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Studies of *Sl/Sl*^{d}↔+/+ mouse aggregation chimaeras

I. Different distribution patterns between melanocytes and mast cells in the skin

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Summary

In spite of their different origin, both melanocytes and mast cells are deficient in the skin of mutant mice of the *Sl/Sl*^{d} genotype. Since the neural crest and the liver of *Sl/Sl*^{d} embryos contain normal precursors of melanocytes and mast cells, respectively, the deficiency is attributed to a defect in tissue environment necessary for migration and/or differentiation of precursor cells. We investigated whether the tissue environment used for differentiation of melanocytes and mast cells was identical by producing aggregation chimaeras from *Sl/Sl*^{d} and +/+ embryos. Chimaeric mice with apparent pigmented and nonpigmented stripes were obtained. In the nonpigmented stripes of these *Sl/Sl*^{d}↔+/+ chimaeras, melanocytes were not detectable in hair follicles but were detectable in the dermis. In contrast, melanocytes were detectable neither in hair follicles nor in the dermis of non-chimaeric *Sl/Sl*^{d} mice. Concentrations of mast cells were comparable in the pigmented and nonpigmented stripes of *Sl/Sl*^{d}↔+/+ chimaeras, but the average concentration of mast cells significantly varied in the chimaeras (from 8 % to 74 % of the value observed in control +/+ mice). The present result suggests that mesodermal cells that support the migration and differentiation of both melanocyte precursors and mast-cell precursors mix homogeneously in the dermis and that ectodermal cells that influence the invasion of differentiating melanocytes into hair follicles make discrete patches.

Key words: aggregation chimaera, melanocyte, mast cell, steel locus, tissue environment, patch size.

Introduction

Melanoblasts originate from the neural crest and, following their migration, differentiate in the mesodermal layer of the skin (reviewed by Rawles, 1953). In mice, most melanocytes are incorporated into hair follicles, but some remain in the dermal connective tissue in the skin of the ear and tail (reviewed by Silvers, 1979). On the other hand, precursors of mast cells are derived from the fetal liver (Kitamura *et al.* 1979), migrate in the bloodstream (Hayashi *et al.* 1983), invade connective tissues and then differentiate into mast cells (reviewed by Kitamura *et al.* 1983). Although most skin mast cells remain in the connective tissue, some mast cells invade the epithelium, especially in the alimentary canal (Enerbäck, 1981). Therefore, in spite of their apparent difference in origin, melanocytes and mast cells seem to have the mesodermal layer of the skin as a common environment for migration and differentiation.

A double gene dose of mutant alleles at either the *W* or the *Sl* locus is known to produce the pleiotropic effects of hypoplastic anaemia, sterility and lack of hair pigmentation (reviewed by Russell, 1979). In addition to the three above-mentioned abnormalities, we found that mast cells are also depleted in both *W/W*^{v} (Kitamura *et al.* 1978) and *Sl/Sl*^{d} mice (Kitamura & Go, 1979). Although the phenotypic expression of the two mutations is very similar, underlying mechanisms are quite different. The depletion of erythrocytes, melanocytes and mast cells in *W/W*^{v} mice is attributed to a defect in their precursor cells. In fact, neural crest cells of normal +/+ embryos can differentiate in the skin of *W/W*^{v} embryos (Mayer &

Green, 1968; Mayer, 1970) and the transplantation of bone marrow cells from congenic +/+ mice normalizes the number of erythrocytes (Russell *et al.* 1959; McCulloch *et al.* 1964; Russell & Bernstein, 1968) and mast cells (Kitamura *et al.* 1978) in W/W^v recipients.

In contrast, the depletion of erythrocytes, melanocytes and mast cells in Sl/Sl^d mice is due to a defect in the tissue environment. Mayer & Green (1968) and Mayer (1970) investigated the effect of the *Sl* mutant gene on the differentiation of melanocytes by transplanting recombined mouse embryonic tissues to the chick coelom. The neural crest of normal +/+ embryos failed to produce melanocytes when combined with Sl/Sl^d embryonic skin, whereas neural crest from Sl/Sl^d embryos produced melanocytes when combined with +/+ neural-crest-free embryonic skin. Moreover, Mayer (1973) recombined the mesodermal and ectodermal components between Sl/Sl^d and +/+ embryos, and demonstrated that both components of Sl/Sl^d embryos were defective.

Haematopoietic tissues of Sl/Sl^d mice contain stem cells that form haematopoietic colonies in the spleen of irradiated +/+ mice, whereas transplanted +/+ bone marrow cells do not produce colonies in irradiated Sl/Sl^d spleens (McCulloch *et al.* 1965; Bernstein, 1970). When +/+ bone marrow cells were injected directly into the skin of W/W^v and Sl/Sl^d mice, mast cell clusters appeared only in the connective tissue of the W/W^v recipients (Sonoda *et al.* 1982). Moreover, mast cells did not develop in Sl/Sl^d skin that was grafted to the back of +/+ mice (Kitamura & Go, 1979; Matsuda *et al.* 1981). Therefore, not only is mast cell differentiation adversely affected by Sl/Sl^d skin but differentiated mast cells cannot invade skin of this genotype.

