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An allelic difference determines reciprocal patterns of expression of binding sites for *Dolichos biflorus* lectin in inbred strains of mice

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SUMMARY

We used staining of tissue sections by lectin conjugates to screen inbred strains of mice for polymorphisms which could be used as histological markers of chimaerism. We found one polymorphism, which involves reciprocal patterns of expression of binding sites for the N-acetyl-galactosamine-binding lectins from *Dolichos biflorus* (DBA), *Helix pomatia* (HPA) and *Wisteria floribunda* (WFA) on intestinal epithelium and vascular endothelium. The polymorphism is due to alleles at a single locus, designated *Dlb-1* (for Dolichos lectin binding). Of 29 inbred strains examined, 3 are $Dlb\text{-}1^a$ (type strain RIII-*ro*; gut epithelium − ve, vascular endothelium + ve), and 26 are $Dlb\text{-}1^b$ (type strain C57BL/6J; gut epithelium + ve, vascular endothelium − ve). In RIII-*ro* and C57BL/6J embryos, the polymorphic difference is not clearly present until day 11 of gestation. Before then, embryos of both strains express binding sites on gut epithelium and on endothelium.

The temporal and tissue-specific patterns of expression of lectin-binding sites may result from differences in expression of an N-acetyl galactosaminosyl transferase. If so, elucidation of the genetic basis of the polymorphism might provide an insight into the mechanisms of developmental regulation of glycosyltransferase activity.

INTRODUCTION

We screened inbred mouse strains for histological markers of chimaerism by staining cryostat sections of tissues from different strains with a panel of peroxidase-conjugated lectins. By analogy with the blood group antigens in man (Kapadia, Feizi & Evans, 1981), we thought that polymorphisms between inbred mouse strains would most likely be carbohydrate based.

We found only one polymorphism (Ponder & Wilkinson, 1983). It is recognized by the lectins from *Dolichos biflorus* (DBA), *Helix pomatia* (HPA) and *Wisteria floribunda* (WFA), which have specificity for terminal non-reducing N-acetyl galactosamine residues (Etzler, 1972; Hammarstrom, 1972; Kurokawa, Tsuda & Sugino, 1976). The polymorphism is confined to intestinal epithelium and vascular

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endothelium, but in those tissues it can be used as a marker for studying clonal development in aggregation chimaeras (Schmidt, Garbutt, Wilkinson & Ponder, in press; Ponder *et al.* 1985). The polymorphism is unusual in that the pattern of expression of lectin-binding sites is reciprocal in the two polymorphic types. In this paper we describe the appearance of the polymorphism during embryonic development, the reciprocal pattern in adult mice, and genetic data which indicate that the reciprocal patterns are determined by alleles at a single locus.

MATERIALS AND METHODS

Mice

CBA/Ca mice were bred in the animal house of the Institute of Cancer Research. DDK, RIII-*ro*, C57BL/6J, B10.A., and C57BL/10ScSn, were bred at the MRC Laboratories, Carshalton. Other mice of the inbred strains shown in Tables 1 and 2 were obtained from Olac (Bicester, UK) or from the Medical Research Council, Mill Hill, London and housed at the MRC Laboratories, Carshalton.

Male and female mice, 4 to 24 weeks old, were used for the screening for polymorphisms and in the study of the strain distribution of DBA binding. Embryos studied were from DDK, RIII-*ro*, and C57BL/6J strains, raised as above. F1, backcross and F2 animals were bred at the MRC Laboratories, Carshalton.

