# Quantitation and histochemical localization of galectin-1 and galectin-1-reactive glycoconjugates in fetal development of bovine organs

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**Summary.** The display of cellular oligosaccharide chains is known to undergo marked developmental changes, as monitored histochemically with plant lectins. In conjunction with endogenous lectins respective ligand structures may have a functional role during fetal development. The assumption of a recognitive, functionally productive interplay prompts the study of the expression of a tissue lectin and of lectin-reactive glycoconjugates concomitantly. Focusing on common ß-galactosides as constituents of oligosaccharide chains and the predominant member of the family of galectins in mammals, namely galectin-1, the question therefore is addressed as to whether expression of lectin and lectin-reactive glycoconjugates exhibits alterations, assessed in three morphologically defined fetal stages and in adult bovine organs. Using a sandwich ELISA, the level of the rather ubiquitous galectin-1 is mostly increased in adult organs relative to respective fetal stages, except for the case of kidney. This developmental course is seen rather seldom, when the amounts of lectin-reactive glyco-proteins or glycolipids are quantitated in solid-phase assays after tissue homogenization. Western blotting, combined with probing by labeled galectin-1, discloses primarily quantitative changes in the reactivity of individual glycoproteins. Performing the same assays on extract aliquots with a plant agglutinin, namely the galactoside-binding mistletoe lectin, whose fine specificity is different from galectin-1, its reduced extent of binding in solid-phase assays and the disparate profile of lectin-reactive glycoproteins reveal a non-uniform developmental alteration within the group of structural variants of ß-galactosides. Although sample preparation can affect ligand preservation and/or presentation and thus restricts the comparability of biochemical and histochemical results, especially for soluble reactants, the histochemical studies on frozen and paraffin-embedded sections of bovine heart, kidney and liver

demonstrate that the localization of the galectin and of lectin-reactive epitopes can show a similar distribution, as seen in liver and heart, with organ-typical quantitative changes of a rather similar staining profile (heart, kidney) or notable changes in the spatial distribution (liver) in the course of development. This report emphasizes the potential value of combined monitoring of the lectin and its potential *in vivo* ligands to contribute to eventually unravel organ-related function(s) of a tissue lectin.

**Key words:** Lectin, Galectin, Glycoproteins, Glyco-lipids, Development

## Introduction

Protein and lipid glycosylation is not a temporarily and spatially uniform aspect of mammalian cells. In contrast, it is widely documented to undergo tissue type- and stage-dependent alterations, which are tempting indications for a presumably functional role (Mann, 1988; Muramatsu, 1988; Bourrilon and Aubery, 1989; Varki and Marth, 1995). The physiological requirement for appropriate glycostructures is underscored by the occurrence of serious defects in correct glycosylation caused by genetic disturbances and manifested in clinically apparent aberrations such as I-cell disease or leucocyte adhesion deficiency syndrome type II (Varki and Marth, 1995). Deliberately engineered loss-of-function mutants in this respect reveal the necessity of presence of complex type N-linked oligosaccharides for early murine developmental processes (Hathaway and Shur, 1997). Since plant lectins have proven expedient as histochemical tools of exogenous origin to monitor the changes in oligosaccharide display (Spicer and Schulte, 1992; Danguy et al., 1994, 1997; Danguy, 1995), it is reasonable to assume that the recognitive interplay of such oligosaccharide sequences with endogenous lectins can partake in mediation of the intricate processes that govern the intriguing accuracy of cell and tissue

development and differentiation. Due to fine specificity differences of plant and mammalian lectins, supportive evidence for this hypothesis can be gathered by the introduction of isolated tissue lectins to the histochemical monitoring of animal specimens (Gabius et al., 1993). Consequently, this issue is one topic of this report.

