelet counts of about $1{\cdot}4 \times 10^9$/ml were present in both normal and mutant mice (Table 1). A strikingly abnormal feature of platelets of the mutant mice were a series of abnormalities in measurements of platelet dense granules (Table 5). Platelet serotonin concentrations in the mutant were only 7% of normal values. Lowered dense granule components are a principal diagnostic feature of storage pool deficiency in both humans and animals with storage pool deficiency (Lages, 1987).

Further evidence for an abnormality in the platelet dense granule environment was obtained by measurement of the uptake of the fluorescent dye mepacrine. Previous studies (Skaer *et al.* 1981) have demonstrated that mepacrine is specifically incorporated into platelet dense granules rather than other platelet subcellular organelles. Also, upon prolonged illumination with ultraviolet light, a flashing phenomenon, thought to be caused by decreased quenching of fluorescence when mepacrine is released from platelet dense granules into the cytosol, occurs (Lorez *et al.* 1975). This flashing phenomenon typically does not occur in platelets from SPD patients or animal models with SPD, probably because of the altered intragranular environment in SPD.

When platelets of normal and mutant mice were treated with mepacrine, equal numbers of fluorescent granules/platelet were apparent by light microscopy (Table 5). Mepacrine uptake (as measured by relative intensity) in mutant platelets was only 67% of normal, however, and an abnormal intragranular environment was apparent in the 10-fold reduction in flashes/granule in mutant platelets.

Another method of directly visualizing the dense granule defect in platelet storage pool deficiency is by electron microscopy of air dried whole mounted platelets (Hui & Costa, 1979; Novak *et al.* 1981). The results of these analyses indicated a striking abnormality in platelet dense granules in that either no or very few dense granules were visible in *sdy/sdy* platelets (Fig. 1). A collation of the distribution of dense granules in 90 individual *sdy/+* and *sdy/sdy* platelets is given in Fig. 2. A very broad distribution of the number of dense granules, with a mean of 10, was found in *sdy/+* platelets. An extreme abnormality was present in mutant *sdy/sdy* in that 83 of 90 platelets had no dense granules, 4 had 1 granule and 3 had 2. No significant differences were noted between the sizes of normal and mutant platelets.

To determine if the increased bleeding times in mutant mice were associated with abnormal aggregation, normal and mutant platelet samples were analyzed by whole blood aggregometry using collagen as agonist (Fig. 3). The release of ATP from dense granules was monitored simultaneously using a firefly luminescence method. At low (1 μg/ml) collagen concentrations (Fig. 3*b*) normal *sdy/+* platelets had significant aggregation and ATP release. These responses were increased at high (4 μg/ml) concentrations (Fig. 3*a*). A reduced rate of aggregation of mutant platelets was apparent at low collagen concentrations (Fig. 3*a*). This rate was restored to normal levels at high collagen concentrations (Fig. 3*b*). A more notable feature of the mutant aggregation was the complete absence of secreted ATP, normally derived from dense granules, at either collagen concentration.

An interesting feature of storage pool deficiency in murine pigment models is a triad of organellar deficiencies in melanosomes, platelet dense granules and lysosomes (Novak *et al.* 1984, 1988). The melanosome deficiency causes pigment dilution, the platelet dense granule deficiency is associated with bleeding upon experimental injury and the lysosomal abnormalities include defects in secretion of lysosomal enzymes. Comparative analyses of secretion of the kidney lysosomal enzymes β-glucuronidase and β-galactosidase into urine are listed in Table 6. Daily secretion from *sdy/sdy* kidneys were reduced to about 50% the normal secretion for both lysosomal enzymes. Consistent with this reduced rate of secretion was the finding (Table 7) that the concentration of both

Table 5. *Characteristics of dense granules of normal* (sdy/+) *and mutant* (sdy/sdy) *platelets as determined by fluorescence microscopy of mepacrine-labelled normal and mutant platelets and serotonin determinations*

Genotype	Granules/platelet	Flashes/platelet	Flashes/granule	Relative intensity (mv)	Serotonin (μg/10^9 platelets)
sdy/+	4·33 ± 0·23 (27)	3·59 ± 0·32 (27)	0·83	39·5 ± 1·5 (37)	2·37 ± 0·41 (3)
sdy/sdy	3·85 ± 0·20 (27)	0·33 ± 0·10 (27)*	0·086*	26·4 ± 1·2 (38)*	0·16 ± 0·004 (3)*

Values are the mean ± S.E.M. of the number of determinations in parentheses. Relative intensity/platelet was determined prior to commencement of the flashing phenomenon. Flashes/granule values were calculated by dividing flashes/platelet by granules/platelet.
* $P \leqslant 0{\cdot}001$.

