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Lectin-histochemical and -cytochemical study of periodic acid Schiff-Positive lysosome granules as a histological feature of the female mouse kidney

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Summary. Renal proximal straight tubules (PST) of the female mouse contain periodic acid Schiff–positive lysosome granules. An excellent example of this is found in the kidneys of female DBA/2Cr mice. In the present study, lectin-histochemistry showed that lectin-positive granules occur in the PST of DBA/2Cr mice. Out of twenty-one lectins studied, the granules bound WGA, s-WGA, LEL, STL, DSL, GSL-II, VVL, RCA-I, ECL, PSA, LCA and PHA-E. Such granules were also observed in the proximal convoluted tubules (PCT). In addition, heterogeneous binding to the SBA or DBA was observed in the PST. Lectin-cytochemistry for s-WGA, STL, VVL, RCA-I, ECL and PSA, showed that: 1) lysosomes bind a higher level of s-WGA or STL than VVL, RCA-I, ECL or PSA; 2) PSA binding is similar in PST and PCT; 3) there are many PCT lysosomes that are negative for s-WGA, STL, VVL, RCA-I, and ECL lectin binding; and 4) s-WGA binding is highly specific to the lysosomes of the PST. Based on the binding specificities of each lectin, it was suggested that the mannose content of PST and PCT lysosomes is similar, and that PST lysosomes have a high level of N-acetylglucosamine, N-acetylgalactosamine, galactose or galactosyl (ß1,4) N-acetylglucosamine.

Key words: Female mouse, Kidney, Lectin, Lysosome, Proximal straight tubule

Introduction

Lysosomes in the proximal straight tubules (PST) of the female mouse kidney are larger in size than their male counterparts and display periodic acid Schiff (PAS)–positive granules that are visible by light microscopy (Yabuki et al., 1999, 2001a). Among five strains of mice, including ICR, BALB/cA, C57BL/6J, C3H/HeN and DBA/2Cr, this feature is most apparent in female DBA/2Cr (Yabuki et al., 2001a). In these mice, PAS-positive giant lysosomes, larger in size than the nucleus, are frequently seen in the PST. Because the lysosome is an important organelle for the cellular metabolic function, we suspected that the PAS-positive granules in the PST reflect an aspect of renal metabolic function that is specific to the female mouse.

Given that the PAS stain binds to carbohydrates, we predicted that the PAS-positive lysosome granules contained a high level of glycoconjugates. Lectins, which bind to specific sugars, have been used as probes for identification of the carbohydrate moieties on the cell surface or in cytoplasmic organelles. In fact, many lectin-histochemical studies for mammalian kidney have been performed (Faraggiana et al., 1982; Holthöfer, 1983; Murata et al., 1983; Schulte and Spicer, 1983; Truong et al., 1988; Coppee et al., 1993; Ojeda and Piedra, 1994). However, these previous reports did not examine the carbohydrate moieties in the lysosome granules. In the present study, we utilized lectin-histochemistry and -cytochemistry to explore lysosomal lectin binding to PAS-positive lysosomes of the female DBA/2 mouse kidney.

Materials and methods

All experimentation proceeded in accordance with the Guideline for Animal Experimentation of the Faculties of Medicine and Agriculture, Kagoshima University, Japan.

Animals

Three- to four-month-old female DBA/2Cr Slc mice (n=6) were housed in an open system room with a one-way airflow system (temperature; 22±1 °C, humidity; 55±10%, light period; 07:00 h - 19:00 h, ventilation; 12

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times/hr) in the Institute of Laboratory Animal Sciences, Faculty of Medicine, Kagoshima University, Japan. The mice received an autoclaved commercial diet (CE-2, Japan CLEA., Japan) and tap water *ad libitum*.

Tissue preparation

For lectin-histochemistry, three animals were sacrificed by dislocation of the cervical vertebrae under anesthesia with a mixture of ketamine and medetomidine. According to a routine fixation procedure for histologic investigation (Yabuki et al., 2001b), they were immediately perfused through the left ventricle with a physiological saline for 30 sec and then with 10% neutral formalin in 0.1 M phosphate buffer (PB, pH 7.4) for 10 min at 4 °C. From the left kidneys, central slices that included the hilum were cut perpendicular to the long axis and immersed in the same fixative for 2 days (4 °C). The slices were then embedded in paraffin and cut in 3 μm-thick sections.

For lectin-cytochemistry, three animals were dissected as described above, and immediately perfused through the left ventricle with a physiological saline for 30 sec and then with 0.25% glutaraldehyde, 4% paraformaldehyde in PB for 10 min at 4 °C. From the left kidneys, small pieces, approximately 1 mm^3, were cut from the cortex or outer medulla and thoroughly washed in PB (4 °C) without further fixation. These samples were dehydrated through a graded ethanol series (4 °C), embedded in LR white resin, and polymerized at 50 °C for 48 hrs. Ultrathin sections were cut and mounted on nickel grids.