Melanocytes and mast cells are deficient in the skin of Sl/Sl^d mice, and the deficiency of both types of cells is attributed to a defect in the tissue environment. However, the tissue environment necessary for differentiation of both types of cells has not been characterized. In the present study, we investigated whether the environment for melanocytes is identical with that for mast cells by producing aggregation chimaeras between Sl/Sl^d and +/+ embryos. Chimaeras with apparent pigmented and nonpigmented stripes were obtained; in spite of the lack of melanocytes in hair follicles of nonpigmented stripes, concentrations of mast cells were comparable between the pigmented and nonpigmented stripes.

Materials and methods

Mice

C57BL/6-Sl^d/+ and WB-*Sl*/+ mice as well as their F_1 hybrids (hereafter WBB6F_1) were raised in our laboratory. The original stocks were derived from the Jackson Laboratory, Bar Harbor, ME. C3H/He (hereafter C3H) and ICR (albinos employed as foster mothers of aggregated embryos) mice were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan. The C3H strain is *A/A* (agouti), and the WB and C57BL/6 strains *a/a* (nonagouti). Agouti hairs and nonagouti hairs are easily identified under the microscope. Mice of C57BL/6-Sl^d/+, WB-*Sl*/+ and C3H have B-type phosphoglycerate kinase (PGK), and we recently established a C57BL/6 congenic strain with the A-type PGK.

Aggregation chimaeras

The method described by Mintz *et al.* (1973) and by Hoppe & Pitts (1973) was used with a slight modification. Briefly, 8- to 16-cell embryos were aggregated in Hoppe's medium containing phytohaemagglutinin (Difco Laboratories, Detroit, MI) after removal of the zona pellucida with acidified Tyrode's solution (pH 2·5; Nicolson *et al.* 1975). Subsequently, the aggregated embryos were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. The resulting chimaeric blastocysts were transferred to the uterus of a 3·5-day *post coitum* pseudopregnant ICR female mouse.

Whole-mount specimen of the dermis

Neonatal mice were killed within 24 h after birth and their skin removed. Melanocytes were identified by their tyrosinase activity (Staricco & Pinkus, 1957). Thus, the whole skin was incubated at 37°C in phosphate-buffered saline (PBS) containing 0·1% 3,4-dihydroxyphenylalanine (DOPA, Wako Pure Chemical Industries, Osaka) for 6 h before being dipped in heated PBS at 50°C for 2 min to separate the epidermis from the dermis (hair follicles remained in the dermis). The specimen was fixed in 10% formalin overnight, and stained with alcian blue. Subsequently, the specimen was dehydrated and cleared. The whole mount of dermis was examined under the microscope; the number of hair follicles with DOPA-positive cells and the number of mast cells (expressed as numbers mm^{-2}) were counted using a square micrometer.

Paraffin sections

The whole skin of chimaeric mice was removed, attached to a hard rubber board with needles and fixed in 10% formalin. White regions and pigmented regions were carefully dissected and embedded in paraffin. Sections (5 μm thick) were stained with acidified toluidine blue (pH 3·0). The number of mast cells was counted under the microscope, and expressed as the number cm^{-1}.

Electrophoresis of PGK

The whole skin of chimaeric mice was removed. White and black regions were carefully dissected. When 'pure white' and 'pure black' regions were selected, it was very difficult to obtain skin fragments larger than 1 mm^2. In one experiment, the epidermis and the dermis of each skin region were separated by incubating in 0·25% trypsin solution (Sigma Chemical Co., St Louis, MO) at 4°C for 14 h (Szabó, 1955; Hirobe *et al.* 1987).

Lysis of skin cells was achieved by freezing and thawing according to the method described previously (Taguchi *et al.* 1984). A tissue fragment inserted between two small pieces of filter paper (3×3 mm) was placed in a small plastic container and kept in a freezer (−80°C). After thawing, the tissue fragment (along with two pieces of filter paper) was inserted into the gel plate (10×20 cm) of starch (Wako Pure Chemical Industries, Osaka). The electrophoresis was carried out according to the method described by Tanooka & Tanaka (1982).

The relative activity of PGK allozymes was estimated visually using known mixtures as standards (Reddy & Fialkow, 1979). The proportion of A-type PGK was scored from 0 % to 100 % with 10 % intervals.

Results

Since mice of the Sl/Sl^d genotype are sterile, WB-Sl/+ and C57BL/6-Sl^d/+ mice were mated to obtain Sl/Sl^d embryos. Therefore, mixing of embryos of three other genotypes (Sl/+, Sl^d/+ and +/+) was inevitable. In the first series of experiments, embryos with four different genotypes were aggregated with embryos of the C3H strain. Since WB and C57BL/6 mice are a/a, and C3H mice are A/A, the genetic compositions of the four different kinds of aggregation chimaeras expected with these parental strains are shown in Table 1.

