Characteristics of Lectin Staining Patterns Assessed by a Modified Sensitive Thermo-Method in Rat Livers with Heterologous Serum-Induced Fibrosis

Makoto MIYAMOTO, Noboru MANABE*, Kozue UCHIO, Keiko KURAMITSU, Kotaro TAMURA, Yoshihiro FURUYA¹⁾, Nobuo NAGANO¹⁾ and Hajime MIYAMOTO

Unit of Anatomy and Cell Biology, Department of Animal Sciences, Kyoto University, Kyoto 606-8502 and ¹⁾Pharmaceutical Research Laboratory, Kirin Brewery Co., Takasaki 370-1295, Japan

(Received 5 February 1998/Accepted 23 April 1998)

ABSTRACT. Lectins are sensitive probes which bind carbohydrate structures specifically. In this study, we modified the lectin staining procedure for sensitive detection of carbohydrate structures in formalin-fixed, paraffin-embedded sections of normal and heterologous serum-induced fibrotic livers. The liver sections were heated in hot distilled water at 100°C for 10 min (thermo-treatment: TT), and then stained with 24 different lectins. In comparison with the results from sections without TT (nonTT), enhanced and/or alternated staining patterns of 19 lectins were demonstrated in sections with TT, and enhanced staining of *Vicia villosa* agglutinin seen in Kupffer cells was noted. Interestingly, no positive staining was seen with *Dolichos biflorus* agglutinin, peanut agglutinin or soybean agglutinin (SBA), which recognize O-linked carbohydrate chains, in Kupffer cells of non-TT sections, but strong positive staining was demonstrated in those of TT sections. SBA-positive staining in the cytoplasm of some scattered hepatocytes located in the periportal and perifibrous zones and central zone of pseudolobules was demonstrated only in the fibrotic liver sections with TT. Such findings indicate the heterogeneity of hepatocytes in the liver with fibrosis. Formalin fixation causes masking of lectin binding sites, especially O-linked carbohydrate chains, and TT may recover such masking reactions. TT improved the staining reactions for many lectins in formalin-fixed, paraffin-embedded liver sections, and new staining patterns appear after TT. Modified TT staining procedures may be useful for the diagnosis and prognosis of liver fibrosis.—**KEY WORDS:** lectin histochemistry, liver fibrosis, O-linked carbohydrate chain, rat, thermo-treatment.

J. Vet. Med. Sci. 60(8): 953–960, 1998

Formalin fixation is commonly used for histological tissue processing and provides good preservation of morphological details, but this procedure produces many types of artifacts in histochemical staining. Formalin fixation produces intra- and/or inter-methylene bridges in tissue proteins (cross-linkage of proteins) [16]. In immunohistochemical staining, such linkage masks epitopes, resulting in a decrease in availability of antibodies applicable to formalin-fixed tissue sections and in an increase in false-negative staining [16]. As the cross-linkage of protein can be reversed by high temperature treatment, retrieval of antigens masked by formalin fixation has been achieved by thermo-treatment (TT) in formalin-fixed, paraffin-embedded tissue sections [17].

Lectins are proteins or glycoproteins which bind cellular carbohydrate chains specifically and have been used as powerful tools for histochemistry to detect carbohydrate structures of various tissues and cells [3, 10, 11, 19]. Formalin-fixed, paraffin-embedded tissue sections are commonly used for lectin histochemical staining. However, lectin-carbohydrate recognition is also considered to be affected by cross-linkage of tissue proteins caused by formalin fixation similarly to antibody-antigen recognition. Previously, we applied 24 different lectins to investigate the localization of carbohydrate structures in formalin-fixed, paraffin-embedded tissue sections of rat liver with