Lectins, lectin conjugates and antibodies

Peroxidase conjugates of *Ricinus communis* agglutinin I and of lectins from *Mangifera indica*, Cotoneaster and Jack fruit were a gift from Dr J. A. Forrester (Institute of Cancer Research). Other lectins were purchased as purified lectin or as peroxidase or fluorescein isothiocyanate (FITC) conjugate from Sigma, Poole, England. Peroxidase conjugates of *Dolichos biflorus* agglutinin (DBA) (Sigma No L1135) were prepared in our laboratory by the periodate method as previously described (Ponder & Wilkinson, 1983). Alkaline phosphatase conjugates of purified lectins from *Helix pomatia, Wisteria floribunda, Vicia villosa* and *Codium fragile* were prepared by glutaraldehyde conjugation (Ponder & Wilkinson, 1983) using alkaline phosphatase from calf intestine (Boehringer, No 567744). Rabbit antiserum to Forssman antigen (Willison *et al.* 1982) was a gift from Dr K. Willison (Institute of Cancer Research). Sugars were obtained from Sigma.

Histological methods

Methods were as described by Ponder & Wilkinson (1983). The initial screening of mouse tissues for polymorphisms was carried out on cryostat sections fixed in 10 % formol saline after cutting. Subsequent studies of the distribution of DBA, HPA and WFA-binding sites in embryonic and adult tissues were made on methacarn fixed, paraffin-embedded tissue (except that tissue from F1 and F2 animals used in the genetic studies and tissues used for enzyme digestions were fixed in 10 % formol saline: see below). Of the fixatives tried (10 % formol saline pH 7, 0·1 % to 2 % buffered glutaraldehyde, benzoquinone, 4 °C acetone, 4 °C absolute ethanol, Carnoy's, Bouin's, and methacarn), only glutaraldehyde above 0·2 % concentration caused loss of DBA binding; but the cleanest and highest density staining was obtained with methacarn. Controls for the specificity of lectin staining were provided in each experiment by the inclusion of slides incubated without lectin conjugate, with lectin conjugate diluted in buffer containing the appropriate inhibiting sugar (final concentration 2 % w/v), and (for DBA) known positive and negative tissues.

Binding of the rabbit anti-Forssman antibody was demonstrated using a goat anti-rabbit alkaline phosphatase conjugate (Sigma, A8025).

Chemistry of the DBA-binding site

For enzyme digestions, cryostat sections fixed in formol saline for 10 min at room temperature, and formalin-fixed paraffin-embedded sections, were incubated as follows: (i) neuraminidase (Behring, *Vibrio cholerae* 1 μl ml^{-1}) diluted 1:10 in 0·2 M-acetate buffer pH 5·5 for 30 min at 37 °C. A positive control was provided by unmasking of sites for peanut lectin–peroxidase conjugates on similarly processed sections of human kidney. (ii) Trypsin (Sigma, type III from bovine pancreas) 1 mg in 20 ml PBS pH 7·5 for 15 min at 37 °C: no control. (iii) Glycosidases (no controls): α-galactosidase (Sigma) 0·025 μl ml^{-1} in 50 mM-acetate buffer pH 4·5 for 30 min at room temperature; β-galactosidase (Sigma) 1 mg ml^{-1} in 50 mM-Tris pH 7·5 at 37 °C for 15 min. Other treatments (on unfixed and formalin fixed cryostat sections): (i) NP40 (Nonidet P40) extraction: NP40 (Sigma) 1 % or 0·1 % in PBS pH 7·5 for 15 min at room temperature; (ii) 10 % trichloroacetic acid (TCA): 2 min at 4 °C; (iii) chloroform/methanol: 2:1 chloroform: methanol for 2 min at room temperature followed by 1:2 chloroform: methanol for a further 2 min at room temperature. In each case, sections were washed in PBS after treatment and then incubated with DBA–peroxidase above.

Genetic studies

Mice were scored for *Dlb-1* polymorphic type by DBA–peroxidase staining of vascular endothelium and intestinal epithelial cells in formalin-fixed paraffin-embedded sections of small intestine and adjacent mesentery.

RESULTS

Screening for polymorphisms

The mouse strains, lectins and tissues used in the initial screen for polymorphisms are shown in Table 1.