In the last decade, the description and characterization of animal lectins has made remarkable progress, enabling the definition of distinct categories with insights into the architecture of the carbohydrate-recognition domains and evolutionary pathways in order to procure the known intra-family diversity (Drickamer and Taylor, 1993; Gabius et al., 1995; Powell and Varki, 1995; Gabius, 1997; Zanetta, 1997). Besides C-type, I-type and P-type lectins the galectins form a family of animal agglutinins with common structural features which explain their shared selectivity to ß-galactosides at oligosaccharide chain termini and in internal poly-N-acetyllactosamine repeats (Caron et al., 1990; Harrison, 1991; Barondes et al., 1994; Kasai and Hirabayashi, 1996; Ohannesian and Lotan, 1997). A rather ubiquitous member of this family is galectin-1. With respect to mammals, it was first isolated from calf heart and lung (De Waard et al., 1986). In these tissues, it is the predominant galectin, conveniently leading to highly purified preparations. Moreover, the nearly exclusive restriction of galectin expression in these organs to this type has ignited the notion of an important, albeit still elusive role of this protein via interaction with abundant carrier-attached ß-galactosides. The ease of accessibility has paved the way to the thorough analysis of its carbohydrate specificity and its crystallographic structure. Besides type I and type II GalGlcNAc-core elements the binding site of bovine galectin-1 tolerates extensions to B/H blood group-like epitopes and the $\alpha$2,3-sialylation (Abbott et al., 1988; Solomon et al., 1991). The two carbohydrate-binding sites of the non-covalently linked dimer are located at the far ends of the jelly roll-like motif established by the ß-sheet topology (Bourne et al., 1994; Liao et al., 1994). The overall architecture predisposes the protein for potent cross-linking capacity, which in model mixtures leads to homogeneous complexes (Gupta and Brewer, 1994). Since carbohydrate recognition requires no marked deviation of the ligand's conformation from a low energy configuration, as shown by co-crystallization of bovine galectin-1 and NMR analysis in solution for a related galectin (Bourne et al., 1994; Siebert et al., 1996), the principal selection of a suitable, weakly populated subset from the abundant array of ß-galactosides appears rather unlikely. Compared to the elaborate status of these structural studies, our knowledge on the function of galectin-1 in conjunction with its *in vivo* ligands is less advanced.

Remarkably, galectin-1 expression is developmentally regulated in mice and rabbits, following organ-typical patterns, as inferred by hemagglutination assays (Levi and Teichberg, 1984; Catt et al., 1987). To exclude

any inhibitory influences of lectin-reactive glyco-conjugates in an activity assay, RNA blot analysis or immunological methods are preferable to determine lectin expression (Catt et al., 1987; Poirier et al., 1992). This reasoning explains the selection of a sandwich ELISA assay to determine the amount of galectin-1 in three morphologically defined fetal stages of bovine development relative to the adult organs. However, our comparative study is deliberately not confined to the aspect of modulation of galectin expression. Since no information is available on the level of presence of galectin-1-reactive glycoconjugates in development, we concomitantly employ labeled galectin-1 for *in vitro* quantitation of sugar-inhibitable binding sites, classified as glycoproteins and glycolipids. Relative to the use of thin-layer chromatography the microtiter plate assay has been described to be preferable in specificity determination (Solomon et al., 1991). This conclusion accounts for the selection of this procedure. To distinguish between a global increase of the presence of ß-galactoside-containing structures and non-uniform alterations within this group of structural variants, a plant agglutinin with slightly different target properties such as preferential affinity to $\alpha$2,6-linked sialyllactose, a reduced discrimination for the subterminal sugar unit and no notable activity for internal disaccharides was tested in parallel assays (Lee et al., 1992; Galanina et al., 1997). Three types of organ were then further analyzed to figure out whether lectin reactivity changes quantitatively or qualitatively at the level of glyco-proteins. The necessity to homogenize tissues inevitably compels one to restrain from devising a correlation between the presence of a receptor and its ligand(s) and their localization *in situ*. Although the processing of the tissue specimens and the accessibility of epitopes in the sections introduce variables into the histochemical analysis which must be reckoned with in comparison with results obtained with inherently different techniques, careful selection of conditions for optimal signal-to-background ratio and concomitant analysis of frozen and paraffin-embedded sections are supposed to constitute an adequate basis to supplement the biochemical data by histochemical results. In addition to answering the technical point of comparability these experiments disclose how the expression of galectin-1 and lectin-reactive glycoconjugates is apparently regulated spatially in the defined fetal stages and the adult organs.

## Materials and methods

### Lectin isolation, antibody preparation and probe labeling

The galactoside-specific lectin from mistletoe (*Viscum album* L. agglutinin, VAA) was purified from extracts of dried leaves by affinity chromatography, using Sepharose 4B (Pharmacia, Freiburg, FRG) to which lactose had been coupled after activation with divinyl sulfone, as described by Gabius (1990). Isolation