heterologous serum-induced fibrosis, but no differences in staining pattern of these lectins were seen between normal and fibrotic livers in bile canaliculi or sinusoidal endothelium [10, 11]. Morphological observation, however, implied changes in carbohydrate structures in bile canalicular and sinusoidal endothelial cells [10, 11]. We presumed that formalin fixation causes “epitope” masking and inhibits lectin binding. In the present study, we added a modified TT procedure for lectin staining to retrieve masked sugar structures in formalin-fixed, paraffin-embedded tissue sections and observed enhanced or altered staining patterns of 19 lectins.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats (initial body weight 135 ± 2 g, 5 weeks old; from SLC, Shizuoka, Japan) were divided randomly into two groups (five rats in each group), housed in individual cages and given water and a standard diet *ad libitum* in an air conditioned room ($22 \pm 1^\circ\text{C}$, RH 50 $\pm$ 5%) under controlled lighting conditions (12L/12D). All animals received humane care as outlined in the “Guide for the Care and Use of Laboratory Animals” (Kyoto University Animal Care Committee according to NIH #86-23; revised 1985). In the heterologous serum-sensitized group (fibrosis group), the rats received intraperitoneal injections of pig serum (3.5 ml/kg of body weight) prepared from fresh blood and sterilized by 0.45 μm -millipore filtration (Millipore Corp., Bedford, MA, U.S.A.) twice a week for 8 weeks [10–12]. In the nonsensitized vehicle control group (normal

* CORRESPONDENCE TO: MANABE, N., Unit of Anatomy and Cell Biology, Department of Animal Sciences, Kyoto University, Kyoto 606-8502, Japan.

group), the rats were injected with 0.9% NaCl in the same way.

Histological evaluation of liver fibrosis: Animals were sacrificed under ether anesthesia, and then the livers were removed, cut into small pieces, and fixed in 10% neutral buffered formalin. The liver specimens were dehydrated through a graded ethanol series and embedded in paraffin. Mirror and serial sections (3 μ m thickness) were cut and mounted on 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO, U.S.A.)-coated slides. After deparaffinization with xylene, they were rehydrated through a graded ethanol series. Some of the sections were stained with hematoxylin-eosin for standard histopathological evaluation and with sirius red (saturated picric acid in distilled water containing 0.1% (w/v) sirius red F3B (BDH Chemicals Ltd., Poole, U.K.), to allow visualization of fibrosis [9, 10]. Total collagen contents of liver sections were measured by a colorimetric method to assess the degree of liver fibrosis [10].

Modified thermo-treatment procedure for lectin staining: Serial deparaffinized sections were set in a stainless steel slide holder and placed in a large beaker filled with hot distilled water (100°C) for 10 min. To avoid bubbles adhering to the sections, hot water was gently stirred on a hot plate (Corning Inc., New York, NY, U.S.A.) during TT. After heating, the sections were rinsed in phosphate buffered

saline (PBS; pH 7.4) for 10 min at room temperature (RT; 20–25°C). Control sections without thermo-treatment (non-TT), were rinsed in PBS after deparaffinization.

Lectin histochemistry: After TT, serial sections were stained with 24 different lectins to identify the localization of specific carbohydrate chains as reported previously [10, 11]. Briefly, the sections were incubated with avidin and biotin blocking solution (Vector Laboratories, Burlingame, CA, U.S.A) at RT for 15 min to block endogenous avidin and biotin-like binding sites, and then washed with PBS. The sections were incubated with biotinylated lectins diluted with PBS (see Table 1) for 24 hr at 4°C, washed with PBS, and then incubated with ABC reagent (Vector) for 60 min at RT. Following washing with PBS, the sections were colorized with 0.002% H₂O₂, 0.1% 3,3'-diaminobenzidine-4HCl (DAB; Wako Pure Chemical Co., Osaka, Japan) in 0.1 M phosphate buffer (pH 7.4) at RT for 1 min, washed with distilled water, and then counterstained with Mayer's hematoxylin. Sections were dehydrated through a graded ethanol series, cleared in xylene, mounted in Entellan (Merck Co., Darmstadt, Germany), and then examined under a light microscope. Negative control sections were incubated without biotinylated lectins or ABC reagent. To confirm the specificity of lectin binding, respective biotinylated lectins were preincubated with the corresponding inhibiting sugars (0.002–0.2 M in PBS; E. Y.