Lectin-histochemistry and -cytochemistry

The present investigation was performed using biotinylated lectins (Vector Laboratories, Inc., USA). Concentrations and specificities of the lectins are shown in Table 1. Concentrations of the lectins for histochemistry were determined in a previous study (Tamura et al., 2000), and those for cytochemistry were determined in the preliminary experiments. In negative control experiments, lectin solutions were preincubated with appropriate inhibitory sugars, according to the manufacturer's procedures; e.g., chitin hydrolysate (Vector Laboratories, Inc., USA) for succinylated wheat germ agglutinin (s-WGA), 0.2 M galactose for *ricinus communis* agglutinin I (RCA-I), or a mixture of 0.2 M α-methyl mannoside and 0.2 M α-methyl glucoside for *pisum sativum* agglutinin (PSA).

Lectin-histochemistry were carried out as follows: 1) deparaffination and rehydration; 2) treatment with 0.3% H_2O_2 in methanol for 30 min; 3) washing in 10mM phosphate-buffered saline (PBS, pH 7.4); 4) incubation with 1% bovine serum albumin (BSA) in PBS for 60 min; 5) treatment with an avidin-biotin blocking kit (Vector Laboratories, Inc., USA); 6) washing in PBS; 7) overnight incubation at 4°C with biotinylated lectins diluted in 0.1% BSA-PBS; 8) washing in PBS; 9) incubation with peroxidase-conjugated avidin-biotin-complex (Elite ABC kit; Vector Laboratories, Inc., USA) for 40 min; 10) washing in PBS; 11) visualization of the reactions with 3,3'-diaminobenzidine (0.25mg/ml)-H_2O_2 (0.003%) in PBS for 5 min; and 12) termination of the reaction in distilled water. The sections were counter

Table 1. Binding specificities of lectins used.

ABBREVIATION	LECTIN	SPECIFICITY	CONCENTRATION (μg/ml)
WGA	Wheat Germ Agglutinin	$(GlcNAc)_n$, Sialic Acid	3 *
s-WGA	Succinylated Wheat Germ Agglutinin	$(GlcNAc)_n$	8 (40) **
LEL (TL)	*Lycopersicon esculentum* (Tomato) Lectin	$(GlcNAc)_n$	2
STL (PL)	*Solanum tuberosum* (Potato) Lectin	$(GlcNAc)_n$	4 (20)
DSL	*Datura stramonium* Lectin	$(GlcNAc)_n$, Galß1-4 GlcNAc	2
GSL-II (BSL-II)	*Griffonia (Bandeiraea) simplicifolia* Lectin II	GlcNAcα, GlcNAcß	6
SBA	*Soybean Agglutinin*	GalNAc	8
VVL (VVA)	*Vicia villosa* Lectin	GalNAc	6 (30)
RCA-I (RCA 120)	*Ricinus communis* Agglutinin I	Gal, GalNAc	4 (20)
DBA	*Dolichos biflorus* Agglutinin	GalNAcα	8
GSL-1 (BSL-I)	*Griffonia (Bandeiraea) simplicifolia* Lectin I	Galα, GalNAcα	6
SJA	*Sophora japonica* Agglutinin	Galß, GalNAcß	8
Jacalin	*Artocarpus integrifolia* (Jackfruit) Seeds	Sialyl-Galß1-3 GalNAc-O-	4
PNA	*Peanut Agglutinin*	Gaß1-3 GalNAc	8
ECL	*Erythrina cristagalli* Lectin	Gal, Galß1-4 GlcNAc	4 (20)
UEA-I	*Ulex europaeus* Agglutinin I	Fucα	8
ConA	Concanavalin A	Manα, Glcα	4
PSA	*Pisum sativum* Agglutinin	Manα	3 (15)
LCA (LcH)	*Lens culinaris* (Lentil) Agglutinin	Manα	3
PHA-E	*Phaseolus vulgaris* Erythroagglutinin	Oligosaccharide	2
PHA-L	*Phaseolus vulgaris* Leucoagglutinin	Oligosaccharide	2 (10)

*: for histochemistry; **: for cytochemistry; GlcNAc: D-N-acetylglucosamine; Gal: D-galactose; GalNAc: D-N-acetylgalactosamine; Fuc: L-fucose; Man: D-mannose; Glu: D-glucose.

stained with hematoxylin and observed by light microscopy at random.

In each segment of the kidney, staining intensities of each lectin were scored from 0 to 4, and the mean score of each segment was calculated for each lectin. These mean scores were estimated as follows: –; mean ≤ 0.5, +1; 0.5< mean ≤ 1.5, +2; 1.5<mean ≤ 2.5; +3; 2.5<mean ≤ 3.5, and +4; 3.5<mean. In addition, in each segment of the tubules, the frequency of the lectin-positive granules were scored from 0 to 4, which based on their number, and estimated from – to +4 as described above.

Lectin-cytochemistry was carried out using the seven biotinylated lectins described in Table 1. The procedure was performed as follows: 1) washing with PBS; 2) incubation with 1% BSA-PBS for 30 min; 3) overnight incubation at 4 °C with biotinylated lectins diluted in 1% BSA-PBS; 4) washing with PBS; 5) incubation with gold (10 nm)-conjugated streptavidin (British Biocell., UK) diluted 1:100 in 1% BSA-PBS for 60 min; 6) washing with PBS; 7) treatment with 1.25% glutaraldehyde in PBS for 5 min; 8) washing with distilled water; 9) air drying; and 10) double staining with uranyl acetate and lead citrate. After air drying, grids were coated with carbon and observed by a transmission electron microscope (H-7000KU, Hitachi, Japan).