of galectin-1 from bovine heart was performed, as described in detail elsewhere (Bardosi et al., 1989; Gabius, 1990), and the lectin was stabilized by carboxy-amidomethylation with 50mM iodoacetamide during elution with 0.3M lactose from the affinity resin. Polyclonal antibodies against the lectin were raised in rabbits, as outlined previously (Gabius et al., 1991). The immunoglobulin G fraction from serum was obtained by application of protein A-Sepharose 4B (Pharmacia, Freiburg, FRG) and affinity-purified antibodies resulted from fractionation on resin-immobilized galectin-1, coupled to divinyl sulfone-activated Sepharose 4B at a density of 1 mg/ml, as given in detail elsewhere (Bardosi et al., 1989). Specificity controls using immunoblotting were also performed, as given previously (Bardosi et al., 1989). Labeling of the lectins was achieved under activity-preserving conditions with biotinyl-N-hydroxy-succinimide ester (Sigma, Munich, FRG), as already described for both agglutinins (Gabius et al., 1991, 1992). To exclude a disruption of dimer formation by this chemical modification, control runs on a Superose-12 column (Pharmacia, Freiburg, FRG) were routinely carried out, using standard markers for molecular weight calibration. Introduction of a label into the galectin-1-specific antibody was facilitated by conjugate formation with periodate-treated horseradish peroxidase, using a common protocol (Nakane and Kawaoi, 1974).

*Quantitation of lectin and lectin-binding glycoproteins in tissue extracts*

Specimens of adult bovine tissues and of three stages of fetal development, grouped on the basis of crown-rump length into distinct age categories, namely F1 = 7-12 cm (week 7-11), F2 = 13-23 cm (week 12-16), and F3 = 24-38 cm (week 17-21), according to Rüsse (1991), were freshly obtained in a local slaughterhouse. Tissue extracts were prepared in a detergent-free medium and subsequently in a 1% Triton X-100-, and 0.1% sodium deoxycholate-containing buffer, as described by Gabius et al. (1991) and Dong et al. (1995). For ELISA measurements 0.1M lactose was added to the extraction buffers. To comparatively assess the quantity of lectin in extracts, a sandwich ELISA assay in microtiter plates (Greiner, Nürtingen, FRG) was performed. In the first step, affinity-purified galectin-1-specific antibody was adsorbed to the surface of the wells from a solution of 100 µl of 0.1M $Na_2CO_3$ (pH 9.6) containing 2 µg immunoglobulin/ml over a period of 12 h at 4 °C. Following blocking of residual protein-binding sites with 20mM phosphate-buffered saline (pH 7.2) containing 0.5% carbohydrate-free bovine serum albumin (blocking solution) for 1 h at 37 °C 100 µl of total tissue extracts (200 µg; diluted in blocking solution containing 0.05% Tween-20 and obtained from various organs of at least four different animals at the same developmental stage after combining salt- and detergent-extractable fractions) were incubated in the antibody-exposing wells for 2 h at 37 °C. Unbound protein was removed by thorough

washing steps with 200 µl of phosphate-buffered saline containing 0.05% Tween-20. The amount of bound galectin-1 was determined with subsequent incubation steps using 100 µl of anti-galectin-1-specific antibody/peroxidase conjugate (5 µg/ml of blocking solution with 0.05% Tween-20) and o-phenylenediamine/$H_2O_2$ as indicator substrates for signal development. The actual quantity of galectin-1 was determined in the linear range of a calibration curve, the detection limit being approximately 2.5 ng/ml.

For determination of the amount of lectin-reactive glycoproteins a constant quantity of salt and detergent extract proteins (2.5 µg), obtained as described for human nerves and bovine testicular tissue (Gabius et al., 1991; Dong et al., 1995), was incubated for 12 h at 37 °C to mediate adsorption to the plastic surface of the ELISA plate wells. Following blocking, washing, incubation with 100 µl of 20mM phosphate-buffered saline containing 0.5% carbohydrate-free bovine serum albumin (blocking solution) and the biotinylated lectin (2 µg galectin-1 or 1 µg VAA per ml, yielding 1 OD per assay with asialofetuin as model ligand) and further washing steps to remove any unbound labeled probe the extent of lectin binding was assessed by successively applying 100 µl of blocking solution with 0.5 mg streptavidin/peroxidase-conjugate/ml for 1 h at 37 °C and 100 µl of 50 mM $Na_2HPO_4$/20mM citric acid at pH 5.0 with o-phenylenediamine (1 mg/ml)/$H_2O_2$ (0.004%; v/v) as color-generating substrates, then by stopping the enzymatic reaction by addition of 100 µl of 0.1M $H_2SO_4$ and by measuring the optical density (OD) of each sample in a plate reader. Total binding was reduced by the extent of binding in the presence of a mixture of 0.2M lactose and 0.1 mg/ml asialofetuin, yielding the level of carbohydrate-inhibitable binding. Using a standard curve with asialofetuin as model glycoprotein ligand, the OD-values of the assays with extract proteins are referred to as amount of asialofetuin in ng, which is required to generate the same signal intensity.