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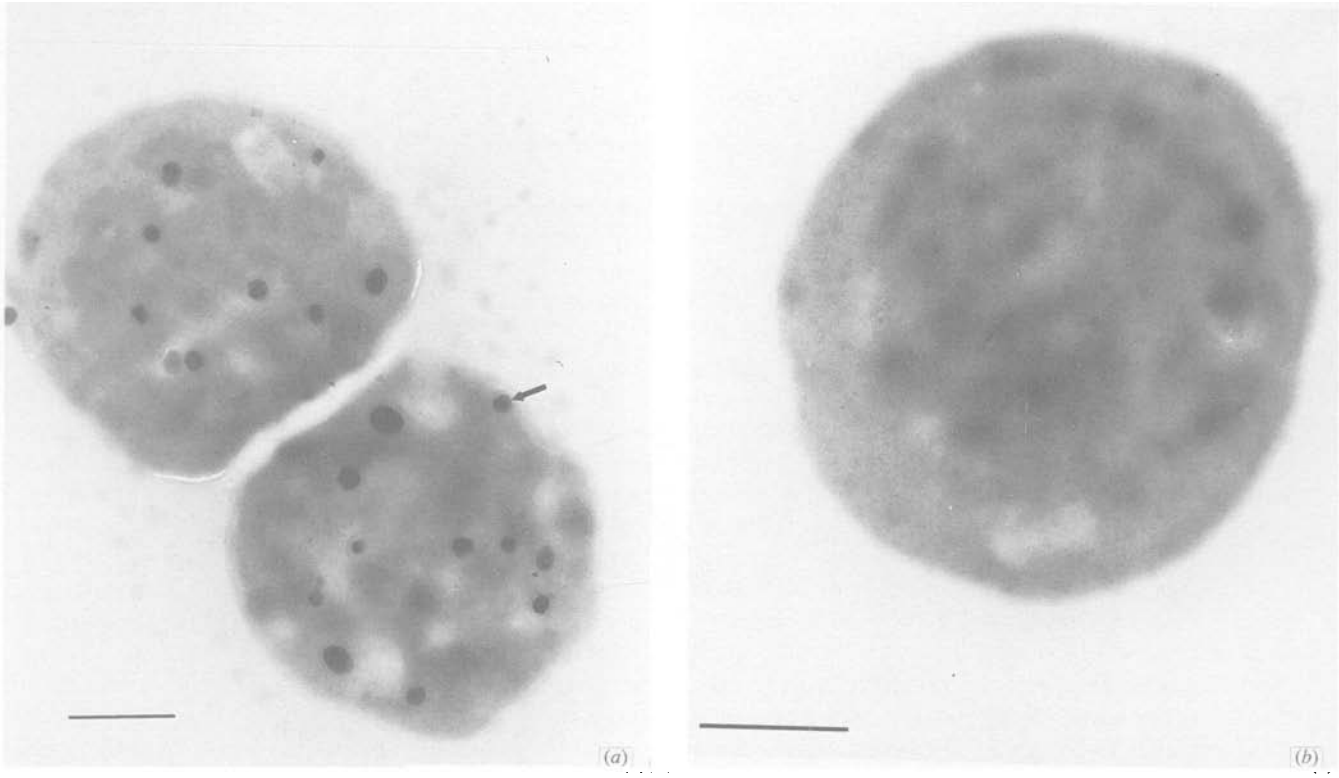

Fig. 1. Electron micrographs of instantaneously air dried and unstained whole platelets from (*a*) normal *sdy*/+ and (*b*) mutant *sdy*/*sdy* mice. The arrow indicates a typical dense granule of a normal platelet. The bar represents 1 μm.