Table 1. Carbohydrate binding specificities of lectins used in this study

Lectin	Source	Dilution ^{c)} (μ g/ml)	Binding specificity ^{d)}
Jacalina ^{a)}	<i>Artocarpus integrifolia</i>	4	α -Gal
LEL ^{a)}	<i>Lycopersicon esculentum</i>	2	β -GlcNAc, Poly LacNAc, GlcNAc oligomer
RCA-I ^{a)}	<i>Ricinus communis</i>	4	β -GalNAc, β -Gal, Gal β 1 GlcNAc on complex glycan
SSA ^{b)}	<i>Sambucus sieboldiana</i>	3	Sia α 2 6Gal/GalNAc
WGA ^{a)}	<i>Triticum vulgare</i>	3	β -GlcNAc, Poly LacNAc, GlcNAc oligomer, Sia
DSL ^{a)}	<i>Datura Stramonium</i>	2	β -GlcNAc, Poly LacNAc, Tri/tetra-antenna complex glycan
STL ^{a)}	<i>Solanum tuberosum</i>	4	β -GlcNAc, Poly LacNAc, GlcNAc oligomer
AAL ^{b)}	<i>Aleuria aurantia</i>	2	Fuc α 1 6GlcNAc
MAM ^{b)}	<i>Maackia amurensis</i>	3	Sia α 2 3Gal
PHA-E ^{a)}	<i>Phaseolus vulgaris</i>	2	Bisected complex glycan
PHA-L ^{a)}	<i>Phaseolus vulgaris</i>	2	Tri/tetra-antenna complex glycan
LCA ^{a)}	<i>Lens culinaris</i>	3	α -Man, Complex glycan with fucosylated core
PSA ^{a)}	<i>Pisum sativum</i>	3	α -Man, Complex glycan with fucosylated core
SJA ^{a)}	<i>Sophora japonica</i>	8	Gal β 1 3GalNAc, β -GalNAc
DBA ^{a)}	<i>Dolichos biflorus</i>	8	α -GalNAc, GalNAc α 1 3GalNAc (Gal)
ECL ^{a)}	<i>Erythrina cristagalli</i>	4	β -Gal> β -GalNAc
PNA ^{a)}	<i>Arachis hypogaea</i>	8	β -Gal, O-linked glycan, Gal β 1 3GalNAc
SBA ^{a)}	<i>Glycine max</i>	8	α -Gal, α -GalNAc, O-linked glycan, GalNAc α 1 3Gal
UEA-I ^{a)}	<i>Ulex europaeus</i>	8	α -Fuc, Fuc α 1 2Gal
BSL-II ^{a)}	<i>Bandeiraea simplicifolia</i>	6	α -, β -GlcNAc
Con A ^{a)}	<i>Canavalia ensiformis</i>	4	α -Man, Oligomannoside glycan, Bi-antenna complex glycan
BSL-I ^{a)}	<i>Bandeiraea simplicifolia</i>	6	α -GalNAc, α -Gal, Gal α 1 3Gal
VVA ^{a)}	<i>Vicia villosa</i>	6	β -GalNAc, O-linked glycan, GalNAc α 1 3Gal
s-WGA ^{a)}	<i>Triticum vulgare</i>	8	β -GlcNAc, Poly LacNAc, GlcNAc oligomer

a) Biotinylated lectins were purchased from Vector Laboratories (Burlingame, CA, U.S.A.).

b) Biotinylated lectins were purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

c) Biotinylated lectins were diluted with PBS.

d) Abbreviation: Fuc: fucose, GlcNAc: N-acetylglucosamine, Gal: D-galactose, LacNAc: N-acetylglucosamine, Man: mannose, GalNAc: N-acetylgalactosamine, Sia: Sialic acid.