Table 1. *Screening mouse strains for lectin-binding polymorphism*

Mouse strains: CBA/CaLac, C57BL/6JLac, BALB/c, GE, HTG, IF, SEA/J, 101/H, RIII-*ro*, DDK

Lectins	*Nominal carbohydrate specificity*
Bandeiraea simplicifolia I *Arachis hypogaea* *Ricinus communis* 120 *Abrus precatorius* *Phaseolus limensis* II	D-galactose
Dolichos biflorus Soybean agglutinin	N-acetyl galactosamine
Wheatgerm agglutinin	N-acetyl glucosamine
Concanavalin A Lens culinaris	Mannose
Lotus tetragonolobus *Ulex europaeus* I	L-fucose

Tissues (unfixed cryostat sections): Skin, brain, spleen, bladder, lung, thyroid and trachea, colon, salivary gland.

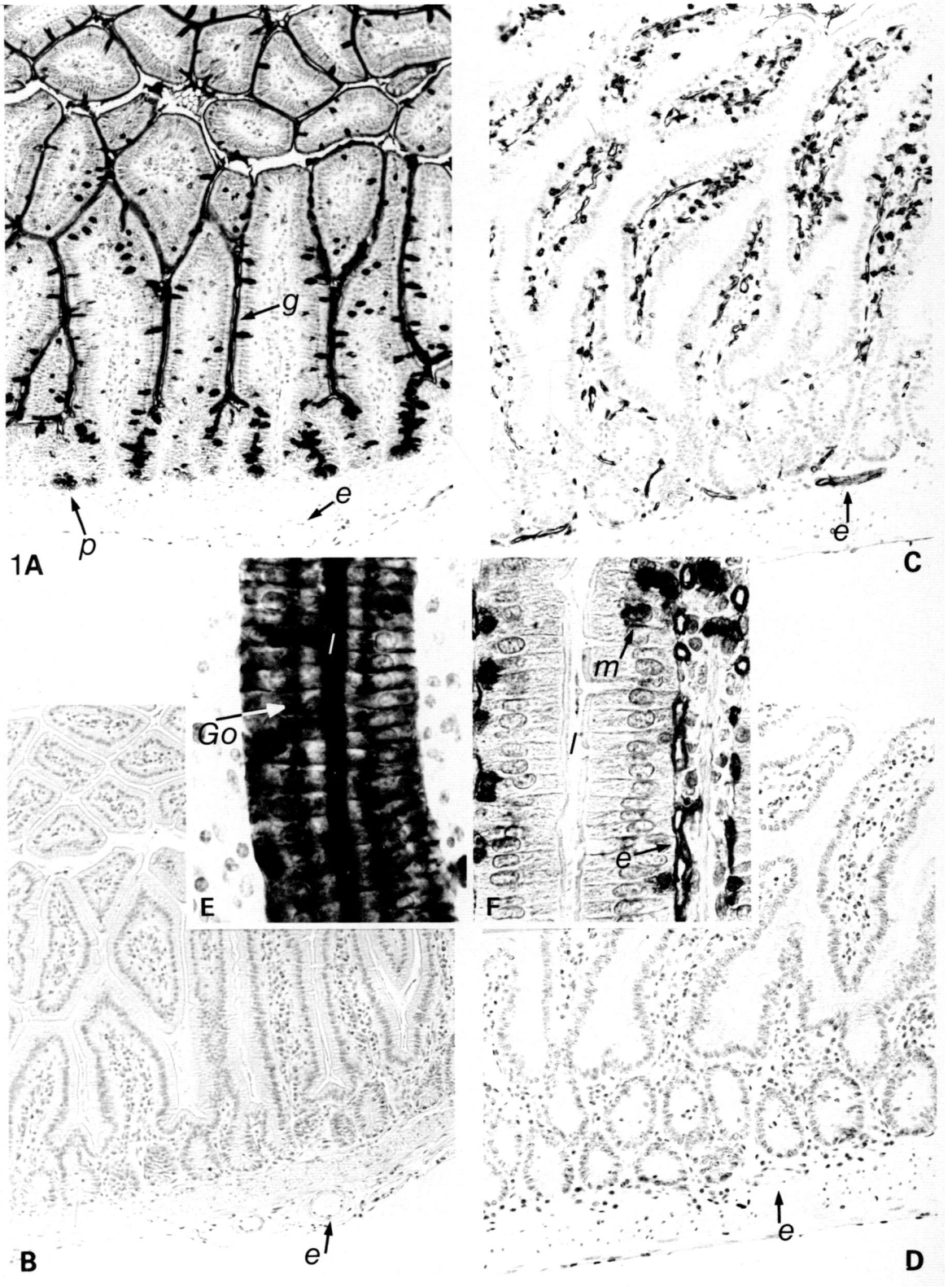

Fig. 1

Table 2. *Classification of inbred strains for DBA-binding pattern*

D1b-1^{a} (Gut epithelium $^{-ve}$; endothelium $^{+ve}$)	DDK, SWR, RIII-*ro*
D1b-1^{b} (gut epithelium $^{+ve}$; endothelium $^{-ve}$)	AKR, A/Nimr, BALB/c, C57L, C57BL/6J, B10.A, C57BL/Ks, C57BL/10ScSn, C57BL/Nimr, CBA/Ca, CBA/HN, C3H/Bi, C3H/He, DBA/1, DBA/2, GE, HTG, IF, LP/Nimr, LT, NZB, NZW, SEA/J, SJL/J, 101/H, 129/RrJ.

The screening revealed only one polymorphism, for binding of DBA–peroxidase to intestinal epithelium and vascular endothelium (Fig. 1). In RIII-*ro* and DDK mice, DBA–peroxidase bound to vascular endothelium but not to intestinal epithelium: in the other 8 strains, the reverse was true. The polymorphism was designated *D1b-1* for Dolichos lectin binding, RIII-*ro* being the type strain for *D1b-1*^{a}, and C57BL/6J the type strain for *D1b-1*^{b}. Subsequently, (see below), the lectins from *Helix pomatia* (HPA) and from *Wisteria floribunda* (WFA), which like DBA have nominal specificity for terminal non-reducing N-acetyl galactosamine, were found to recognise the same polymorphism.