*Analysis of lectin and lectin-reactive glycoproteins on blots*

Extract (glyco)proteins (20 µg per lane for immuno-logical detection and 5 µg per lane for detection of lectin ligand(s)) were electrophoretically separated on a 15% polyacrylamide gel under denaturing and reductive conditions (immunological procedure) or on a 12% polyacrylamide gel (lectinological procedure) and transferred to nitrocellulose by tank blotting (Schleicher and Schuell, Dassel, FRG; 0.2 µm), where further probing with affinity-purified anti-galectin-1-specific antibody (1µg/ml of 20mM phosphate-buffered saline (pH 7.2) containing 0.5% carbohydrate-free bovine serum albumin and 0.1% Tween-20) or biotinylated lectins (10 µg/ml of the same solution) in conjunction with either horseradish peroxidase/goat anti-rabbit immunoglobulin G-conjugate or streptavidin/alkaline phosphatase-conjugate (0.2 µg/ml) was performed, as

described by Gabius et al. (1986, 1991). Carbohydrate-dependent binding of the lectins was inhibited by the presence of 0.3M lactose and 0.25 mg/ml asialofetuin. Control experiments included omission of the incubation step with the marker to visualize staining, for which indicator reagents (second antibody, kit components), but not the probes are responsible.

## Quantitation of lectin-binding neutral glycolipids and gangliosides in tissue extracts

Each tissue sample (1 g wet weight) was homogenized in 4 ml chloroform/methanol (2:1; v/v) with an Ultra-Turrax (Janke & Kunkel, Staufen, FRG). Following centrifugation at 5,000 rpm for 15 min at room temperature extraction of the insoluble pellet and centrifugation were repeated and the two supernatants were combined. This fraction was added to a mixture with 22 ml of chloroform/methanol (2:1; v/v) and 8 ml of aqueous 0.1M NaCl solution in a separation funnel. The resulting solution was shaken vigorously and the two phases were separated. After reextraction of the organic phase with 8 ml of the 0.1M NaCl solution the water from the combined aqueous phases was cautiously evaporated at a temperature below 40 °C and the solid substance was resuspended in 5 ml methanol, referred to as ganglioside fraction (Ledeen and Yu, 1982; Zimmer et al., 1992). The organic phase was dried with $Na_2SO_4$, and washed with chloroform/methanol after filtration, and gentle evaporation yielded a solid substance, which was also resuspended in 5 ml methanol, referred to as the neutral glycolipid fraction (Ledeen and Yu, 1982; Zimmer et al., 1992). 100 µl aliquots of methanol with either neutral glycolipid or ganglioside were kept for adsorption of the lipid part of the glycoconjugate to the plate well surface (Greiner, Nürtingen, FRG). Each well was washed with 200 µl of 20mM phosphate-buffered saline (pH 7.2) and residual binding sites were blocked during 2 h at 37 °C with 200 µl of this buffer containing 2% carbohydrate-free bovine serum albumin. Following thorough washes 100 µl aliquots of lectin-containing 20mM phosphate-buffered saline with 0.05% Tween-20 (10 µg biotinylated galectin-1/ml, 1 µg biotinylated VAA/ml) were kept for 2 h at 37 °C in the wells. Signal development and assessment were performed, as already described with respect to the assays for lectin-reactive glycoproteins. Each series of measurements with triplicates was accompanied by control incubations in the presence of 0.3M lactose and 0.2 mg/ml asialofetuin to determine the extent of sugar-inhibitable binding. Similar to the aforementioned enzyme-linked lectin-binding assay (ELLA) background values in the absence of ligand during the coating step or in the absence of biotinylated lectin in the respective incubation step were also taken into account to exclude any influence of non-specific binding reactions. For comparison between the individual samples the OD-values for carbohydrate-mediated binding were normalized per 20 µg wet weight of each tissue sample.