Laboratories, San Mateo, CA, U.S.A.) for 1 hr at RT prior to incubation of the tissue sections [10]. In all cases of lectin staining, control tests were negative after omission of either biotinylated lectin or the ABC reagent, and preincubation of lectins with corresponding inhibiting sugars commonly abolished the staining reaction. For instance, preincubation of soybean agglutinin (SBA) with appropriate inhibitory sugar (N-acetyl-D-galactosamine) resulted in the disappearance of the staining reaction.

RESULTS

No histological damage was caused by the TT procedure in control or fibrotic liver sections. Preliminary experiments (data not shown) indicated that 10 min heat treatment was adequate for histopathological evaluation, because prolonged TT, especially for more than 30 min, caused histological damage. TT improved histochemical staining by the lectins used in this study, except for *Bandeiraea simplicifolia* lectin-II (BSL-II), concanavalin A (Con A) and succinylated wheat germ agglutinin (s-WGA). The characteristics of staining reactions of the 24 different lectins in normal and fibrotic liver sections with TT are summarized in Table 2.

Sinusoidal endothelium: Sinusoidal endothelial cells were stained with 13 lectins in non-TT sections from both normal

and fibrotic rat livers. TT enhanced staining intensity with these lectins. For instance, these cells were weakly stained with *Solanum tuberosum* lectin (STL) in the non-TT sections from both normal (Fig. 1a) and fibrotic livers. TT markedly enhanced the intensity of the STL staining in both normal (Fig. 1b, mirror section of 1a) and fibrotic tissue sections.

Bile canaliculi: Bile canaliculi were specifically stained with 7 lectins on non-TT sections in both normal (Fig. 1a) and fibrotic livers. TT enhanced the lectin staining intensity in both normal (Fig. 1b) and fibrotic liver sections. No differences were seen in localization of positive staining with these lectins between normal and fibrotic livers.

Hepatocyte cytoplasm: No significant changes of lectin staining in normal or fibrotic liver sections, except for SBA and *Bandeiraea simplicifolia* lectin-I (BSL-I), were seen between non-TT and TT. BSL-I exhibited positive staining of the cytoplasm of scattered hepatocytes (Fig. 2a). TT enhanced the intensity of BSL-I staining reaction (Fig. 2b, serial section of 2a). Interestingly, no positive reaction was detected with SBA in either control or fibrotic (Fig. 2c, serial section of 2a) livers without TT, but marked strong staining was seen in the fibrotic liver sections with TT (Fig. 2d, serial section of 2a). However, no such positive staining was seen in the normal liver sections with TT. These SBA-positive hepatocytes were scattered in the periportal and

Table 2. Characteristics of thermo-treatment in lectin staining of normal and fibrotic rat livers

	Sinusoidal endothelium		Bile canaliculi		Kupffer cells		Hepatocyte cytoplasm		Fibrotic septa
	NL ^{a)}	FL ^{b)}	NL	FL	NL	FL	NL	FL	FL
Jacalin	c)				—	—	—	—	—
LEL					—	—	—	—	—
RCA-I					—	—			—
SSa					—	—	—	—	—
WGA					—	—	—	—	—
DSL					—	—			—
STL					—	—	~	~	—
AAL			—	—	—	—	—	—	~
MAM			—	—	—	—	—	—	
PHA-E			—	—	—	—	—	—	~
PHA-L			—	—	—	—	—	—	
LCA			—	—	—	—	—	—	~
PSA			—	—	—	—	—	—	~
SJA	—	—	—	—	—	—	—	—	
DBA	—	—	—	—	+	+			
ECL	~	~	—	—	—	—			
PNA	—	—	—	—	+	+	—	—	
SBA	—	—	—	—	+	+	—	—(+)	
UEA-I	—	—	—	—	—	—	—	—	
BSL-II	—	—	—	—	—	—			—
Con A	—	—	—	—	—	—			—
BSL-I	—	—	—	—	—	+	~	()	—
VVA	—	—	—	—	—	—	—	—	—
s-WGA	—	—	—	—	—	—	—	—	—

a) NL: Normal liver. b) FL: Fibrotic liver. c) : Enhancement of staining intensity, : Decrease of staining intensity, : No changes, —: No reaction +: New positive staining, (): Staining reaction restricted to some scattered hepatocytes.