A total of 64 mice was obtained after the transfer of the aggregated embryos to foster mothers; 14 of 64 mice had apparent white stripes. Since only embryonic aggregates that included Sl/Sl^d embryos would be expected to display some unpigmented areas in their coat and since this number ($14/64 = 0{\cdot}22$) was not significantly ($P > 0{\cdot}5$, by χ^2 test) different from the expected probability ($1/4 = 0{\cdot}25$), we considered mice with apparent white stripes to be $(Sl/Sl^d;a/a) \leftrightarrow (+/+;A/A)$ chimaeras. Moreover, this assumption was confirmed by mating two such male mice with WB-+/+;a/a females. Thus, the phenotypes of the resulting 160 progenies included 131 nonagouti, dilute mice (considered to be either Sl/+;a/a or Sl^d/+;a/a) and 29 agouti +/+;A/A mice. Since none of the offspring had nonagouti hairs without dilution (i.e. none were +/+;a/a), this indicates that the testes of such chimaeras included only $Sl/Sl^d;a/a$ and +/+;A/A spermatogonia.

Fig. 1 shows the coat colour patterns of three typical chimaeric mice with apparent white stripes. The pattern was characterized by fine transverse stripes of various widths, extending from the mid-dorsum to the mid-ventrum. The striping continued down the length of the head, body and tail with frequent asymmetry. Although it was not easy to count the number of stripes accurately, about 15 to 40 stripes were detectable on a side.

The pigmented regions of most chimaeric mice with apparent white stripes had both agouti and black hairs. Microscopical examination revealed that these black hairs did not contain phaeomelanin. Since the agouti locus acts *via* the mesodermal component of the hair follicle (Mayer & Fishbane, 1972), the genotype of this component in the black hairs of $(Sl/Sl^d;a/a) \leftrightarrow (+/+;A/A)$ chimaeras must be $Sl/Sl^d;a/a$. In other words, $Sl/Sl^d;a/a$ papilla cells did not inhibit the migration, invasion and differentiation of melanoblasts into hair follicles.

Aggregation chimaeras were produced by using the same combination of parental strains, but some resulting neonatal mice were killed within 24 h after birth. The whole skin was removed and melanocytes were demonstrated by the DOPA reaction. In about one fourth of the neonatal mice, the distribution pattern of hair follicles that were positively stained with the DOPA reaction was quite similar to the

Table 1. *Parental mice and expected genetic compositions of aggregation chimaeras*

Experiment*	Parental mice†	Embryos: Genotype	Embryos: Coat colour	Genetic composition of chimaeras
1	WB-Sl/+;a/a × C57BL/6-Sl^d/+;a/a	$Sl/Sl^d;a/a$	White	$(Sl/Sl^d;a/a) \leftrightarrow (+/+;A/A)$
		Sl/+;a/a	Steel‡	$(Sl/+;a/a) \leftrightarrow (+/+;A/A)$
		Sl^d/+;a/a	Steel‡	$(Sl^d/+;a/a) \leftrightarrow (+/+;A/A)$
		+/+;a/a	Black	$(+/+;a/a) \leftrightarrow (+/+;A/A)$
	C3H-+/+;A/A × C3H-+/+;A/A	+/+;A/A	Agouti	
2	WB-Sl/+;*Pgk-B* × C57BL/6-Sl^d/+;*Pgk-B*	Sl/Sl^d;*Pgk-B*	White	$(Sl/Sl^d;Pgk\text{-}B) \leftrightarrow (+/+;Pgk\text{-}A)$
		Sl/+;*Pgk-B*	Steel‡	$(Sl/+;Pgk\text{-}B) \leftrightarrow (+/+;Pgk\text{-}A)$
		Sl^d/+;*Pgk-B*	Steel‡	$(Sl^d/+;Pgk\text{-}B) \leftrightarrow (+/+;Pgk\text{-}A)$
		+/+;*Pgk-B*	Black	$(+/+;Pgk\text{-}B) \leftrightarrow (+/+;Pgk\text{-}A)$
	C57BL/6-+/+;*Pgk-A* × C57BL/6-+/+;*Pgk-A*	+/+;*Pgk-A*	Black	

* In experiment 1, all mice had B-type PGK; in experiment 2, all mice had the a/a genotype.

† The *Pgk-1* locus is linked to the X chromosome. Both $Pgk\text{-}1^a/Pgk\text{-}1^a$ and $Pgk\text{-}1^a$/Y are designated as *Pgk-A*, and both $Pgk\text{-}1^b/Pgk\text{-}1^b$ and $Pgk\text{-}1^b$/Y are designated as *Pgk-B*.

‡ Either the Sl or the Sl^d gene (chromosome 10) induces the dilution of coat colour. A, a; the agouti locus (chromosome 2).