A further 19 strains and substrains have been examined for their *D1b-1* type, the majority falling into the *D1b-1*^{b} group (Table 2).

In order to assess the use of the polymorphism for studying clonal development in gut epithelium and vascular endothelium during embryogenesis, we also examined a series of embryos of C57BL/6J and RIII-*ro* strains at different ages (see below).

Distribution of DBA binding sites in RIII-ro and C57BL/6J tissues (Tables 3 and 4)

The distributions reported below were consistent for 20 adult mice of each strain (males and females, aged from 4 to 24 weeks), and four embryos of each strain at days 8, 9, 10, 11, 13 and 18 of gestation. DBA–peroxidase (rather than HPA or WFA) was used throughout these studies.

Fig. 1. Polymorphism in expression of DBA-binding sites in adult small intestine. (A, E) C57BL/6J small intestine (A, × 160; E, detail of epithelium and lamina propria of villus × 400). (B) adjacent section to (A): sugar control (× 160). (C, F) RIII-*ro* small intestine (C, × 160; F, detail of epithelium and lamina propria of villus × 400). (D) adjacent section to (C): sugar control (× 160).

Methacarn fixed, paraffin-embedded 4μm sections of tissue from 8-week-old mice. Counterstained with haemalum. DBA-binding sites appear black. *g*, gut epithelium; *l*, lumen between villi; *m*, unidentified DBA-positive cells (see Table 4): *p*, Paneth cells; *e*, endothelium; *Go*, Golgi apparatus.