Fig. 1. Mirror sections of normal livers without TT (a) or with TT (b) stained with STL. Although weak reactivity with STL was observed in sinusoidal endothelium (arrows) and bile canaliculi (arrowheads) in non-TT sections, TT enhanced staining intensity in both sites. cv: central vein, and p: portal zone. a and b: $\times 115$.

Fig. 2. BSL-I and SBA histochemistry in fibrotic liver. Four serial sections were stained with BSL-I (a and b) and SBA (c and d). BSL-I showed positive staining in the cytoplasm of some scattered hepatocytes in sections without TT (a), TT clearly enhanced the BSL-I staining (b). No positive staining was seen with SBA in the sections without TT (c), but strong positive staining with SBA was demonstrated in sections with TT (d). p: portal zone. a, b, c and d: $\times 115$.

perifibrous zones and the central zones of pseudolobules. Moreover, in the fibrotic liver sections with TT, some hepatocytes showed positive reactions with both BSL-I (Fig. 3a) and SBA (Fig. 3b, mirror section of 3a). Mitotic nuclear morphology was observed in many of the positive hepatocytes (Fig. 3c, d).

Kupffer cells: In both normal and fibrotic (Fig. 4a) liver sections without TT, Kupffer cells reacted with *Vicia villosa* agglutinin (VVA). TT markedly enhanced the staining intensity of these cells (Fig. 4b, mirror section of 4a). On the other hand, no *Dolichos biflorus* agglutinin (DBA)-, peanut agglutinin (PNA)- or SBA-positive staining of

Fig. 3. Mirror fibrotic liver sections with TT (a and b: SBA and BSL-I, respectively). These sections indicated that SBA-positive hepatocytes coincided with those positive for BSL-I, and some proliferating hepatocytes (arrow) were stained with both lectins (c and d: SBA and BSL-I, respectively). a and b: $\times 230$. c and d: $\times 575$.

Kupffer cells was demonstrated in the non-TT sections of either normal (Fig. 4c) or fibrotic livers. When TT was applied to both normal (Fig. 4d, mirror section of 4c) and fibrotic liver sections, very strong staining of Kupffer cells was achieved with these lectins. These lectin-positive Kupffer cells were localized in the periportal zone of normal livers, but were scattered throughout the pseudolobular zone in the fibrotic livers.

Fibrotic septa: In fibrotic liver sections without TT, two distinct staining patterns were observed with 12 lectins. *Aleuria aurantia* lectin (AAL), *Lens culinaris* agglutinin (LCA), *Maackia amurensis* lectin (MAM), *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Phaseolus vulgaris* leucoagglutinin (PHA-L) and *Pisum sativum* agglutinin (PSA) reacted with all of fibrotic septa. DBA, PNA, SBA, *Erythrina cristagalli* lectin (ECL), *Saphora japonica* agglutinin (SJA) and *Ulex europaeus* agglutinin-I (UEA-I) reacted with only the centrilobular part of fibrotic septa. TT had no significant effect on the staining reactivities of AAL, PHA-E, LCA or PSA. Slightly diminished reactions of MAM, PHA-L, DBA, PNA, SBA, ECL, SJA and UEA-I were seen in the sections with TT.

DISCUSSION

Modified TT enhanced staining intensity or appearance of positive staining by many lectins used in this study in sections of both normal and fibrotic rat liver. Our findings were in agreement with the results of lectin staining in bovine brain tissue sections with TT using a microwave oven [7]. However, the microwave method often causes severe tissue damage, and determination of the suitable conditions for microwave TT is difficult. Our modified TT method is very simple and effective for lectin staining in liver specimens.