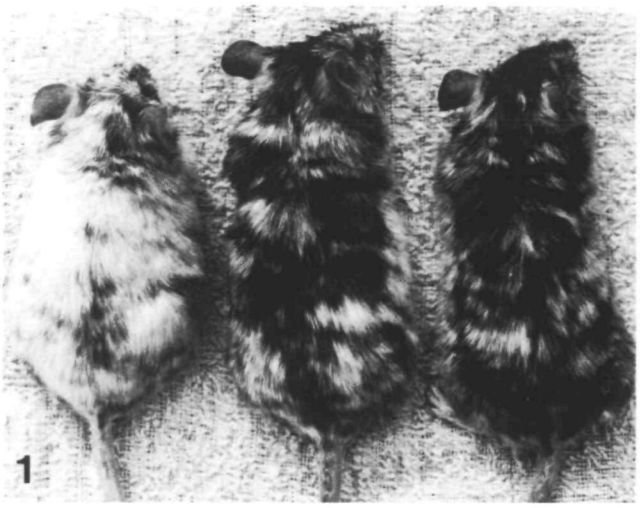

Fig. 1. Typical $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaeras with apparent white stripes.

distribution pattern of pigmented hairs in adult $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaeras. We assumed these neonatal mice to be $(Sl/Sl^d;a/a)\leftrightarrow(+/+;$melanocytes alternated with nonpigmented hair follicles, producing a $A/A)$ chimaeras. As shown in Fig. 2A, hair follicles with striped pattern. Despite the abundance of nonpigmented hairs in the white patches, a considerable number of melanocytes were present in the dermis (Fig. 2B). Since the epidermis was removed during DOPA staining, these melanocytes were considered to be located in the mesodermal layer of the skin. As controls, the dermis of neonatal WBB6F$_1$-$+/+$ and -Sl/Sl^d mice was also examined after DOPA staining. A considerable number of melanocytes was observed in the dermis of all four neonatal WBB6F$_1$-$+/+$ mice, but no melanocytes were detectable in the dermis of any of the four neonatal WBB6F$_1$-Sl/Sl^d mice.

Skin from neonatal $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaeras was not only incubated in DOPA solution but was also stained with alcian blue. The distribution pattern of mast cells stained with alcian blue in areas in which melanocyte-positive hair follicles were rich ($\geq 30\,mm^{-2}$) was compared with the distribution of mast cells in regions deficient in melanocyte-positive hair follicles ($<10\,mm^{-2}$). In both areas, mast cells were distributed uniformly, with no groups or clusters detectable (Fig. 2A,B). Differences in mast cell numbers were also not detectable between the white and pigmented regions of each $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaeric mouse (Table 2). However, the average numbers of mast cells in the skin of $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaeras, while significantly greater than the numbers observed in WBB6F$_1$-Sl/Sl^d mice (Table 2), were significantly less than the average numbers observed in the skin of normal neonatal WB, C57BL/6, WBB6F$_1$ and C3H mice.

The proportion of white regions to the whole skin area was not measured exactly in individual chimaeras. However, the proportion of white regions was greatest and the average mast cell number least in the chimaera no. 1-1 of Table 2, whereas the proportion was least and the mast cell number greatest in the chimaera no. 1-3.

In the second series of experiments, we used embryos of the C57BL/6 congenic strain with the A-type PGK. These embryos were produced by mating C57BL/6-*Pgk-1*^{a}/*Pgk-1*^{a} female mice with

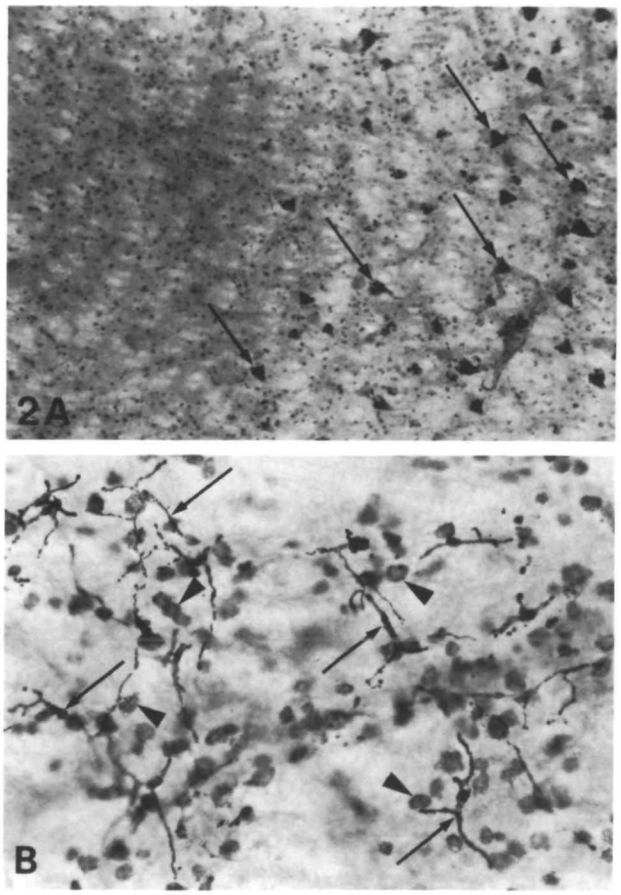

Fig. 2. (A) Pigmented (right) and nonpigmented (left) stripes in the skin of a neonatal $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaera. The whole dermis was stained with the DOPA reaction and alcian blue. Only hair follicles with melanocytes are visible (arrows). Small dots scattered over the whole field are mast cells, the cytoplasmic granules of which were stained with alcian blue. The number of mast cells in the pigmented stripe is comparable to the number in the nonpigmented stripe. ×37. (B) Higher magnification of a nonpigmented stripe. Although hair follicles without DOPA-positive cells are not visible, a considerable number of free melanocytes (arrows) are seen in the dermis. Mast cells with alcian-blue-positive granules are marked by arrowheads. ×184.