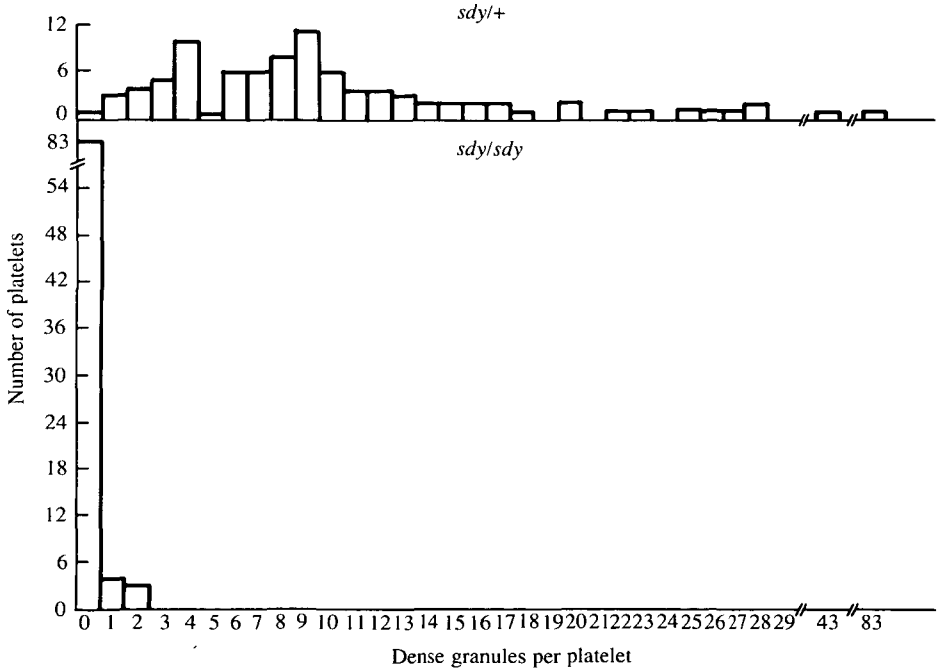

Fig. 2. Histograms of number of dense granules per individual platelet. The number of platelets surveyed were 91 *sdy*/+ and 90 *sdy*/*sdy*. The two platelets with 43 and 83 dense granules per platelet were approximately twice the size of normal platelets.

lysosomal enzymes was doubled in kidneys of mutant mice. That the effect on kidney lysosomal enzymes is specific and likely due to the secretion abnormality is evidenced by the lack of any mutant effect on lysosomal enzyme levels in liver (Table 7), an organ from which significant lysosomal enzyme secretion does not occur.

It was also of interest to determine if there is abnormal secretion of granule contents of platelets of the *sdy*/*sdy* mutant mice, as has been observed in the

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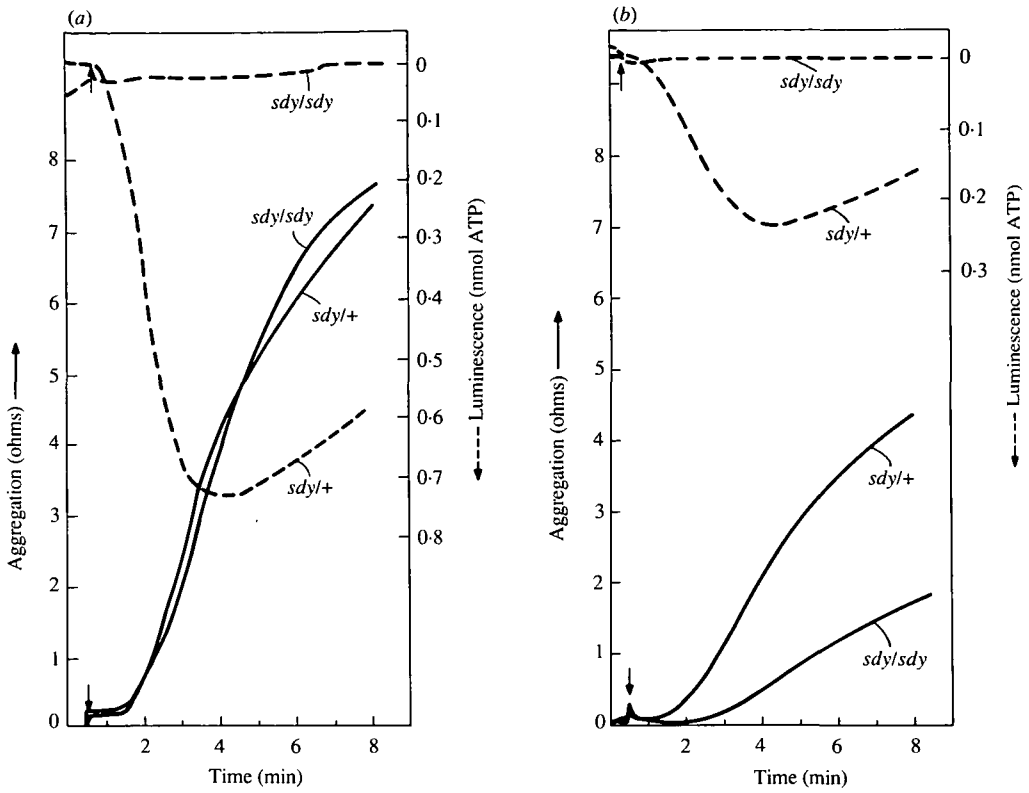

