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Splenic pigment deposition in C57BL mice - an age-related phenomenon ?

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Introduction

Pigment deposition in the spleen, seen as dark brown discolouration predominantly in the cranial part of the spleen, is a common finding in many mice of C57BL sublines. The coat colour of C57BL mice is black. The condition has been named splenic haemosiderosis, splenic lipofuscinosis, splenic ceroidosis, and splenic melanosis (*Danse and Crichton* 1990). There is yet no conclusive evidence for which pigment being responsible for this discolouration. *Crichton et al.* (1978a, 1978b, 1980), *Crichton & Shire* (1982) identified the pigment as lipofuscin, *Veninga et al.* (1989) described it as splenic haemosiderosis, and *Weissman* (1967), *Hill et al.* (1977), *Sundberg* (1991) and *van der Heijden et al.* (1995) suggested that melanin was the principal pigment causing spleen discolouration in mice. A simultaneous occurrence of a combination of several pigments in the same spleen has also been proposed (*Danse & Crichton* 1990).

Many of the mice examined for splenic pigmentation have been relatively young animals (from 5-10 weeks of age). The oldest mice with pigmented spleens described were 187 days old (*Crichton et al.* 1978a). The pigment in that study was identified as lipofuscin, which often has been referred to as an age pigment as the rate of accumulation within internal organs appears to be a function of chronological age (*Crichton et al.* 1978a). As macrophages usually store debris with advancing age, inclusive lipofuscin from membrane lipids, haemosiderin from degradation of erythrocytes, and melanin from degraded melanocytes (*van der Heijden et al.* 1995), an increase in the distribution of splenic pigmentation by increasing age would not be unexpected.

In contrast to this speculation, the present paper describes the observation of an age-related reduction of pigment deposition in the spleens from mice of C57BL sublines that took part in a series of studies of varying length, up to 84 weeks of age.

Materials and Methods

A full list of the mouse C57BL sublines used is given in Table 1. The mice came from seven different studies of varying length. The mice were obtained from IFFA Credo, Lyon France (C57BL/6ByA), the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands (C57BL/6ByA Wild Type and Transgenic), and Bomholtgård Breeding and Research Centre Ltd., Ry, Denmark (C57BL/6J). In all the studies, except no. III, some of the mice had been exposed to one of the two heterocyclic amines present in cooked foods 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) or 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP). As no differences between the incidence of splenic pigmentation in dosed and in control groups were seen, the data presented are the pooled data from control and dosed groups.

The mice were housed two females/cage and one male/cage. All animals were kept under controlled environmental conditions (temperature 21 ± 1°C, relative humidity 55 ± 5%, 12/12 hours light/dark cycle, air changed 10 times/h). The mice had free access to feed (Altromin 1314, Altromin GmbH u., Co KG, Lage, Germany) and tap water. All surviving animals were decapitated and bled in CO_2-anaesthesia and all animals were subjected to a detailed necropsy. Selected organs were fixed in 4% neutral buffered formaldehyde, and paraffin-

Table 1. Incidence of macroscopic splenic pigmentation in C57BL mice sublines at different ages.

Study No.	Subline	Sex	Terminal sacrifice, weeks	No. affected	Total No.	Incidence (%)
I	C57BL/6ByA	Female	8	21	80	26.3
II	C57BL/6J	Female	11	14	16	87.5
	C57BL/6J	Female	17	27	47	57.4
III	C57BL/6J	Female	22	18	44	40.9
	C57BL/6J	Female	24	1	3	33.3
IV	C57BL/6ByA TG[a]	Female	37	2[b]	30	6.7
	C57BL/6ByA TG	Male	37	1	39	2.6
V	C57BL/6ByA WT[c]	Female	40	5	33	15.2
	C57BL/6ByA WT	Male	40	5	33	15.2
	C57BL/6ByA TG[d]	Female	40	5	33	15.2
	C57BL/6ByA TG	Male	40	5	33	15.2
VI	C57BL/6ByA WT[c]	Female	41	0	30	0
	C57BL/6ByA WT	Male	41	0	30	0
	C57BL/6ByA TG[d]	Female	41	0	30	0
	C57BL/6ByA TG	Male	41	0	30	0
VII	C57BL/6ByA WT[c]	Female	84	0	20	0
	C57BL/6ByA WT	Male	84	2	20	10
	C57BL/6ByA TG[d]	Female	84	0	13	0
	C57BL/6ByA TG	Male	84	1	20	5

a: TG: Transgenic *Apc1638N* mice
b: 10 wks and 37 wks old
c: WT: Wild Type mice
d: TG: Transgenic Eμ *pim*-1 mice

embedded sections, 4-6 µm thick, were prepared for histopathological examination. For differentiation of the pigment in sections of the spleen the following staining techniques were used: haematoxylin and eosin (HE), autofluorescence, acid fastness by the long Ziehl-Neelsen technique, sudanophilia by the Sudan Black B method, bleaching by 0.25% $KMnO_4$ followed by 1% oxalic acid, Prussian blue reaction, Schmorl's ferri ferricyanide method, periodic acid-Schiff (PAS) reaction, Lillie (ferrous iron for melanin), Oil red O, and DOPA reaction (*Stevens* 1990).