Results

Lectin-histochemistry

The semi-quantitative binding pattern of each lectin is shown in Table 2. In female DBA/2Cr mice, we could detect the following twelve lectins in the cytoplasmic granules of the PST: wheat germ agglutinin (WGA), s-WGA, *Lycopersicon esculentum lectin* (LEL), *Solanum tuberosum* lectin (STL), *Datura stramonium* lectin (DSL), *Griffonia simplicifolia* lectin II (GSL-II), *Vicia*

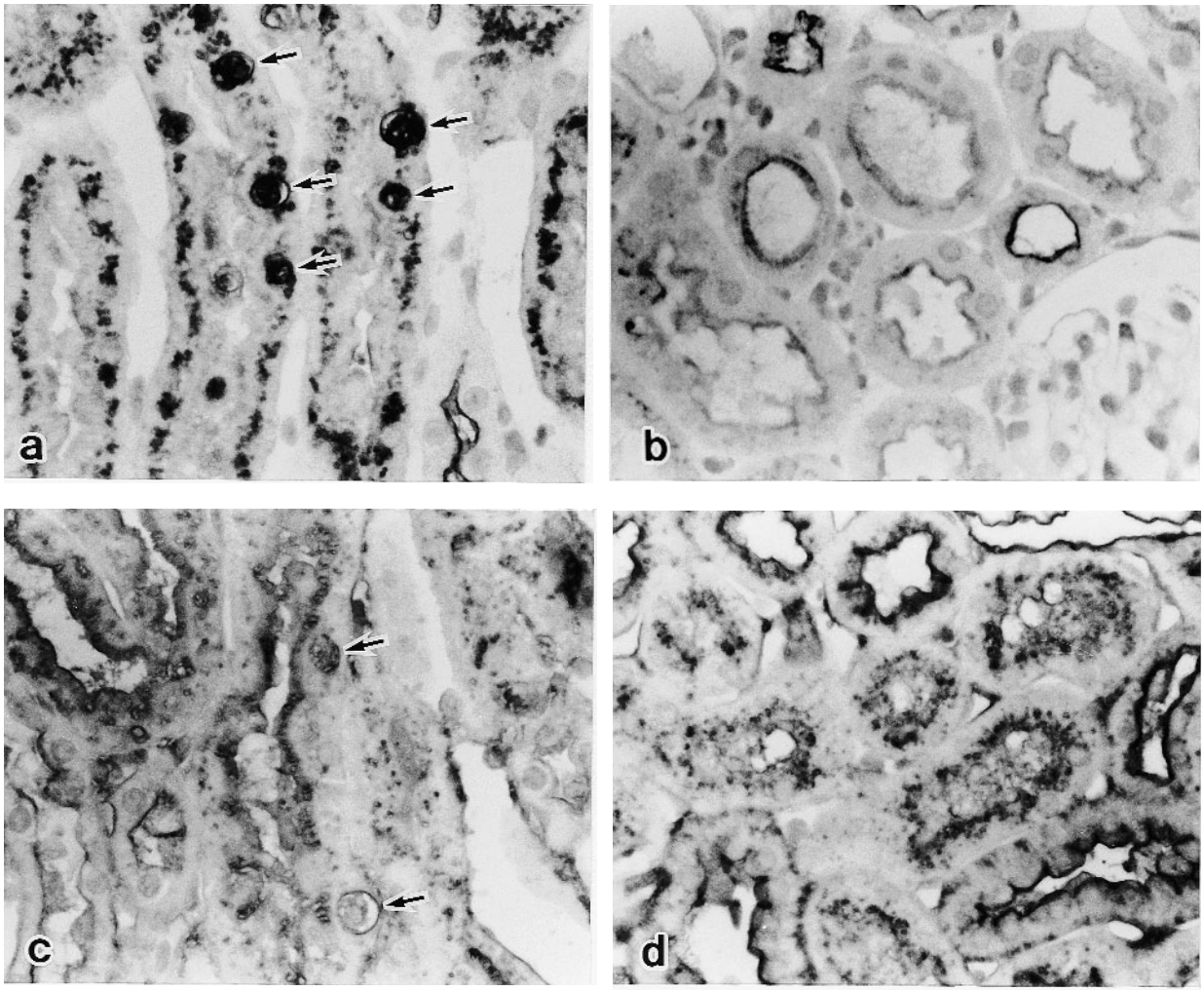

Fig. 1. Histochemical binding pattern for s-WGA **(a and b)** and RCA-I **(c and d)**. **a and c**: outer medulla. **b and d:** cortex. Arrows indicate giant granules. Especially in s-WGA, positive granules are abundant in the PST **(a)**. x 600

villosa lectin (VVL), RCA-I, *Erythrina cristagalli* lectin (ECL), PSA, *Lens culinaris* agglutinin (LCA) and *Phaseolus vulgaris* erythroagglutinin (PHA-E). Similarly, the giant granules of the PST bound all twelve of these lectins. In addition, these same lectins bound to the cytoplasmic granules of the proximal convoluted tubules (PCT). The frequency of granules that bound WGA, s-WGA, LEL, STL, GSL-II, VVL, PSA, LCA and PHA-E was higher in PST than in PCT. This difference was particularly apparent for s-WGA (Fig. 1). Binding of DSL was the same in PST and PCT, and for RCA-I and ECL, levels were lower in the PST than in the PCT. Binding of VVL, GSL-I, ConA, PSA, LCA or *Phaseolus vulgaris* Leucoagglutinin (PHA-L) revealed that, except for the proximal tubules, few granules occur in the Henle's loops, thick ascending limbs, or collecting ducts. Furthermore, heterogeneous binding was observed in the soybean agglutinin (SBA) and *Dolichos biflorus* agglutinin (DBA), especially in the PST. Bindings for SBA and DBA were observed in the brush border, cytoplasm, granules or giant granules (Fig. 2). The binding of each lectin was eliminated by pre-incubation with the appropriate inhibitory sugar, showing that the positive reaction was specific.