Fig. 3. Collagen-mediated aggregation of mutant *sdy/sdy* and normal *sdy/+* platelets and release of ATP. Platelet aggregation was determined by the impedance method in whole blood in response to 4 μg/ml (*a*) and 1 μg/ml (*b*) collagen. ATP release was simultaneously determined by luminescence methods. The small arrow indicates the time of addition of collagen.

Table 6. *Daily secretion of lysosomal enzymes into urine of normal and mutant mice. Each metabolism cage contained 3 female mice which had been treated with testosterone for 22 days*

Genotype	β-Glucuronidase (units/mouse)	β-Galactosidase (units/mouse)
sdy/+	12·6 ± 0·80	1·29 ± 0·097
sdy/sdy	6·1 ± 0·60*	0·69 ± 0·050*

Values represent the mean ± S.E.M. of 4 determinations on consecutive days.
* $P \leqslant 0{\cdot}01$.

cases of other mutant mice with storage pool deficiency. Upon thrombin stimulation of isolated platelets, secretion of three types of organelles including dense granules, α granules and lysosomes, rapidly occurs (Hawiger, 1989). When secretion of [^{3}H]serotonin, a dense granule marker, was monitored (Table 8), an abnormality was apparent in that even with no thrombin treatment, a large percentage of labelled serotonin was lost from *sdy/sdy* platelets during the 3 min incubation and subsequent centrifugation step (40% *vs.* the 5% lost from normal *sdy/+* platelets). In fact, the amount of serotonin secreted from *sdy/sdy* platelets was not significantly increased with low (0·25 U) thrombin. At high (2·5 U) thrombin, the small nominal increase (to 53%) in secretion was also not statistically significant. In contrast, 78 and 91% of total incorporated serotonin was secreted from normal *sdy/+* platelets treated with low and high thrombin concentrations, respectively.

Thrombin-stimulated secretion of the two platelets lysosomal enzymes β-glucuronidase and β-galactosidase was also considerably reduced in mutant as compared with normal platelets (Table 8). Secretion of 20–25% of both enzymes occurred after treatment of normal platelets with high thrombin. In contrast, high thrombin stimulated the secretion of only 6–8% of each enzyme from normal *sdy/+* platelets. Similar reductions in secretion were noted at low thrombin concentrations.

Table 7. *Concentrations of lysosomal enzymes in kidney and liver of testosterone-treated* sdy/+ *normal and* sdy/sdy *mutant mice*

Tissue	Genotype	β-Glucuronidase (units/g)	β-Galactosidase (units/g)
Kidney	*sdy/+*	80 ± 9·2	9·13 ± 0·85
	sdy/sdy	137 ± 6·5*	20·2 ± 0·73†
Liver	*sdy/+*	17·1 ± 2·0	3·38 ± 0·34
	sdy/sdy	13·6 ± 0·9	3·54 ± 0·20

Values are the mean ± S.E.M. of 3 mice.
* $P \leqslant 0{\cdot}02$; † $P \leqslant 0{\cdot}001$.