In C57BL/6J intestine, columnar, goblet and Paneth cells are DBA-positive, and vascular endothelium is DBA-negative. In RIII-*ro* intestine the reverse is true: the endothelium of arteries, veins and blood capillaries (but not lymphatics) is DBA-positive; intestinal epithelium is DBA-negative.

Table 3. *Polymorphic patterns of binding of DBA-peroxidase in tissue sections of RIII-*ro *and C57BL/6J mice*

	RIII-*ro* (D1b-1^a)	C57BL/6J (*D1b-1*^{b})
Adult		
Small intestine		
columnar cells	−	+
goblet cells	−	+
Paneth cells	−	+*
entero endocrine cells	−	?
colon†		
columnar cells	−	+
goblet cells	−	+
vascular endothelium‡	+	−
Embryo (8, 9, 10, 11, 13, and 18 days)		
gut epithelium	+ve up to ca. 11 days gestation, then −ve	
vascular endothelium	+	patchy +ve in major vessels up to ca. 11 days gestation, then −ve

* Paneth cells DBA^{-ve} in some *D1b-1*^{b} strains (e.g. CBA/Ca, C3H/Bi)

† Some *D1b-1*^{b} strains show transition to DBA^{-ve} distally

‡ Consistent tissue-specific patterns of DBA^{+ve} endothelium are seen in adult mice of *D1b-1*^{a} strains. The endothelial location of DBA binding sites has been confirmed by electron microscopy. Lymphatic endothelium is DBA-negative. (Ponder & Wilkinson, 1983 for details).

Adult mice

The polymorphism in DBA-binding sites in the adult was confined to intestinal epithelium and vascular endothelium (Fig. 1; Table 3). Non-polymorphic binding sites are listed in Table 4.

Notable departures from the general pattern of polymorphism in the adult mice were (i) that the colonic epithelium was DBA-negative distally in some *D1b-1*^{b} strains (CBA/Ca, C3H/Bi, BALB/c, AKR, DBA/2, but not C57BL/6J, C57B10, NZB, NZW, 129/RrJ) which were otherwise DBA-positive in intestinal epithelium; (ii) that in otherwise DBA-positive colonic epithelium, crypts adjacent to lymphoid follicles were often DBA-negative, but this was not so for crypts adjacent to lymphoid follicles in small intestine; and (iii) in adult *D1b-1*^{a} strain mice (endothelium-positive), DBA binding was not present on endothelium in certain tissues in which endothelial cells were DBA positive in 13-day embryos (see Ponder & Wilkinson, 1983).

Table 4. *Non-polymorphic patterns of binding of DBA-peroxidase in tissue sections of RIII-*ro *and C57BL/6J mice*

Adult	*Embryo only*
epithelium of biliary duct pancreatic duct } (patchy) stomach caecum salivary gland acini collecting tubules of kidney (patchy) fallopian tube oocytes unidentified cells in epithelium, lamina propria and lymphoid follicles of small intestine (see Figure 1).	yolk sac cell population in mid/late gestation thymus* medial and dorsal quadrant of developing otocyst

* described in detail by Kasai, Takashi, Takahashi & Tokunaga, 1983.

Note: The following tissues are DBA-negative in adult mice of RIII-*ro* and C57BL/6J strains: central and peripheral nervous system; skeletal and cardiac muscle; thyroid, parathyroid, adrenal, ovary (except oocytes), testis, pancreatic islets; skin and appendages; squamous epithelia; non-pregnant mammary gland ducts; lung parenchyma and bronchi; connective tissue; adipose tissue; ureter; bladder epithelium; kidney (except as above); uterine epithelium; lymphoid tissue (except as listed above); bone marrow and peripheral blood; hepatocytes: pancreatic acini.