Table 2. *Number of mast cells in the skin of neonatal* (Sl/Sl^d;a/a)↔(+/+;A/A) *chimaeras*

Mice	Number of mast cells per mm^2 (mean ± S.E.)*	
	Area with <10 pigmented hair follicles mm^{-2}	Area with ≥30 pigmented hair follicles mm^{-2}
WB-+/+;*a/a*	–	2500 ± 140 (11)†
C57BL/6-+/+;*a/a*	–	2510 ± 80 (8)†
WBB6F$_1$-+/+;*a/a*	–	2530 ± 50 (8)†
WBB6F$_1$-*Sl/Sl^d*;*a/a*	100 ± 10 (10)†	–
C3H-+/+;*A/A*	–	2560 ± 110 (8)†
Chimaera no. 1-1	690 ± 40 (36)‡§	770 ± 60 (16)¶
Chimaera no. 1-2	1540 ± 320 (10)‡§	1270 ± 120 (9)¶
Chimaera no. 1-3	1620 ± 320 (7)‡§	1850 ± 270 (15)

* The number of samples is shown in parenthesis.
† More than two areas were sampled from each of four mice.
‡ $P<0.01$, when compared to the value of WBB6F$_1$-*Sl/Sl^d* mice by *t*-test.
§ $P>0.4$, when compared to the value observed in the areas with ≥30 pigmented hair follicles mm^{-2} of the same chimaera.
¶ $P<0.01$, when compared to the value of WB-+/+ mice.

C57BL/6-*Pgk-1^a*/Y male mice. Although, as a result of the fact that *Pgk-1* is X-linked, female embryos were *Pgk-1^a*/*Pgk-1^a* and male embryos *Pgk-1^a*/Y, we did not distinguish sexes and designated the genotype of all embryos with the *Pgk-1^a* gene as *Pgk-A*. Since both parental strains used to obtain *Sl/Sl^d* embryos had the B-type PGK, the genetic compositions of expected aggregation chimaeras are as shown in Table 1.

In total, 66 mice were obtained after the transfer of the aggregated embryos to foster mothers; 13 mice (20%) had apparent white and black stripes, and so were assumed to be (*Sl/Sl^d*;*Pgk-B*)↔(+/+;*Pgk-A*) chimaeras. Since all chimaeras were nonagouti (*a/a*), no agouti hairs developed. Blood samples were removed from the 13 mice with white stripes as well as from 10 mice that were randomly selected from the remaining 53 mice born at the same time. Electrophoresis of erythrocytes of all these 23 mice showed both A-type and B-type PGK.

Seven (*Sl/Sl^d*;*Pgk-B*)↔(+/+;*Pgk-A*) chimaeras were killed. Although we had originally planned to isolate all of the 'pure white' and 'pure black' regions of the skin from these chimaeras and divide each region into two parts for PGK analysis and determination of mast cell number, we were unable to dissect more than five 'pure white' and 'pure black' regions from each (*Sl/Sl^d*;*Pgk-B*)↔(+/+;*Pgk-A*) animal. Moreover, it was impossible to obtain large enough pieces to analyse both parameters at the same time. Therefore, we examined only the proportion of the A-type PGK in each 'pure white' and 'pure black' isolate. In five (*Sl/Sl^d*;*Pgk-B*)↔(+/+;*Pgk-A*) chimaeras, the proportion of the A-type PGK was obtained by using the whole layer of the skin (i.e. the epidermis and the dermis). Both types of PGK were detectable in all 'pure white' and 'pure black' regions examined (Table 3). However, the proportion of A-type PGK was significantly higher in 'pure black' than in 'pure white' areas.

In the remaining two (*Sl/Sl^d*;*Pgk-B*)↔(+/+;*Pgk-A*) chimaeras, the epidermis of 'pure white' and 'pure black' pieces of skin was separated from the dermis, and examined for PGK electrophoretic pattern. The epidermis of most white regions showed only B-type PGK (Table 4). Although the

Table 3. *Proportion of A-type PGK in erythrocytes, and white and black regions of the skin of* (Sl/Sl^d;Pgk-B)↔(+/+;Pgk-A) *chimaeras*

Chimaera no.	Proportion of A-type PGK (%, mean ± S.E.)*			Comparison between white and black regions†
	Erythrocytes	White regions	Black regions	
2-1	20	20 ± 3 (9)	39 ± 3 (9)	$P<0.01$
2-2	30	19 ± 2 (9)	39 ± 2 (9)	$P<0.01$
2-3	30	23 ± 9 (9)	51 ± 4 (8)	$P<0.01$
2-4	50	20 ± 7 (2)	53 ± 3 (3)	$P<0.05$
2-5	50	21 ± 2 (10)	49 ± 4 (10)	$P<0.01$

* The number of samples is shown in parenthesis.
† Comparison by *t*-test.