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Table 8. *Thrombin-stimulated secretion of platelet dense granule contents and lysosomal enzymes. Platelets (1×10^8 cells/ml) were treated with thrombin for 3 min at 37 °C. The reaction was stopped with thromstop. Values represent the mean ± S.E.M. of three determinations. Total cpm [^3H]serotonin initially incorporated in* sdy/+ *and* sdy/sdy *platelets were 10000 and 7130, respectively, per 10^8 platelets*

	Thrombin (U)			
	0	0·25	2·5	2·5+ADP
	Secretion (% total)			
[^{3}H]serotonin				
sdy/+	5·3 ± 1·4	78·3 ± 3·2	90·6 ± 1·0	90·5 ± 0·3
sdy/sdy	40·0 ± 4·4*	43·7 ± 3·7*	53·0 ± 5·1*	54·8 ± 3·4†
β-Glucuronidase				
sdy/+	4·5 ± 0·90	12·5 ± 3·5	21·5 ± 0·75	23·0 ± 0·76
sdy/sdy	2·9 ± 0·36	2·3 ± 0·67§	6·2 ± 1·6*	6·8 ± 2·0*
β-Galactosidase				
sdy/+	5·1 ± 0·75	21·7 ± 4·2	24·0 ± 1·3	27·0 ± 3·0
sdy/sdy	3·8 ± 1·3	6·4 ± 1·1§	7·9 ± 1·4*	9·0 ± 0·50*

† $P \leqslant 0{\cdot}001$; * $P \leqslant 0{\cdot}01$; § $P \leqslant 0{\cdot}05$.

An intriguing common feature of melanosomes, lysosomes and platelet dense granules is that all have acidic interiors (Anderson & Orci, 1988; Dean *et al.* 1984; Wolff & Schreiner, 1970). To test if the abnormalities in the structure or processing of these organelles in sandy and the other pigment mutants were due to defective acidification, the proton pumping activity of platelet acidic vesicles was measured as the initial rate of accumulation of acridine orange within the vesicles (Colbaugh *et al.* 1989). It was found (Table 9) that acridine orange accumulated reproducibly at a decreased rate (25% less than normal) in platelet vesicles of sandy mice. However, by measurements of sedimentation (100000 ***g***, 30 min) of β-glucuronidase activity of normal and sandy platelets extracts, it was apparent that sandy extracts had 23% fewer intact lysosomes than normal (not shown). The likely explanation for the lowered proton pumping activity of sandy platelet extracts therefore is not a defective proton pump but rather a greater susceptibility of sandy lysosomes to disruption during homogenization. Normal proton pumping activity was found in platelet extracts of other pigment mutants with platelet SPD (Novak *et al.* 1984, 1988) including pearl, maroon, ruby-eye, light-ear, pale ear and cocoa (not shown).

Lages *et al.* (1988) and Rendu *et al.* (1987) have recently observed that the abnormally low lysosomal enzyme secretion from platelets of human SPD patients can be restored by inclusion of ADP in the secretion buffer. This result suggests that the dense granule component ADP is required for normal lysosomal enzyme secretion. No effect of ADP on secretion of either lysosomal enzymes or serotonin was apparent, however, in the case of platelets from *sdy/sdy* mice (Table 8). We have observed no effect on secretion of lysosomal enzymes from platelets of *sdy/sdy* mice when 1, 5, 10 or 20 μM-ADP was included in the secretion buffer. Also, inclusion of serotonin, another dense granule component present at very low levels in *sdy/sdy* mice, did not affect lysosomal enzyme secretion at 0·1, 1 or 5 μM, nor did the simultaneous addition of 1 μM serotonin and 10 μM-ADP.

Table 9. *ATP-stimulated acidification activity of extracts of normal* (sdy/+) *and mutant* (sdy/sdy) *platelets*

Genotype	Initial slope/mg protein ($\times 10^3$)
sdy/+	5·7 ± 0·03 (3)
sdy/sdy	4·3 ± 0·20* (5)

The initial slope is expressed as the absorbance 492–540 nm per minute. Numbers in parentheses are the number of separate determinations. Each determination was made on platelet extracts pooled from three mice.
* $P \leqslant 0{\cdot}001$.