Lectin-cytochemistry

Electron microscopy showed binding of s-WGA,

Table 2. Semi-quantitative determination of lectin binding intensity in the female DBA/2 mouse kidney.

	WGA	s-WGA	LEL	STL	DSL	GSL-II	SBA	VVL	RCA-I	DBA	GSL-I	SJA	Jacalin	PNA	ECL	UEA-I	ConA	PSA	LCA	PHA-E	PHA-L
RCA[a]																					
Gl[b]	+4[c]	-	+4	+2	+2	-	-	+2	+3	-	+1	-	+3	-	+2	-	+2	+2	+3	+3	+2
PL	+3	-	+3	+2	+2	-	H[e]	+2	+3	H	+1	-	+3	+2	+1	-	+2	+2	+3	+3	+2
PCT																					
BB	+2	+1	-	-	+2	+1	-	+1	+3	-	-	-	+1	+1	+3	-	+1	+3	+3	+3	+4
Cy	-	-	-	-	+1	-	-	-	-	-	-	-	-	-	-	-	+1	-	+1	-	-
Gr	(+2)d)	(+1)	(+2)	(+2)	(+2)	(+3)	(-)	(+3)	(+3)	(-)	(-)	(-)	(-)	(-)	(+4)	(-)	(+1)	(+1)	(+1)	(+1)	(+1)
BS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BM	+2	-	+3	+2	+2	-	-	+1	+3	-	-	-	+3	-	-	-	+1	+2	+3	+3	+2
PST																					
BB	+1	+1	-	-	+3	+2	H	+1	+2	H	-	-	+1	+1	+3	-	+1	+1	+3	+2	+4
Cy	-	-	-	-	-	-	H	-	-	H	-	-	-	-	-	-	+1	-	-	-	-
Gr	(+3)	(+4)	(+4)	(+4)	(+2)	(+4)	(H)	(+4)	(+2)	(H)	(-)	(-)	(-)	(-)	(+2)	(-)	(-)	(+3)	(+2)	(+2)	(-)
GGr	+2	+4	+4	+4	+3	+3	H	+3	+2	H	-	-	-	-	+1	-	+1	+3	+3	+2	-
BS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BM	+2	-	+2	+1	+2	-	-	+2	+2	-	+1	-	+3	-	-	-	+1	+2	+3	+3	+2
HL																					
LS	+4	+4	+4	+4	+4	+4	H	+4	+4	H	-	H	+2	+4	+4	-	+1	+1	-	+2	+1
Cy	+2	+1	+2	+2	+2	+1	-	+3	+2	H	+1	-	+1	+3	+3	-	+1	+1	+1	+1	+1
Gr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+1)	(-)	(-)	(-)	(-)	(-)	(+1)	(-)	(+1)	(-)	(-)
BM	+1	-	-	-	+2	-	-	+1	-	-	+2	-	+3	-	-	-	+1	+2	+2	+2	+1
TAL																					
LS	+3	+2	+4	+2	+3	-	-	-	+1	H	-	-	-	+2	+2	-	+2	-	-	+1	+1
Cy	-	-	+1	+1	+1	-	-	-	-	H	-	-	-	-	-	-	+1	-	-	-	-
Gr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+2)	(-)	(-)	(-)	(-)	(-)	(-)	(+2)	(+2)	(-)	(-)
BS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+1	-	-	-	-
BM	+2	-	-	+2	+1	-	+1	+2	+2	-	+3	-	+3	-	-	-	+1	+2	+3	+2	+2
DCT																					
LS	+4	+4	+4	+4	+3	+3	H	+4	+4	H	+2	H	+2	+4	+4	-	+2	+2	+2	+3	+1
Cy	+2	+1	+2	+2	+1	-	-	+1	+2	H	+1	-	-	+2	+2	-	+1	+1	+1	+1	+1
Gr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
BS	+3	-	+3	+2	+1	-	-	-	+1	-	+1	-	-	-	+2	-	+1	-	+1	+2	-
BM	+2	-	+2	-	-	-	-	+2	+1	-	+3	-	+3	-	-	-	+1	+2	+3	+3	+2
CD																					
LS	+4	+4	+4	+4	+4	+2	H	+3	+4	H	+3	-	+3	+4	+4	-	+2	+3	+3	+3	+3
Cy	+2	-	+2	+2	-	+1	-	-	+1	H	+1	-	-	+1	+1	-	+1	+2	+1	+1	+1
Gr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+1)	(-)	(-)	(+1)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+1)	(-)	(+1)
BS	+4	-	+4	+3	+2	-	-	-	+2	-	+2	-	-	-	+3	-	+1	+1	+2	+3	+3
BM	+2	-	+2	-	+1	-	-	+2	+1	-	+3	H	+3	-	-	-	+1	+2	+3	+3	+2