Embryos

In C57BL/6J and RIII-*ro* embryos before 11 days gestation, the polymorphism in DBA-binding sites was not as clearly expressed as in the adult. Both endothelium and gut epithelium were stained with DBA–peroxidase. In each strain, however, the staining was stronger and more widely distributed on the tissue which would remain DBA-positive in the adult. Thus, (Figure 2) the DBA–peroxidase staining of gut epithelium in C57BL/6J embryos extended outside the basal lamina into the surrounding mesenchyme. Conversely, endothelial staining was widespread in RIII-*ro* embryos, but confined in C57BL/6J embryos to patchy staining in the large vessels. By 13 days of gestation, the adult polymorphic pattern was established, and it was maintained in 18-day embryos.

Chemistry of the DBA-binding site

Complete inhibition of DBA–peroxidase staining was obtained with 0·2 M-N-acetyl galactosamine, and slight reduction in staining intensity with 0·2 M-L-fucose. 0·2 M-D-glucose, D-galactose, D-mannose and N-acetyl glucosamine were without detectable effect.

To investigate whether DBA-binding sites were borne on glycoprotein or glycolipid, we extracted unfixed cryostat sections with chloroform–methanol (see Methods). The distribution and intensity of DBA–peroxidase staining was unaltered. To investigate whether masking by changes in membrane conformation

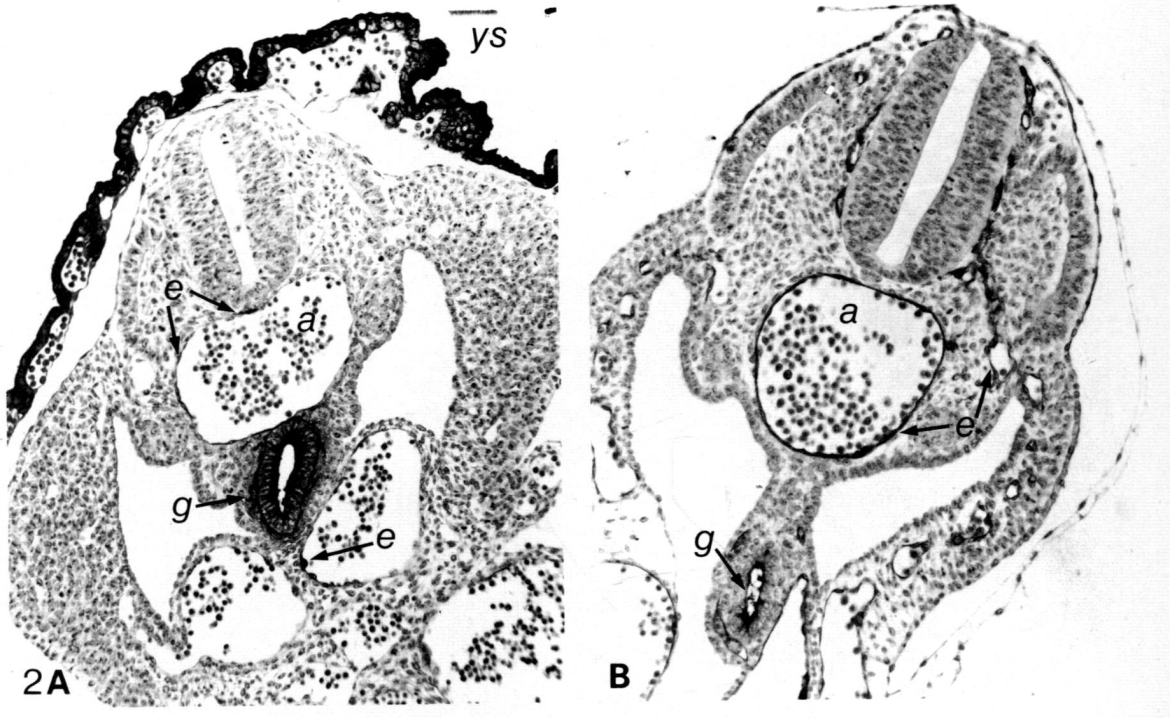

Fig. 2. Polymorphism in expression of DBA binding sites in embryonic tissue. (A) C57BL/6J embryo: estimated 9½ days gestation. Transverse section through posterior body region (× 100). (B) RIII-*ro* embryo: estimated 9½ days gestation. Transverse section through posterior body region (× 100).