Table 4. *Proportion of A-type PGK in the epidermis and the dermis of each skin portion of* (Sl/Sl^d;Pgk-B)↔(+/+;Pgk-A) *chimaeras*

	Proportion of A-type PGK in each skin region					
	White regions			Black regions		
Chimaera no.	Sample no.	Epidermis	Dermis*	Sample no.	Epidermis	Dermis*
2-6	1	0	20	1	60	40
	2	0	20	2	70	50
	3	0	30	3	80	40
	4	0	30	4	90	50
	5	0	40	5	100	50
	6	0	40			
	7	10	30			
2-7	1	0	10	1	60	20
	2	0	10	2	60	30
	3	0	20	3	70	20
	4	0	20	4	70	30
				5	80	30

* The epidermis was separated from the dermis by trypsin treatment. Since hair follicles remained in the dermis, the contribution of the epidermal component to the PGK pattern of the dermis was not removed.

proportion of the A-type PGK was higher in the epidermis than in the dermis of 'pure black' skin, 100% A-type PGK was observed in only one 'pure black' isolate (Table 4). This may be attributed to the fact that detection of white hairs on a black background is more difficult than detection of black hairs on a white background. In other words, whereas the 'pure white' isolate probably did not contain black hairs, the 'pure black' isolate probably did include some white hairs. Moreover, since each hair follicle may itself be a mixed population (reviewed by McLaren, 1976), 'pure black' skin may contain hair follicles with a fairly large minor component of *Sl/Sl^d;Pgk-B* cells. A mixed hair follicle of this type may well be able to support melanocyte differentiation but, of course, will have some contamination of B-type PGK.

Finally, another five (*Sl/Sl^d;Pgk-B*)↔(+/+;*Pgk-A*) chimaeras were killed and their skins removed and fixed in 10% formalin. 'Pure white' and 'pure black' regions were carefully cut from these pelts and histological sections were prepared from each region for enumeration of mast cells. In each (*Sl/Sl^d;Pgk-B*)↔(+/+;*Pgk-A*) chimaeric mouse, the numbers of mast cells in 'pure white' regions were comparable to the numbers of mast cells in 'pure black' regions (Table 5). Although the average numbers of mast cells varied among individual chimaeras, the numbers were significantly less than the numbers observed in the skin of normal WB, C57BL/6 and WBB6F$_1$ mice, but significantly greater than the numbers observed in the skin of WBB6F$_1$-*Sl/Sl^d* mice. The proportion of white regions to the whole skin area was not measured exactly in this experiment, either. However, the proportion of white regions appeared to be inversely proportional to the mast cell number; the proportion was greatest and number least in chimaera no. 2-11 of Table 5.

Table 5. *Number of mast cells in white and black regions of* (Sl/Sl^d;Pgk-B)↔(+/+;Pgk-A) *adult chimaeras*

	Number of mast cells cm^{-1} (mean ± S.E.)*	
Mice	White regions	Black regions
WB-+/+;*Pgk-B*	–	297 ± 7 (11)†
C57BL/6-+/+;*Pgk-B*	–	302 ± 11 (13)†
C57BL/6-+/+;*Pgk-A*	–	293 ± 13 (8)†
WBB6F$_1$-+/+;*Pgk-B*	–	280 ± 19 (8)†
WBB6F$_1$-*Sl/Sl^d;Pgk-B*	<1 (10)	–
Chimaera no. 2-11	30 ± 4 (11)‡§	23 ± 7 (11)¶
Chimaera no. 2-12	82 ± 6 (10)‡§	96 ± 6 (10)¶
Chimaera no. 2-13	88 ± 9 (9)‡§	96 ± 11 (7)¶
Chimaera no. 2-14	93 ± 15 (9)‡§	106 ± 29 (7)¶
Chimaera no. 2-15	145 ± 23 (16)‡§	196 ± 20 (14)¶

* The number of mast cells in the section (5 μm thick) of the skin. The number of samples is shown in parenthesis.

† More than two samples were examined in each of four mice.

‡ $P<0.01$, when compared to the value of WBB6F$_1$-*Sl/Sl^d;Pgk-B* mice by *t*-test.

§ $P>0.1$, when compared to the value observed in the black regions of the same chimaera.

¶ $P<0.01$, when compared to the value of WB-+/+;*Pgk-B* mice.

Discussion

We produced $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ and $(Sl/Sl^d;Pgk\text{-}B)\leftrightarrow(+/+;Pgk\text{-}A)$ aggregation chimaeras. In both cases, mice with apparent white stripes were assumed to represent such chimaeras because (1) in the present combination of parental mice, only Sl/Sl^d animals are nonpigmented; (2) melanocytes are absent in hair follicles of Sl/Sl^d mice (Mayer & Green, 1968) and the hair follicles of white stripes also lacked pigment; (3) the proportion of chimaeras displaying white striping was 27/130 [14/64 in the first series experiment for production of $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaeras and 13/66 in the second series experiment for production of $(Sl/Sl^d;Pgk\text{-}B)\leftrightarrow(+/+;Pgk\text{-}A)$ chimaeras] and this percentage is consistent with the expected frequency ($P>0{\cdot}25$, when compared to 1/4 by χ^2 test); and (4) when two putatively $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ male chimaeras were mated with $+/+;a/a$ females, the phenotypes of their offspring indicated that their testes contained spermatogonia of only the $Sl/Sl^d;a/a$ and $+/+;A/A$ genotypes.