[a]: segment: RC, renal corpuscle; PCT; proximal convoluted tubule; PST, proximal straight tubule; HL, Henle's loop, TAL, thick ascending limb; DCT; distal convoluted tubule, CD; collecting duct. [b]: structure: Gl; glomerulus, BB, brush border; Cy; cytoplasm; Gr: granule; BM, basement membrane; GGr, giant granule; LS, luminal surface; BS, basement striation. [c]: lank of the staining intensity. [d]: ( ), frequency of the lectin-positive Gr. [e]: H: heterogeneous binding.

STL, VVL, RCA-I, ECL and PSA to proximal tubules. Furthermore, gold particle densities showed differential binding of the various lectins in the PST. For example, binding of s-WGA and STL was highly localized in the lysosomes, including giant lysosomes (Fig. 3). Binding of VVL, RCA-I and ECL was less specific to the lysosomes than s-WGA or STL, and they bound not only in the lysosomes but also in the brush border, apical vesicles and apical tubules (Fig. 4). In the PCT, the lysosomes bound STL, VVL, RCA-I and PSA. However, many lysosomes did not bind STL, VVL or RCA-I. In contrast, most of the lysosomes bound PSA (Fig. 5a). Binding of STL was mainly observed in lysosomes, but binding of VVL, RCA-I, PSA and ECL was also

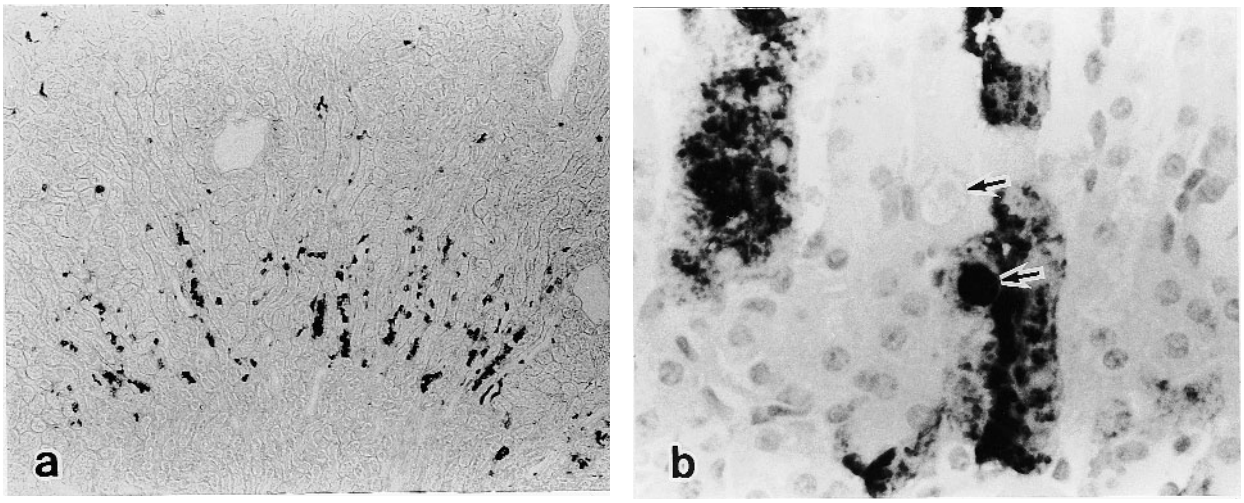

Fig. 2. Histochemical binding pattern for DBA. **a:** outer medulla, x 60. **b:** higher magnification view, x 600. Positive reactions are heterogeneously observed in the proximal straight tubules. Arrows indicate giant granules.