The staining intensities of most lectins, which recognize N-linked carbohydrate chains on sinusoidal endothelial and bile canalicular cells were markedly enhanced by TT, indicating that many lectin binding sites hidden by formalin fixation could be recovered by TT. The reaction of VVA, which recognizes O-linked carbohydrate chains, in Kupffer cells was also enhanced by TT. For instance, TT enhanced the staining intensity of VVA in Kupffer cells, suggesting that these cells had glycoconjugates with terminal GalNAc α 1-3Gal or GalNAc α 1-6Gal [23]. A part of such

Fig. 4. VVA and PNA histochemistry in mirror sections of normal or fibrotic liver. VVA showed positive staining in Kupffer cells in fibrotic sections without TT (a), TT clearly enhanced staining (b). Kupffer cells were not stained with PNA on normal sections without TT (c), but were strongly stained with PNA in those with TT (d). p: portal zone. a, b, c and d: $\times 230$.

VVA binding sugar structures is masked by cross-linkage of tissue proteins by formalin fixation, and TT removes these masking structures. On the other hand, Kupffer cells did not stain with DBA, PNA or SBA, which recognize O-linked carbohydrate chains, in non-TT sections of either normal or fibrotic livers. TT of the tissue sections resulted in the appearance of Kupffer cells positive for staining with these lectins. Some studies indicated that Kupffer cells did not react with DBA, PNA or SBA in formalin-fixed or Bouin's-fixed, paraffin-embedded sections of normal rat livers [24, 26]. In liver sections digested with neuraminidase, PNA and SBA bound to Kupffer cells, indicating that terminal sialic acid masked the binding sites of these lectins [24]. However, it is unlikely that TT resulted in the dissociation of terminal sialic acid, since sinusoidal endothelium and bile canaliculi showed enhanced staining with MAM and *Sambucus sieboldiana* agglutinin (SSA), which recognize terminal Sia α 2-3Gal and Sia α 2-6Gal, respectively [14, 18], after TT. TT is considered to loosen the cross-linkage between proteins caused by formalin fixation and to alter the three-dimensional structures of proteins and sugar chains [2, 15, 17, 21], allowing the lectins to approach hidden binding sites. It is difficult to detect these carbohydrate chains by conventional lectin histochemical techniques without TT.

Positive staining was also observed in the cytoplasm of some scattered hepatocytes located on the periportal and perifibrous zones, and central zone of pseudolobules in fibrotic liver sections following TT. Although the cytoplasm of the hepatocytes did not react with SBA in sections without TT, positive staining appeared in those with TT. TT breaks cross-linkage between glycoproteins in the tissue sections, and so SBA will easily bind to the carbohydrate sites. SBA shows relatively broad affinity to terminal GalNAc and Gal [5]. The BSL-I-positive reaction shown in hepatocyte cytoplasm was markedly enhanced by TT. The same hepatocytes were double positive for BSL-I and SBA, demonstrated on the mirror sections with TT. BSL-I reacts with terminal α Gal and α GalNAc [4, 6]. PNA and SJA, which bind to terminal Gal β 1-3GalNAc [8, 13, 25], did not react with hepatocyte cytoplasm in the fibrotic liver sections with TT. These results indicate that SBA and BSL-I double positive hepatocytes have cytoplasmic glycoproteins which possess carbohydrate chains with terminal α Gal and/or α GalNAc. O-Glycosylation occurs by the simple step-wise addition of sugars to a nascent peptide when it has moved to the smooth endoplasmic reticulum-Golgi apparatus region of the cells [1, 3, 20, 22]. The positive staining of hepatocyte cytoplasm may represent the O-glycosylation pathway of some glycoproteins.