The striped pattern observed in $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ and $(Sl/Sl^d;Pgk\text{-}B)\leftrightarrow(+/+;Pgk\text{-}A)$ chimaeras is consistent with the result of Mintz & Cronmiller (1978), although they produced $Sl^J/+\leftrightarrow+/+$ chimaeras by injecting $Sl^J/+$ teratocarcinoma cells into +/+ blastocysts. In a monograph on the coat colour of mice, Silvers (1979) anticipated that $Sl/Sl\leftrightarrow+/+$ chimaeras might display pigment patterns different from the white spotted pigment pattern of $W/W\leftrightarrow+/+$ chimaeras (Mintz, 1970, 1971). Indeed, Silvers suggested that $Sl/Sl;+/+$ mice might display pigment patterns resembling those produced by hair follicle phenoclones (Mintz, 1971). The present results bear this out.

Melanoblasts originate from the neural crest, migrate and differentiate in the mesodermal layer of the skin, and invade hair follicles that develop from the ectoderm (Silvers, 1979). When the future white stripes of neonatal $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaeras were investigated, considerable numbers of melanocytes were detectable in the mesodermal layer, despite the absence of pigment in the hair follicles. This suggests that melanoblasts may migrate and differentiate in the mesodermal layer that is located beneath the epidermis of melanocyte-deficient hair follicles. In fact, the development of black (nonagouti) hairs in $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ chimaeras indicates that melanocytes can invade and deposit pigment in $Sl/Sl^d;a/a$ hair follicles; this is consistent with the report of Poole & Silvers (1979).

Precursors of mast cells originate from the fetal liver (Kitamura *et al.* 1979; Hayashi *et al.* 1985), migrate in the bloodstream, invade the mesodermal layer of the skin and differentiate into mast cells. The average number of mast cells in both $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ and $(Sl/Sl^d;Pgk\text{-}B)\leftrightarrow(+/+;Pgk\text{-}A)$ chimaeras was less than the value observed in the skin of normal +/+ mice but higher than the value observed in the skin of WBB6F$_1$-Sl/Sl^d mice. Since the concentration of mast cell cluster-forming cells in the liver of WBB6F$_1$-Sl/Sl^d embryos was comparable to that of WBB6F$_1$-+/+ embryos (Hayashi *et al.* 1985), the quantity and quality of mast-cell precursors in the chimaeras would be expected to be normal. Therefore, the moderate but significant decrease of mast cells in the chimaeras may be attributable to the mixing of Sl/Sl^d and +/+ mesodermal cells. Although the proportion of white regions to the whole skin area was not measured exactly, it appeared that the proportion of white regions was inversely proportional to the average number of mast cells within particular chimaeras. When the proportion of cells of the Sl/Sl^d genotype increases, the efficiency of mast-cell differentiation will decrease.

The number of mast cells in nonpigmented regions was comparable to the number observed in pigmented areas of $(Sl/Sl^d;a/a)\leftrightarrow(+/+;A/A)$ and $(Sl/Sl^d;Pgk\text{-}B)\leftrightarrow(+/+;Pgk\text{-}A)$ chimaeras. This suggests that the mixing of Sl/Sl^d cells and +/+ cells is homogeneous in the mesodermal layer of the skin. The presence of cells of the +/+ genotype in white regions of the skin was directly demonstrated in $(Sl/Sl^d;Pgk\text{-}B)\leftrightarrow(+/+;Pgk\text{-}A)$ chimaeras by using the PGK electrophoretic pattern as a marker.

However, the proportion of A-type PGK was significantly lower in white than in black regions. This uneven distribution between the $Sl/Sl^d;Pgk\text{-}B$ and $+/+;Pgk\text{-}A$ components was attributable to the fact that the ectodermal layer of the white portion is mostly composed of $Sl/Sl^d;Pgk\text{-}B$ cells, whereas mesodermal cells appeared to mix homogeneously. The result of two chimaeras, in which the dermis and epidermis were separated, does not appear to support the above-mentioned notion of the homogeneous mix of mesodermal layer cells. The proportion of A-type PGK was less in the dermis of white regions than in that of black regions (Table 4). However, since hair follicles were not removed from the dermis in the present study, we attribute the difference in the proportion of A-type PGK to contaminations of ectodermal cells located in the remaining hair follicles. If the proportions of A-type and B-type PGK were to be compared between purified mesodermal cells from white regions and those from black regions, we predict that nearly equal values might well be obtained.

Mayer (1973) demonstrated that both the ectodermal and mesodermal components of Sl/Sl^d

embryos are defective in their ability to support melanoblast differentiation. The different distribution pattern between melanocytes and mast cells observed in $Sl/Sl^d \leftrightarrow +/+$ chimaeras may be attributable to developmental differences in the ectodermal and mesodermal layers of the skin. Cells of the Sl/Sl^d genotype and cells of the $+/+$ genotype might mix homogeneously in the mesodermal layer, whereas each of them might make discrete patches in the ectodermal layer. Indeed, according to Mintz (1970, 1971), each such discrete patch has a clonal origin.