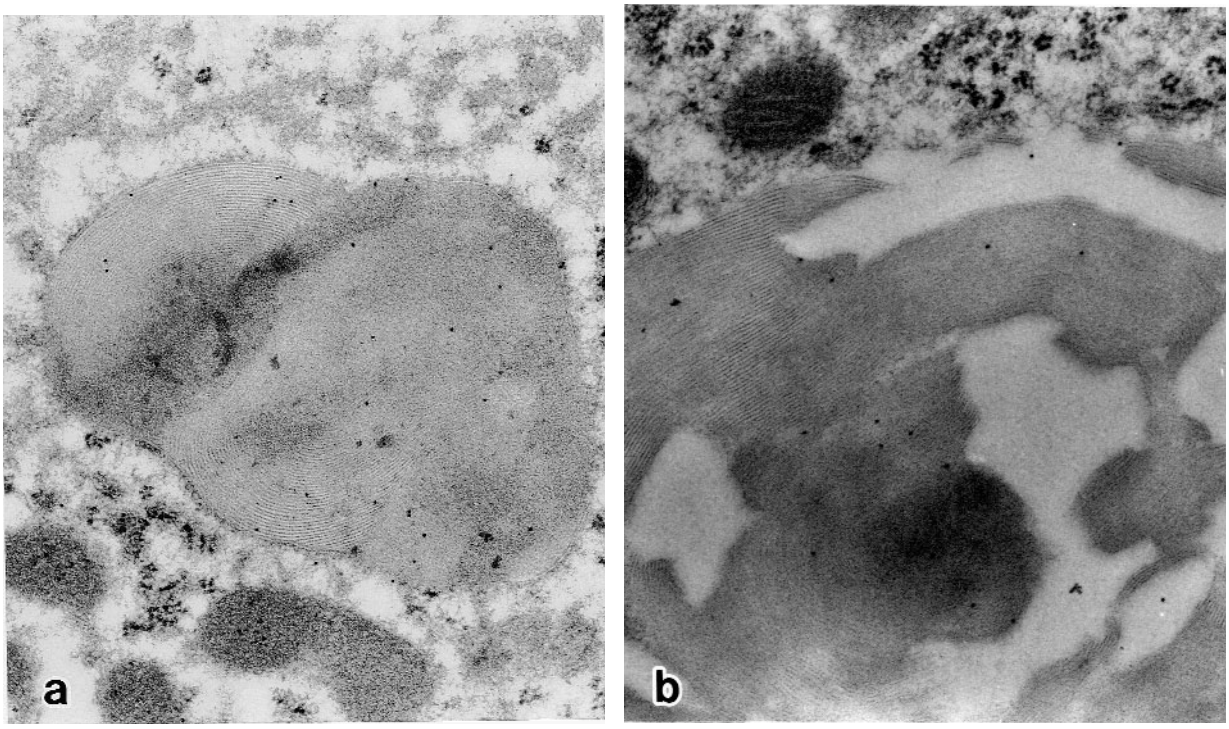

Fig. 3. Cytochemical binding pattern for s-WGA in the proximal straight tubular cells. The gold-particles localize in the large **(a)** or giant **(b)** lysosomes. x 40,000

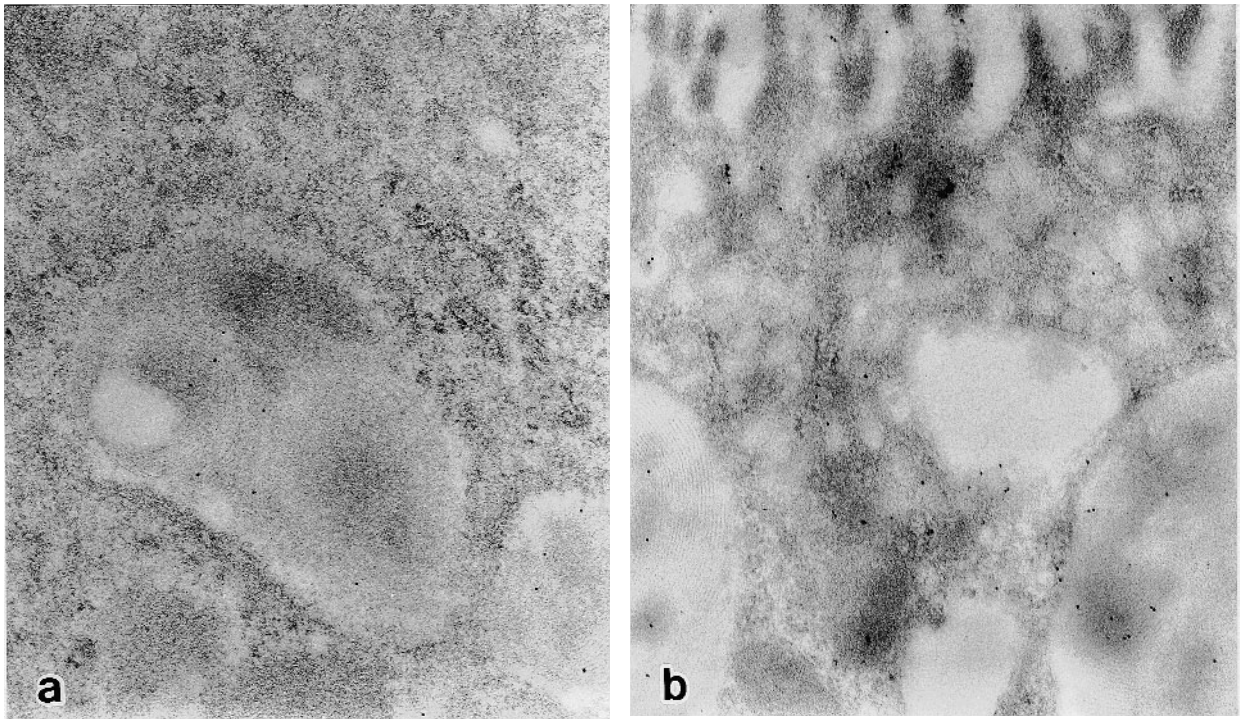

Fig. 4. Cytochemical binding pattern for PSA **(a)** and RCA-I **(b)** in the proximal straight tubular cells. The distribution of gold particles is wide, and they are observed in the brush borders, apical tubules and lysosomes. x 40,000