Moreover, SBA and BSL-I also reacted with M-phase hepatocytes, or proliferating hepatocytes. It is considered that SBA- and BSL-I-binding glycoproteins may play an important role in proliferation of hepatocytes. Interestingly, all of the lectins with which positive staining appeared following TT preferentially recognize O-glycosidically linked carbohydrate chains. These results indicate not only that formalin fixation causes masking of lectin binding sites of O-linked carbohydrate chains but also that such obstructions are removed by TT.

In conclusion, TT may be a useful novel method for analysis of *in situ* carbohydrate chain structure. The TT procedure provides different findings from those of enzymatic treatment such as neuraminidase digestion. Modified TT staining procedures are considered to be useful tools for the diagnosis and prognosis of liver fibrosis.

ACKNOWLEDGEMENTS. This work was supported by a Grant-in-Aid to N. M. from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to Drs. K. Ogawa and K. Taniguchi (Iwate University, Morioka, Japan) for adequate advice on lectin histochemistry.

REFERENCES

1. Carraway, K. L. and Hull, S. R. 1991. Cell surface mucin-type glycoproteins and mucin-like domains. *Glycobiology* 1: 131–138.
2. Cattoretti, G., Pileri, S., Parravicini, C., Becker, M. H. G., Poggi, S., Bifulco, C., Key, G., D'Amato, L., Sabatini, E., Feudale, E., Reynolds, F., Gerdes, J. and Rilke, F. 1993. Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J. Pathol.* 171: 83–98.
3. Damjanov, I. 1987. Biology of disease: lectin cytochemistry and histochemistry. *Lab. Invest.* 57: 5–20.
4. Goldstein, I. J. and Hayes, C. E. 1978. The lectins: carbohydrate-binding proteins of plants and animals. *Adv. Carbohydr. Chem. Biochem.* 35: 127–340.
5. Hammarström, S., Murphy, L. A., Goldstein, I. J. and Etzler, M. E. 1977. Carbohydrate binding specificity of four *N*-acetyl-D-galactosamine-“specific” lectins: *Helix pomatia* A hemagglutinin, soy bean agglutinin, lima bean lectin, and *Dolichos biflorus* lectin. *Biochemistry* 16: 2750–2755.
6. Hayes, C. E. and Goldstein, I. J. 1974. An α -D-galactosyl-binding lectin from *Bandeiraea simplicifolia* seeds. *J. Biol. Chem.* 249: 1904–1914.
7. Hewicker-Trautwein, M., Schultheis, G. and Trautwein, G. 1995. Effects of trypsinization and microwave treatment on lectin labelling of microglial cells in paraffin-embedded sections from pre- and postnatal bovine brains. *Acta Histochem.* 97: 455–461.
8. Lotan, R., Skutelsky, E., Danon, D. and Sharon, N. 1975. The purification, composition, and specificity of the anti-T lectin from peanut (*Arachis hypogaea*). *J. Biol. Chem.* 250: 8518–8523.
9. Manabe, N., Chvallier, M., Chossegros, P., Causse, X., Guerret, S., Trépo, C. and Grimaud, J. A. 1993. Interferon- α_{2b} therapy reduces liver fibrosis in chronic non-A, non-B hepatitis: a quantitative histological evaluation. *Hepatology* 18: 1344–1349.
10. Miyamoto, M., Manabe, N., Kuramitsu, K., Kuribayashi, Y., Tamura, K., Furuya, Y., Nagano, N., Fukumoto, M. and Miyamoto, H. 1997. Lectin histochemistry in rat liver fibrosis induced by heterologous serum sensitization. *J. Vet. Med. Sci.* 59: 681–687.