## Visualization of lectin and lectin-reactive binding sites in tissue sections

Fresh tissue specimens from at least three individual animals for each developmental stage were either rapidly frozen in isopentane at -80 °C or fixed in different systems, i. e. methanol with 30% acetic acid, 4% buffered paraformaldehyde or in Bouin's solution. These samples were dehydrated by routine passage through graded ethanol solutions, cleared in xylene and embedded in paraffin. Serial sections (5 µm thick) of fixed tissues were mounted on glass slides that had been coated with a solution of 0.5% gelatin and 0.05% potassium chromium sulfate. After deparaffination endogenous peroxidase activity was impaired by incubation for 30 min with methanolic $H_2O_2$ solution and non-specific protein-binding sites were blocked with different reagents of optimal background-reducing performance for each probe, i. e. 3% normal goat serum (Camon, Wiesbaden, FRG) for immunohistochemistry, 2% carbohydrate-free bovine serum albumin for the plant lectin and a solution with 0.1% carbohydrate-free albumin and 0.5% Triton X-100 for galectin-1. Any binding of the biotinylated probes to endogenous biotin-specific sites or of the kit reagent avidin to endogenous biotin was excluded by application of the commercial blocking kit according to the instructions of the commercial supplier (Camon, Wiesbaden, FRG). The processed sections were then incubated overnight at 4 °C with 10mM phosphate-buffered saline containing either 5 µg anti-galectin-1-specific antibody/ml, 5 µg biotinylated galectin-1/ml or 1 µg biotinylated VAA/ml. As indicator reagent for the primary antibody biotinylated goat anti-rabbit immunoglobulin G (Camon, Wiesbaden, FRG) was used at a concentration of 7.5 µg/ml for 1 h at room temperature. Further steps for signal development followed common protocols (Bardosi et al., 1989; Gabius et al., 1991). The cryostat sections (10 µm thick), prepared from the frozen specimens, were thawed on the gelatin-coated coverslips and fixed for 5 min with 2% buffered paraformaldehyde at 4 °C. Further processing starting with the treatment with phosphate-buffered saline/0.3% $H_2O_2$ followed the series of steps given above. Control reactions included preabsorption of the antibody with purified galectin-1, using coincubation of 40 µg lectin with 5 µg immunoglobulin G for 1 h at room temperature and centrifugation prior to application of aliquots to test sections, preincubation of the labeled lectins with 0.1M lactose or a mixture of 0.2M lactose and 0.65 mg/ml asialofetuin for 1 h at room temperature prior to the application of the lectin-containing solution to test sections and omission of the incubation step with primary antibody or the biotinylated lectin to verify the antigen- or carbohydrate-dependent binding and the absence of a contribution to staining by carbohydrate-independent cell binding of kit reagents or the biotin label.

## Statistical analysis

For statistical evaluation the commercial PC-program (GraphPad Software, San Diego, USA) was used, namely the one-way analysis of variance, Bonferroni's multiple comparison test for calculation of significance of sets of values in comparison between different developmental stages and Student's t-test for calculation of significance in inter-lectin comparison.

## Results

Affinity chromatography with immobilized lactose yielded galectin-1 with no indication of the presence of further galectin-like ß-galactoside-binding proteins upon gel electrophoretic analysis from adult heart, liver and colon. Using galectin-1 as antigen, polyclonal antibodies were raised in rabbits which showed no cross-reactivity to other extract proteins, as exemplarily documented with an immunoblot in the case of different developmental stages of bovine heart (Fig. 1). This antibody fraction was crucial to establish a sandwich ELISA assay with a limit of sensitivity at approximately 2.5 ng lectin/ml and a recovery ratio of lectin, which had been added in known quantities to extracts, of above 85%. Parallel control experiments in solid-phase assays ascertained that ligand-bound lectin could still be detected in this assay to ensure determination of the total lectin quantity. 0.1M lactose was added during extraction to prevent association of galectin-1 with insoluble material. Control experiments with extracts from adult bovine heart ascertained that galectin-1 was not detectable in this fraction after treatment with sodium dodecyl sulfate.