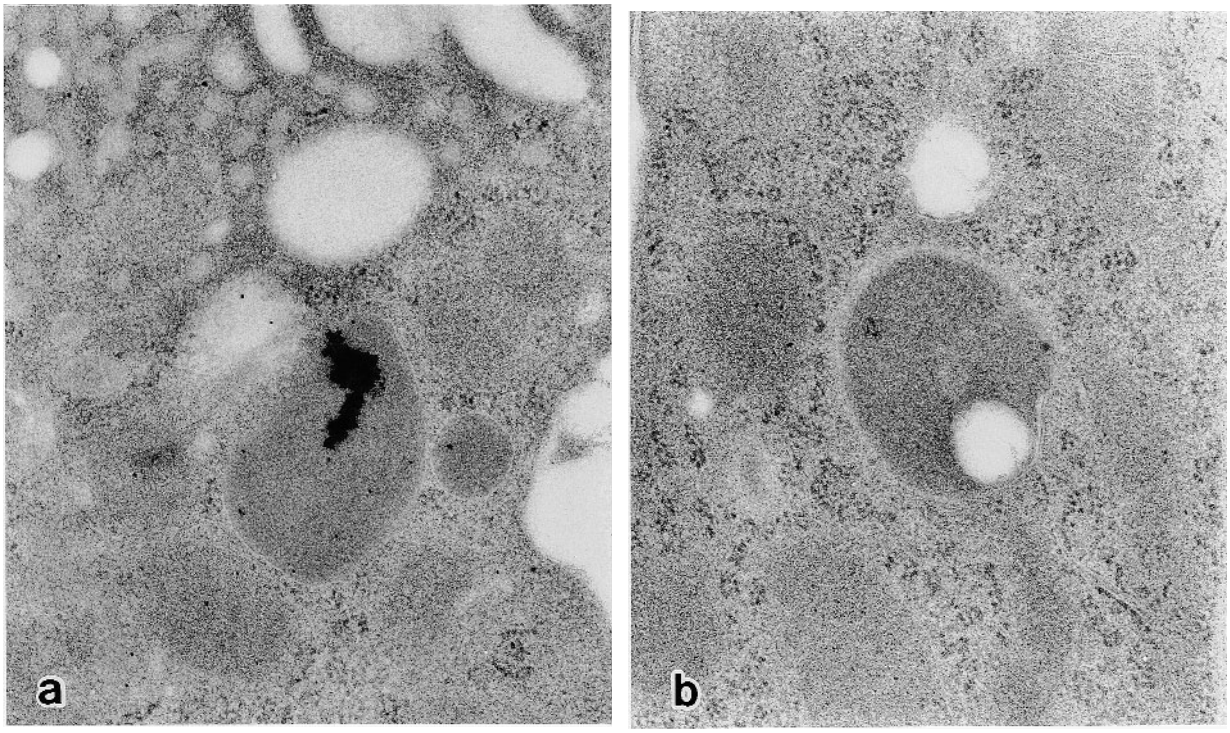

Fig. 5. Cytochemical binding pattern for PSA **(a)** and s-WGA **(b)** in the proximal convoluted tubular cells. In s-WGA **(b)**, gold particles are not observed in the lysosomes. x 40,000

observed in the brush border, apical vesicles and apical tubules. Binding of s-WGA was rarely observed, and most of the lysosomes showed negative reaction for this lectin (Fig. 5b).

The pattern of PHA-L binding was similar between PCT and PST. Figure 6 shows the binding pattern of PHA-L in the PST epithelium. Gold particles were mainly observed in the brush border, and were rarely observed apical vesicles, apical tubules or lysosomes.

The positive reactions for each lectin were eliminated by pre-incubation with the appropriate inhibitory sugar, showing that the positive reaction was specific for the indicated lectin.

Discussion

The PAS-positive granule in the PST is a histological feature of the female mouse kidney (Yabuki et al., 1999, 2001a). Because this PAS staining suggests differences in carbohydrate content, we investigated the carbohydrate content of PST lysosomes by lectin-histochemistry and -cytochemistry.

At a light microscopic level, small or giant granules were detected in the PST by binding of the following twelve lectins: WGA, s-WGA, LEL, STL, DSL, GSL-II, VVL, RCA-I, ECL, PSA, LCA and PHA-E. These granules appeared to be the same as the PAS-positive granules. Based on the binding specificities of each lectin (Table 1), it appears that these granules contained N-acetylglucosamine, N-acetylgalactosamine, galactose, galactosyl (ß 1,4) N-acetylglucosamine, or mannose.

Binding of these twelve lectins was also found in the small granules of the PCT. PAS-positive small or giant granules in the PST are lysosomes, and their detection by light microscopy depends on their size (Yabuki et al., 2001a). Because we utilized the sensitive ABC method for lectin-histochemistry, it appeared that the granules in the PCT were also lysosome granules. Whether lectin-positive granules were truly lysosomes could not be determined by light microscopy. Therefore, we performed further observations at an electron microscopic level.