Because of similarities between the sandy mutant and the beige (Chediak–Higashi) mouse mutant in lysosomal secretion abnormalities and in features of platelet SPD, retinal pigment epithelial cells were examined for the giant granules which are characteristic of the Chediak–Higashi syndrome (Brandt *et al.* 1981). No enlarged granules were observed in the sandy mutation. However, ceroid-like pigments, a hallmark of human Hermansky–Pudlak syndrome (Witkop *et al.* 1989; Depinho & Kaplan, 1985), were detected in much higher than normal quantities in proximal tubules when frozen sections of kidney of sandy mice were examined by ultraviolet fluorescence microscopy.

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Discussion

Sandy is a recently discovered pigment dilution mutation which arose spontaneously in the DBA/2J inbred mouse strain. It is caused by an autosomal recessive gene located on mouse Chr 13, and this gene is independent of previously described mouse pigment genes causing SPD. Because it arose spontaneously on the DBA/2J inbred background, sandy mice likely are identical to mice of the parental inbred strain except for the sandy mutation.

Besides causing pigment dilution, the sandy mutation has a number of effects on platelets identical to those found in human Hermansky–Pudlak syndrome (HPS) (Witkop *et al.* 1989; Depinho & Kaplan, 1985). It causes, like HPS, an obvious pigment dilution and an accumulation of ceroid-like pigment. Also, it causes increased bleeding time with no effect on platelet numbers. Platelet dense granule contents including serotonin and ATP are very much reduced below normal levels. Further evidence for a dense granule abnormality was apparent in morphological studies. No dense granules were apparent by whole mount electron microscopic studies, but near normal levels were visible by mepacrine analyses. Our interpretation of these results is that there are normal numbers of immature dense granules which are unable to retain normal quantities of dense granule components. Another interpretation of these results is that mepacrine is actually accumulating in other acidic subcellular organelles, such as lysosomes, in the sandy mutant and therefore that sandy platelets have no dense granules. This would require, however, that the numbers of this other organelle fortuitously are equal to the number of dense granules in normal platelets. Resolution of this issue will require enumeration of dense granules by a specific membrane marker (Lages, 1987). Aggregation is normal with high but somewhat reduced with low collagen concentrations, a characteristic also seen with the mouse cocoa mutation (Novak *et al.* 1988). Collagen-induced aggregation and release of ATP is also reduced in human HPS (Weiss *et al.* 1979; Rendu *et al.* 1987).

It is intriguing that a single mutation simultaneously affects three subcellular organelles, melanosomes, platelet dense granules, and lysosomes. This indicates that a single gene affects the biogenesis and/or processing of all three organelles. In fact, at least ten unique genes (Novak *et al.* 1984, 1988), including beige, pearl, pallid, maroon, ruby-eye, light ear, pale ear, cocoa, reduced pigmentation (Ahmed *et al.* 1989; Gibb *et al.* 1981) and sandy, of the mouse have been found to simultaneously effect the above organelles. Human patients with storage pool deficiency likewise often have abnormalities in other organelles including melanosomes (Witkop *et al.* 1989) and platelet α granules (Weiss *et al.* 1979). The ten mouse genes are scattered throughout the genomes. Three of the mutants (beige, pearl and sandy) are on separate sites on mouse Chr. 13. Although the primary gene defect has not been identified in any case, the pigment mutants are a rich source of separate genes that control the biogenesis, structure and/or processing of three organelles.

The finding of a large number of genes involved in the biogenesis/processing of the above organelles is consistent with genetic studies of related systems. Novick *et al.* (1980) have identified 23 genes involved in the secretion of organellar proteins from yeast. Also, a minimum of 49 distinct genes whose products are required to sort, transport and/or retain lysosomal proteins have been identified in yeast (Rothman *et al.* 1989).