11. Miyamoto, M., Manabe, N., Kuramitsu, K., Kuribayashi, Y., Tamura, K., Furuya, Y., Nagano, N., Fukumoto, M. and Miyamoto, H. 1998. Lectin histochemistry in fibrotic liver disease. pp. 483–490. *In: Reproductive Biology Update: Novel Tools for Assessment of Environmental Toxicity* (Miyamoto, H. and Manabe, N. eds.), Nakanishi Pub. Co., Kyoto, Japan.
12. Paronetto, F. and Popper, H. 1966. Chronic liver injury induced by immunologic reactions. *Am. J. Pathol.* 49: 1087–1101.
13. Pereira, M. E. A., Kabat, E. A., Lotan, R. and Sharon, N. 1976. Immunochemical studies on the specificity of the peanut (*Arachis hypogaea*) agglutinin. *Carbohydr. Res.* 51: 107–118.
14. Sata, T., Lackie, P. M., Taatjes, D. J., Peumans, W. and Roth, J. 1989. Detection of the Neu5Ac (α 2,3) Gal (β 1,4) GlcNAc sequence with the leucoagglutinin from *Maackia amurensis*: light and electron microscopic demonstration of differential tissue expression of terminal sialic acid in α 2,3- and α 2,6-linkage. *J. Histochem. Cytochem.* 37: 1577–1588.
15. Shi, S.-R., Coté, C., Kalra, K. L., Taylor, C. R. and Tandon, A. K. 1992. A technique for retrieving antigens in formalin-fixed, routinely acid-decalcified, celloidin-embedded human temporal bone sections for immunohistochemistry. *J. Histochem. Cytochem.* 40: 787–792.
16. Shi, S.-R., Cote, R. J. and Taylor, C. R. 1997. Antigen retrieval immunohistochemistry: past, present, and future. *J. Histochem. Cytochem.* 45: 327–343.
17. Shi, S.-R., Key, M. E. and Kalra, K. L. 1991. Antigen retrieval in formalin-fixed, paraffin embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 39: 741–748.
18. Shibuya, N., Tazaki, K., Song, Z. W., Tarr, G. E., Goldstein, I. J. and Peumans, W. J. 1989. A comparative study of bark lectins from three elderberry (*Sambucus*) species. *J. Biochem. Tokyo* 106: 1098–1103.
19. Spicer, S. S. and Schulte, B. A. 1992. Diversity of cell glycoconjugates shown histochemically: a perspective. *J. Histochem. Cytochem.* 40: 1–38.
20. Strous, G. J. A. M. 1979. Initial glycosylation of proteins with acetylgalactosaminyl serine linkages. *Proc. Natl. Acad. Sci. U. S. A.* 76: 2694–2698.
21. Suurmeijer, A. J. H. and Boom, M. E. 1993. Notes on the application of microwaves for antigen retrieval in paraffin and plastic tissue sections. *Eur. J. Morphol.* 31: 144–150.
22. Takahashi, K., Viviano, C. J., Elwell, M. R., Bakewell, W. E., Kuwahara, M., Nakashima, N., Blackwell, B.-N. and Maronpot, R. R. 1995. Bile duct-specific lectins, *Dolichos biflorus* agglutinin and peanut agglutinin, as probes in mouse hepatocarcinogenesis. *Lab. Invest.* 73: 424–432.
23. Tollefsen, S. E. and Kornfeld, R. 1983. The B4 lectin from *Vicia villosa* seeds interacts with *N*-acetylgalactosamine residues α -linked to serine or threonine residues in cell surface glycoproteins. *J. Biol. Chem.* 258: 5172–5176.
24. Witt, M. and Klessen, C. 1989. Lectin binding sites in normal and phenobarbital/halothane treated rat liver. *Histochemistry* 90: 391–397.

25. Wu, A. M., Kabat, E. A., Gruezo, F. G. and Poretz, R. D. 1981. Immunochemical studies on the reactivities and combining sites of the D-galactopyranose- and 2-acetamido-2-deoxy-D-galactopyranose-specific lectin purified from *Sophora japonica* seeds. *Arch. Biochem. Biophys.* 209: 191–203.
26. Yamamoto, K., Makino, Y., Yoshioka, T., Kobashi, H., Tomita, M. and Tsuji, T. 1992. Quantitative analysis of activated Kupffer cells in viral hepatitis: application of computer image analysis for lectin histochemistry. *Liver* 12: 199–204.