Among twelve lectins described above, we examined six lectins by electron microscopy, including s-WGA, STL, VVL, RCA-I, ECL, and PSA. In the PST, although most of the lysosomes bound all six lectins, binding of VVL, RCA-I, ECL and PSA was less specific to the lysosome than staining for s-WGA or STL. Such a lack of specificity of VVL, RCA-I, ECL or PSA binding for the lysosome was also observed in the PCT. Moreover, in the PCT, there were many lysosomes that were negative for binding STL, VVL, RCA-I or ECL, and especially s-WGA. Together, these results suggest that: 1) light microscopic granules that bind VVL, RCA-I, ECL and PSA consist not only lysosomes but also other organelles (e.g. endosomes), but binding of s-WGA and STL is highly specific for lysosomes; 2) the lysosomes of PST and PCT contain similar amounts of

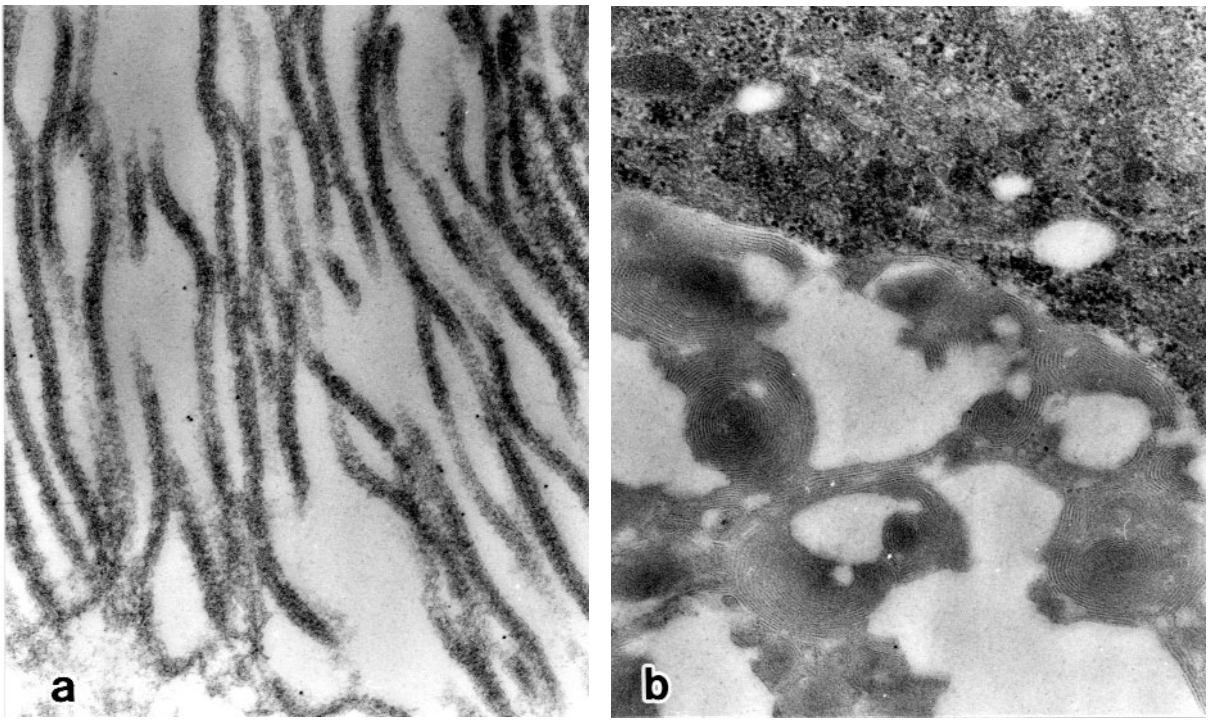

Fig. 6. Cytochemical binding pattern for PHA-L in the proximal straight tubular cells. The gold particles were mainly observed in the brush border **(a)**, whereas they were rare in the cytoplasmic organelles including giant lysosomes **(b)**. x 40,000

mannose, but compared to PCT, the lysosomes of PST contain a higher level of N-acetylglucosamine, N-acetylgalactosamine, galactose or galactosyl (ß1,4) N-acetylglucosamine, especially N-acetylglucosamine (see Table I for a summary of lectin binding specificities).

Among carbohydrate components of glycoproteins contained in lysosomes, asparagine (Asn)-linked oligoschcarides of the lysosome-associated membrane proteins (Lamps) have been well studied (Fukuda, 1991). Important functional roles of Asn-linked oligosccarides of the Lamps were demonstrated by Kundra and Kornfeld (1999). In this report, an absence of Asn-linked oligosaccharides in cultured normal rat kidney cells induced the morphologic alterations, specifically swollen vacuoles that appear to be fusion complexes of late endosomes and dense lysosomes. In the Lamps, polylactosamine had been identified as a major sugar chain of Asn-linked oligosaccharides (Carlsson et al., 1988; Fukuda et al., 1988, Fukuda, 1991), and we propose that there is a relationship between the localization of polylactosamine in lysosomes and the development of giant lysosomes in the PST of the female DBA/2 mouse. Positive binding of PHA-L to polylactosamine has been previously demonstrated (Laferte and dennis, 1989; Amos and Lotan, 1990; Cella et al., 1996). In the present study, light and electron microscopic studies showed only a low level of PHA-L binding in the lysosomes of PST. Although a low amount of polylactosamine appeared to be in the lysosomes of PST, a similar finding was also obtained in the PCT. The functional implications of polylactosamine in the development of giant lysosomes in the PST were not explored in the present study; however, analyses of other lectins showed that some of the carbohydrates described above were contained higher in the lysosomes of PST, and that there were more of them in the PST than in the PCT. Further investigations will be required to determine the functional significances of these differences in the lysosomes of the proximal tubules of the female DBA/2 mouse kidney.

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