Despite their similarities in affecting the same three subcellular organelles, the above mouse pigment genes usually produce unique phenotypic effects. For example, with the exception of the light ear and pale ear and the ruby-eye and ruby-eye-2 mutations that mimic each other, the effects on coat colour are distinguishable. Light ear and pale ear are distinguished from the other mutants in that they have increased rather than decreased secretion of platelet lysosomal enzymes upon thrombin stimulation (Novak *et al.* 1984) and have abnormally low rates of secretion of lysosomal enzymes from macrophages treated with ammonia (Brown *et al.* 1985). The cocoa mutation (Raymond & Dodds, 1975) causes pigmentation and platelet dense granule abnormalities like all other mutants but has no apparent effect on lysosomal functions. Differences in phenotypes of human patients with SPD have also been reported (Witkop *et al.* 1989; Weiss *et al.* 1979). It is thus probable that in humans, as in mice, a large number of genes can cause SPD. The availability of several animal models of human SPD should, therefore, be advantageous in modelling human SPD. A further advantage to study of the mouse mutations including sandy are that they are genetically identical to the normal parental strain except for the chromosomal site of the pigment mutation. Therefore any abnormality observed can be attributed with high probability to the mutant gene rather than to differing background genes.

Because sandy and other mouse pigment mutants have simultaneous abnormalities in the structure and/or processing of several acidic organelles, including melanosomes, lysosomes and platelet dense granules, and because Dean *et al.* (1984) have demonstrated that serotonin accumulation in platelet dense granules is driven by a proton pumping ATPase, the ATP-dependent proton pumping capability of platelet acidic organelles was tested. The small (25%) loss in acidification activity of sandy platelet organelles was found to be associated with a corresponding increase in fragility of platelet lysosomes rather than to defective proton pumping activity. The increased fragility of sandy lysosomes is interesting since it suggests an abnormality in the structure of the lysosomal membrane.

Homozygosity for the sandy mutation causes an

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unusually low content of platelet dense granule components in comparison to all other mouse mutants with SPD (Novak *et al.* 1984, 1988). By whole mount electron microscopy, only 10 granules were apparent in a total of 90 platelets examined. In contrast, the mouse SPD mutant pallid, which previously had the lowest recorded number of dense granules (Novak *et al.* 1984), had 25 granules in 52 platelets. Sandy and pallid have both the greatest degree of pigment dilution and the most severe form of SPD among the mouse mutants. Similarly, serotonin and ATP levels in sandy mice were barely above background levels. Further evidence of the extreme severity of the dense granule defect in the sandy mutant derives from tests of thrombin-stimulated serotonin secretion. A very high percentage (40%) of serotonin was spontaneously released from sandy platelets which had not been treated with thrombin. Also, upon thrombin stimulation, only 53% of serotonin was released compared to 91% in normal controls.

The severity of the secretion defect in sandy platelets was likewise evident when thrombin-stimulated lysosomal enzyme release was measured. Even at high (2·5 U) thrombin, only 6–9% of lysosomal enzymes were released compared with 22–27% release from normal platelets. Secretion of lysosomal enzymes from a second cell type, the kidney proximal tubule, was also depressed. The impaired secretion of platelet lysosomal enzymes was not corrected by exogenous ADP or serotonin. Lages *et al.* (1988) and Rendu *et al.* (1987) found, in contrast, that added ADP corrected the impaired secretion of lysosomal enzymes from platelets of several SPD patients. Presumably ADP, found in normal dense granules and secreted prior to secretion of lysosomal enzymes, stimulates the subsequent secretion of lysosomal enzymes. Whether this result represents differences in species or in the particular SPD genes compared is uncertain. Another difference between human SPD platelets and sandy platelets is that treatment with high concentrations of thrombin corrects the lysosomal enzyme secretion defect in human SPD (Lages *et al.* 1988) but not in sandy platelets.

Variable levels of both dense granule and alpha granule components are found in platelets of SPD patients (Lages, 1987; Weiss *et al.* 1979). Of relevance to the severe deficiency of dense granule components in platelets of the sandy mutant is the finding that HPS patients have significantly greater deficiencies of dense granule components than other types of SPD patients who have no associated pigment dilution (Lages, 1987). The abnormally low levels of dense granule components in the sandy pigment mutant are consistent with this trend. It is also apparent from both human (Weiss *et al.* 1979) and mouse (Novak *et al.* 1984, 1988) studies that variations in levels of dense granule constituents will also be found within HPS patients and their corresponding animal models.

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