Tissue processed as in Fig. 1. DBA-binding sites appear black. *g*, gut; *a*, dorsal aorta; *e*, endothelial cells; *ys*, yolk sac:

In the C57BL/6J embryo there is strong DBA binding to gut epithelium and surrounding tissue, and scattered DBA-positive endothelial cells in large vessels. In the RIII-*ro* embryo, the gut epithelium is weakly DBA-positive (most of the staining is at the luminal surface), but endothelium in aorta and other vessels is strongly positive.

(Willison *et al.* 1982) might be responsible for the lack of binding sites in DBA-negative tissues, we treated unfixed cryostat sections with 10 % cold trichloracetic acid (TCA) and 0·1 % or 1 % NP40. 10 % TCA and 0·1 % NP40 were without effect. After 1 % NP40 extraction staining was lost, but again without the appearance of DBA-positive areas in previously negative tissue. To investigate the possibility of masking of DBA-binding sites by sialic acid or by galactose residues [the sequence N-acetyl galactosamine-galactose is common in carbohydrate chains of glycoproteins (Beyer *et al.* 1981)], we digested tissue sections with neuraminidase and with α and β galactosidases. None of these altered the pattern of DBA–peroxidase staining.

Finally, as another approach to examine whether the DBA-positive/DBA-negative difference was due to addition or loss of a sugar, and if so, which, a further 10 lectins [from *Helix pomatia* (HPA) (Hammarstrom, 1972), *Wisteria floribunda*

(WFA) (Kurokawa *et al.* 1976), *Vicia villosa, Codium fragile, Sofora japonica* and Jack fruit (all with specificity for N-acetyl galactosamine), *Pisum sativum* (α-D-glucose), *Maclura pomifera, Mangifera indica* and Cotoneaster (α-D-galactose)] were examined for tissue-binding pattern. Only two of the lectins with nominal specificity for N-acetyl galactosamine residues, HPA and WFA, gave a pattern identical to DBA. The other lectins showed different patterns, and no polymorphism. Antibody to Forssman antigen, which contains a terminal N-acetyl galactosamine residue (Willison *et al.* 1982), also gave a different staining pattern, again without differences between C57BL/6J and RIII-*ro* intestinal epithelium or vascular endothelium.

Genetics of the polymorphism

Each of the 29 inbred strains and substrains (see Table 2) tested was either gut-epithelium-positive, endothelium-negative (G^+E^-) or gut-epithelium-negative, endothelium-positive (G^-E^+). None was G^+E^+, and none G^-E^-. Similarly, of seven C57L × SWR recombinant inbred (RI) strains (generously provided by Dr B. Taylor, Jackson Laboratory) six were G^+E^- and one was G^-E^+.

All of 24 *D1b-1*^{a} × *D1b-1*^{b} F1 animals (RIII-*ro* × DBA/2; RIII/*ro* × C57BL/6J; SWR × C57L; RIII/*ro* × CBA/CaLac) were G^+E^+. Of 36 F1 × *D1b-1*^{b} backcrosses, all were G^+ and 15/36 were E^+; and of 40 F1 × *D1b-1*^{a} backcrosses, all were E^+ and 19/40 were G^+. The ratio of $G^-{\times}E^+ : G^+E^+ : G^+E^-$ phenotypes in 78 F2 animals was 16 : 45 : 17.