Results

One mouse with pigmented spleen died from anaemia during the study period in study IV. The death had no relation to the condition pigmented spleen, but was related to the increased susceptibility for tumours of the digestive tract in this specific type of mice. The age of this mouse is indicated in Table 1. None of the animals with splenic pigmentation exhibited clinical symptoms.

The gross appearance of splenic pigmentation in mice up to the age of 24 weeks was a dark brown/black discolouration, usually occupying much of the cranial part of the spleen. The number of mice with splenic discolouration and the extent of the area of the spleen occupied by pigment was markedly reduced in mice sacrificed after 37 weeks of age. At 84 weeks of age the discolouration was even more reduced and most often faintly light brown at gross examination (Table 1). Splenic pigmentation displayed an interindividual variation and excessive pigment deposition was not seen macroscopically in other internal organs.

Table 2. Properties and straining reactions of splenic pigment in C57BL mice sublines.

Staining	**Result**
Haemotoxylin and Eosin	Dark brown pigment
Autofluorescence	Negative
Long Ziehl-Neelsen technique	Negative + dark brown pigment
Sudan Black B	Negative + dark brown pigment
Bleaching with $KMnO_4$	Bleaching
Prussian blue reaction	Positive (Blue) + dark brown pigment
Schmorl's ferri ferricyanide method	Positive (Dark blue) + dark brown pigment
Periodic Acid-Schiff (PAS) reaction	Positive (Red) + dark brown pigment
Lillie (ferrous iron for melanin)	Positive (Dark green) + dark brown pigment
Oil red O	Negative + dark brown pigment
DOPA reaction	Positive (Brown)

Histopathological examination of young animals showed that the major part of the pigment was deposited along and in close vicinity to the fibrous trabeculae. Some of the pigment was deposited in the red pulp and to a minor extend in the white pulp. In older animals a few clusters of pigment granules were found in the red pulp and in the white pulp. The amount of pigment deposited along the trabeculae was markedly reduced.

The histochemical reactions of the pigment are summarised in Table 2. In general, apart from the bleaching reaction, all sections contained a certain amount of pigment granules which maintained their original dark brown colour. The predominant findings were: the pigment was dark brown in HE, and did not exhibit autofluorescence. The pigment gave negative staining reaction by the long Ziehl-Neelsen method. Likewise, the pigment was not stained by the Sudan Black B method. The pigment was readily bleached by $KMnO_4$. A positive reaction in Perl's Prussian blue was seen together with dark brown pigment. Schmorl's ferri ferricyanide, periodic acid-Schiff (PAS) and Lillie (ferrous iron for melanin) all gave a positive reaction in some of the deposited pigment. The pigment was negative in Oil red O and pigmented cells gave a positive DOPA reaction.

Discussion

The present observation of an age-related reduction in splenic pigmentation in mice of C57BL sublines has not been described earlier.

In the first half year of age the incidence was relatively high (Table 1) and much of the cranial part of the spleen was occupied by pigment. The incidence of mice with splenic discolouration and the extent of the area of the spleen occupied by pigment was markedly reduced in mice sacrificed after 37 weeks of age. At 40 weeks of age the incidence was higher than after 37 weeks, which could be due to genetic differences between the mice in the two studies. At 84 weeks of age the discolouration was even more reduced and most often faintly light brown at gross examination. Splenic pigmentation displayed an interindividual variation and excessive pigment deposition was not seen macroscopically in other internal organs.

The finding of a deposition of the pigment along and in close vicinity to the fibrous trabeculae of the spleen is similar to what was found in the study by *van der Heijden et al.* (1995). The staining properties of the pigment observed suggest that the pigment predominantly consists of melanin. The pigment did not exhibit autofluorescence suggesting that the pigment is melanin rather than lipofuscin, but lipofuscin has earlier been described not to exhibit autofluorescence (*Crichton et al.* 1978b, 1980). The negative staining reactions for acid fastness by the long Ziehl-Neelsen technique, and for sudanophilia by the Sudan Black B method are suggestive of melanin or late lipofuscin, as these older, more oxidised lipofuscins lose their sudanophilia, and only a few demonstrate acid fastness (*Stevens* 1990). The positive staining reaction for Lillie favours melanin and for DOPA melanocytes, and the positive reaction in Schmorl's ferri ferricyanide is suggestive for melanin and lipofuscin. Perl's Prussian blue reaction for haemosiderin - together with unstained pigment - was positive, which is a common finding in spleens due to haemoglobin degradation. Finally the consistent finding of dark brown pigment maintaining the original colour also is indicative of melanin (*Sundberg* 1991).