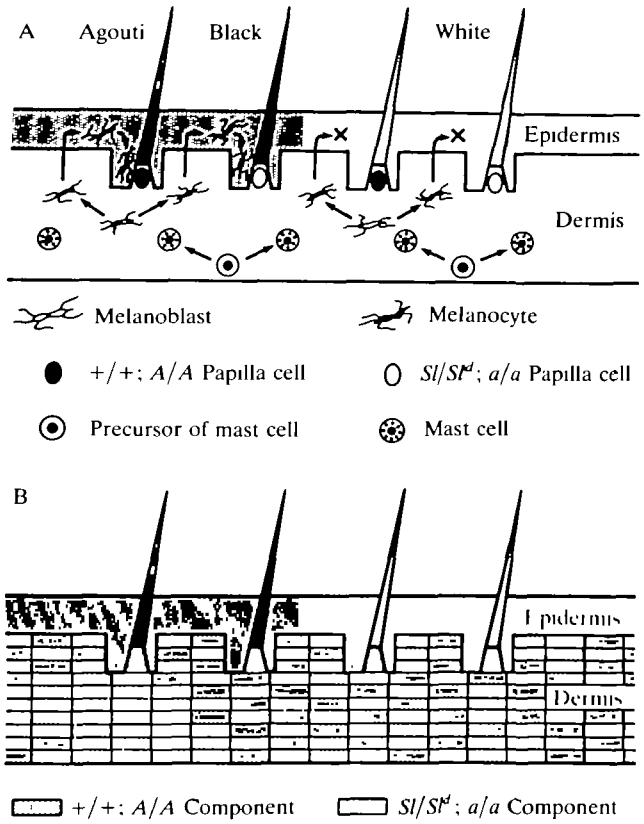

Fig. 3. Diagrams representing the skin of a $(Sl/Sl^d;a/a) \leftrightarrow (+/+;A/A)$ chimaera. (A) Precursors of mast cells migrate in the bloodstream, invade the dermis, proliferate and then differentiate within the dermis. Melanoblasts proliferate and differentiate in the dermis. The resulting melanocytes successfully invade the shaded regions of epidermis, which are composed of $+/+;A/A$ cells, and settle in hair follicles. If papilla cells in the follicle are of $+/+;A/A$ genotype, an agouti hair will develop. In contrast, a black hair will develop from the hair follicle if the papilla cells are of $Sl/Sl^d;a/a$ genotype. Melanocytes cannot successfully invade the white region of the epidermis composed of $Sl/Sl^d;a/a$ cells. (B) This shows the difference in patch sizes between the epidermis and the dermis. Probably mesodermal cells in the dermis do not make discrete patches and mix homogeneously. However, apparent patches are drawn in the dermis as well as in the epidermis to emphasize the difference in patch sizes. There is a possibility that papilla cells may make patches that are smaller than those of epidermal cells, but this possibility is not shown in this diagram, which is kept simple.

The present results indicate that aggregation chimaeras have a unique usefulness for clarifying the actions of genes at the *Sl* locus. Information obtained from experiments employing tissue recombination procedures is somewhat different from that obtained from aggregation chimaeras. For example, although mast cells never appear in the skin grafted from Sl/Sl^d to $+/+$ mice (Kitamura & Go, 1979), we cannot predict from this result the even distribution of mast cells in the dermis of $Sl/Sl^d \leftrightarrow +/+$ chimaeras, in which mesodermal cells of Sl/Sl^d and $+/+$ origins mix homogeneously (or intermingle by making very small patches).

Mast-cell precursors and melanoblasts seem to use the mesodermal layer of the skin as a common environment for migration and differentiation. However, the former completed differentiation in the mesodermal layer, whereas the latter have to invade hair follicles to complete differentiation (Fig. 3A). In the mesodermal layer of $Sl/Sl^d \leftrightarrow +/+$ chimaeras, migration and differentiation of mast cell precursors and melanoblasts may be supported insufficiently because of the mixing of Sl/Sl^d and $+/+$ cells (Fig. 3B). In the ectodermal layer, however, melanoblasts can invade $+/+$ epidermal sheath and then hair follicle groups (clones) composed of $+/+$ epidermal cells, but cannot become incorporated into hair follicles (clones) composed of Sl/Sl^d epidermal cells (Fig. 3A,B).

Both agouti and black (nonagouti) hairs were detectable in pigmented regions of $(Sl/Sl^d;a/a) \leftrightarrow (+/+;A/A)$ chimeras. Since papilla cells are considered to be of mesodermal origin (Searle, 1968; Silvers, 1979), two types of papillas may be present under the $+/+;A/A$ ectodermal layer; (1) papillas chiefly composed of $+/+;A/A$ cells and (2) papillas chiefly composed of $Sl/Sl^d;a/a$ cells (Fig. 3A,B). Hairs from the former papillas are agouti, whereas hairs from the latter papillas are black (nonagouti). Even if papilla cells are of mesodermal origin, these particular cells may form patches, which are smaller than those of epidermal cells. In such a case, small groups of agouti hairs and those of black hairs may appear in pigmented areas of $(Sl/Sl^d;a/a) \leftrightarrow (+/+;A/A)$ chimaeras. This is consistent with our present observation.

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