DISCUSSION

Our description of the distribution of DBA-binding sites in embryonic and adult tissue amplifies those of Watanabe, Muramatsu, Shirane & Ugai, (1981) and Noguchi, Noguchi, Watanabe & Muramatsu (1982), who used only *D1b-1*^{b} strains. Although Van der Valk & Hageman (1982) used mice of *D1b-1*^{a} and *D1b-1*^{b} strains, they reported both intestinal epithelium and vascular endothelium to be DBA-negative. The polymorphism which we now describe is of particular interest because of the reciprocal pattern in the two polymorphic types, which our results suggest is determined by alleles at a single locus.

The F1 and backcross results are those expected if the expression of DBA-binding sites in endothelium and in gut epithelium are each determined by a single dominant gene. The results suggest further that these genes are alleles at a single locus. Two unlinked loci would be expected to give four phenotypes: G^+G^+, G^+E^-, G^-E^+ and G^-E^-. In this case, the likelihood of finding in inbred strains only the two phenotypes which we have observed would be extremely small. Discounting substrains, we have examined 23 inbred strains and 7 RI strains. If the loci were unlinked and all phenotypes fully viable, the chance of observing only the G^+E^- and G^-E^+ phenotypes in these 30 strains would be $(1/2)^{30} = < 10^{-9}$. Even if G^-E^- were lethal (we know from the F1 mice that G^+E^+ is viable), the chance

would be only $(2/3)^{30} = 5 \times 10^{-6}$. The F2 results strengthen the argument. The 16 : 45 : 17 ratio of phenotypes in the F2 animals does not depart significantly ($P = 0{\cdot}66$) from the 1 : 2 : 1 ratio expected for a single locus. While these results might also fit the 9 : 3 : 3 ratio that would be expected with two unlinked loci with dominance at each locus but with the double recessive homozygote ($G^-G^-E^-E^-$) being lethal, it would be very unusual for such a double recessive genotype to be lethal when neither G^-G^- nor E^-E^- is in itself lethal. We conclude that the *D1b-1*^{a}/*D1b-1*^{b} polymorphism is due to an allelic difference at a single locus.

The stability of the DBA-peroxidase staining to chloroform/methanol extraction suggests that some at least of the DBA-receptor carbohydrate is carried on glycoprotein. It is likely that the polymorphic differences and the changes in DBA-binding patterns during embryonic development are due to tissue-specific and temporal patterns of activity of glycosyltransferases (Hakamori, 1981; Kapadia *et al.* 1981). The failure of detergent, trypsin or trichloroacetic acid treatment of unfixed sections to influence the DBA-staining patterns suggests that they are not due to changes in cell membrane conformation which influence the accessibility of the DBA-binding site (Willison *et al.* 1982).

If differences in glycosyltransferase activity are indeed the basis for the polymorphism, DBA-negative tissues might either contain N-acetyl galactosamine (GalNac) residues masked by addition of a further sugar residue, or they might lack GalNac because of failure to transfer the sugar to its acceptor. The failure to unmask DBA-binding sites by enzyme digestion of tissue sections argues against masking as the basis of the DBA-binding polymorphism. Furthermore, one would expect masking to be dominant, which would produce a G^-E^- phenotpye in F1 tissues, but this was not found. It is most probable, therefore, that the polymorphism results from the presence or absence of tissue-specific expression of an N-acetyl galactosaminosyl transferase. This would be predicted to give the G^+E^+ phenotype in F1 animals which we have observed.

The *D1b-1*^{a} strains DDK, SWR and RIII are all of European origin or derived from European stock, as are GRS/A, LIS/A, STS and MAS/A, which are also of *D1b-1*^{a} type (G. Uiterdijk, personal communication). The *D1b-1*^{b} strains were mostly derived in the USA from mice originally from China, through English fanciers and then to American and English laboratories (Festing, 1979). The reciprocal pattern of *D1b-1*^{a}/*D1b-1*^{b} polymorphism may therefore have arisen from a single genetic event in an ancestor of one of these groups of strains. Identification of the genetic mechanism of the polymorphism might provide insight into the mechanisms of temporal and tissue-specific regulation of glycosyltransferase activity.

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