Veninga et al. (1989) who designated splenic pigmentation as haemosiderosis have reported that this condition increases with age, and that the aggregates of yellow-brown pigment are scattered throughout the spleen. The mechanism behind this condition has been proposed to be an inborn error of metabolism in this strain of mice, suggesting a genetic origin, comparable to the genetic disease idiopathic haemochromatosis in humans.

Lipofuscin is an indigestible lysosomal product of oxidized unsaturated fatty acids, probably derived from membrane lipids. The mechanism behind the accumulation of lipofuscin in the spleen is suggested to be a lysosomal defect within the macrophage which causes decomposition of membrane lipids (*Crichton et al.* 1980). The effect of lipofuscin accumulation on cellular function has yet not been established. Lipofuscin often has been referred to as an age pigment as the rate of accumulation within internal organs appears to be a function of chronological age (*Crichton et al.* 1978a). Splenic lipofuscinosis, however, has been observed in relatively young animals as well as in neonatal mice (*Crichton et al.* 1980), and in this particular study

no appreciable increase in concentration of splenic lipofuscinosis in the more mature mice (up to six months of age) was seen. Therefore, the authors suggested that lipofuscin deposition within these mouse spleens was not a consequence of age-related processes (*Crichton et al.* 1980).

Both *Weissman* (1967) and *Sundberg* (1991) have reported that the black pigment of spleens in C57BL mice usually is melanin. Splenic melanosis has been reported to occur in black mice exclusively (*Hill et al.* 1977). Genetic studies suggested that this trait is either not inherited or there is genetic transmission with variable penetrance (*Weissman* 1967). This theory could explain the common finding that only a certain percentage of the mice have black pigmentation of the spleen, but still the mechanism and condition eliciting this interindividual variation in C57BL mice remain unexplained (*van der Heijden et al.* 1995). In addition *Weissman* (1967) has shown that the mouse must be capable of forming melanin pigment if it is to have black spots on its spleen. As melanocytes originate from neural crest cells, the finding of localized clusters of melanocytes in the spleen was suggested to be due to either a migration potential of these cells, a true metaplastic condition following localized prenatal trauma, or perhaps to the transport of melanin by leucocytes (*Weissman* 1967). *van der Heijden et al.* (1995) who examined mice up to 8-10 weeks of age concluded that melanin was the principal pigment with a predominantly accumulation in melanophores. The genesis of this spleen pigmentation was unclear, but the possibility of an association with skin pigmentation and thus a strain characteristic was proposed.

Melanin is regarded to be produced from tyrosine by the action of the enzyme tyrosinase. The initial step is an oxidation to dihydroxyphenylalanine (DOPA) followed by oxidative polymerization to produce melanin. The details of the later stages in this process remain largely speculative (*Stevens* 1990).

A common problem in splenic pigmentation is the simultaneous occurrence of several pigments in the same spleen. The staining properties of the splenic pigment in the present study suggested that melanin was the predominant pigment, but did not excluded the simultaneous occurrence of other pigments. Thus the positive Perl's Prussian blue reaction indicated the presence of haemosiderin, probably due to erythrocyte degradation, and indications for presence of lipofuscin were also seen among the staining reactions.

An explanation to the present observation of an age-related reduction in splenic pigmentation in mice of C57BL sublines remains obscure. It is most likely that splenic pigmentation is of no significance to the C57BL mice, and seemingly somehow the mice are able to get rid of the pigment as they age. The mice with pigmented spleens had normal body weight and no clinical symptoms or behavioral abnormalities. Apart from the pigment deposition, the spleens appeared normal at the histopathological examination. The genesis behind the condition splenic pigmentation needs to be expanded by more detailed mechanistic and ultrastructural studies shedding light on what is going on in the spleen cells as the mice age.

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Summary

Pigment deposition in the spleen, seen as dark brown discolouration predominantly in the cranial part of the spleen, is a common finding in many mice of C57BL sublines. In a series of studies with C57BL mice the incidence of mice with this condition were observed to be reduced with age. The staining properties of the pigment observed suggested that the pigment predominantly consisted of melanin. The pathogenesis of spleen pigmentation is still unclear. To further shed light on the mechanism behind the present observation of an age-related reduction in splenic pigmentation in mice of C57BL sublines more advanced studies is needed.

Sammendrag

Pigmentaflejring i milten, visende sig som sortbrun misfarvning særligt i den forreste del, påvises ofte hos mange linier af C57BL musestammen. I en række forsøg, hvori C57BL mus indgik, blev det observeret, at incidensen af mus med pigmenteret milt faldt, efterhånden som musene blev ældre. Pigmentets reaktioner i forskellige diagnostiske farvemetoder viste, at det hovedsageligt drejede sig om melanin. Miltpigmenteringens patogenese er

ikke klarlagt, og det vil kræve mere avancerede undersøgelser, hvis mekanismen bag observationen af aldersafhængig reduktion i miltpigmentering hos linier af C57BL musestammen skal klarlægges.

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