Having validated the conditions of the assay, its application revealed a non-uniform pattern of regulation. Expressed as ng lectin/mg protein, no major alteration of lectin content in developing and adult kidney was observed relative to the significantly increased lectin concentrations in adult heart and liver (Table 1). When the amount of detectable lectin of adult heart was compared to the actual yield after affinity chromato-graphical fractionation, 45-60% of the theoretically attainable quantity of galectin-1 was purified. Further obviously rich sources of galectin-1 were adult lung and spleen. By monitoring the lectin content of several other organs, the level in adult organs was found to be generally higher than at fetal stages of development, a gradual increase or no apparent change being measurable between tissue specimens from week 7-11 (F1) and week 17-21 (F3) (Table 2). These data thus underscore the occurrence of a relative increase in the
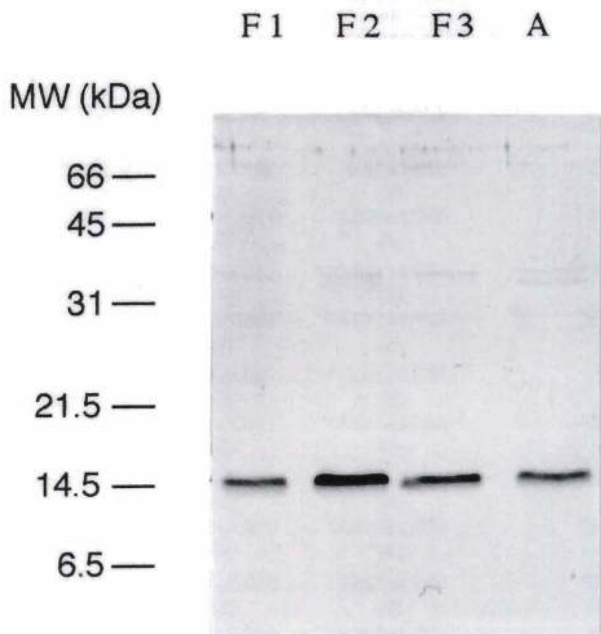


**Fig. 1.** Immunoblotting analysis of extract proteins (20 μg per lane) from fetal bovine heart at the three developmental stages (F1-F3) and the adult organ (A). A polyclonal immunoglobulin G preparation against galectin-1 is used for probing.

**Table 1.** Determination of the galectin-1 content (ng/mg protein) in extracts of fetal and adult bovine heart, kidney and liver tissue

| TISSUE TYPE | DEVELOPMENTAL STAGE | | | |
|---|---|---|---|---|
| | F1 | F2 | F3 | A |
| Heart | 133.1±27.7*** (4) | 160.4±22.21*** (5) | 183.4±26.4*** (5) | 447.9±76.9 (6) |
| Kidney | 125.4±30.6 (5) | 134.7±12.7 (5) | 119.0±21.6 (4) | 147.1±55.8 (5) |
| Liver | 121.7±23.9*** (4) | 162.5±47.0*** (5) | 162.4±80.3*** (5) | 404.0±81.8 (6) |

***; $p \geq 0.001$ significantly different from adult; numbers in brackets denote the number of individual animals for each developmental stage.

**Table 2.** Determination of the galectin-1 content (ng/mg protein) in extracts of fetal and adult bovine tissues.

| TISSUE TYPE | DEVELOPMENTAL STAGE | | |
|---|---|---|---|
| | F1 | F3 | A |
| Lung | 195.3±83.8*** (5) | 190.4±65.7*** (5) | 526.0±113.0 (5) |
| Spleen | 225.1±98.4** (5) | 219.3±75.0** (5) | 539.2±149.2 (5) |
| Colon | 113.5±28.5*** (5) | 135.3±51.9*** (5) | 393.4±34.2 (5) |
| Intestine | 124.5±28.1** (5) | 152.7±45.7*** (4) | 296.5±32.7 (4) |
| Pancreas | 113.6±28.0** (4) | 162.0±17.2** (6) | 317.3±118.9 (6) |
| Thymus | 32.4±12.8*** (5) | 50.9±13.9*** (5) | 194.0±23.2 (5) |

**: $0.01 \geq p > 0.001$; ***: $p \geq 0.001$ significantly different from adult; number in brackets denotes the number of individual animals for each developmental stage.

concentration of galectin-1 in adult organs.

To assess the quantity of lectin-binding glycoproteins, a solid-phase assay was established and calibrated with asialofetuin as model ligand of known high affinity, bearing three triantennary N-linked oligosaccharide chains predominantly with a terminal N-acetyllactosamine unit and three O-linked Galß1,3 GalNAcα-R structures. Labeled galectin-1 maintained

**Table 3.** Determination of the content of lectin-binding glycoproteins in salt and detergent extracts of fetal and adult bovine heart, kidney and liver, expressed as ng model ligand (asialofetuin)/mg protein.

| TISSUE TYPE | EXTRACT TYPE | GALECTIN-1 Developmental stage | | | | Viscum album AGGLUTININ Developmental stage | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | F1 | F2 | F3 | A | F1 | F2 | F3 | A |
| Heart | S | 820.5±331.5*,b (5) | 864.5±313.9*,c (6) | 920.2±139.4*,c (6) | 462.5±97.4 | 205.4±61.0* (5) | 231.2±39.5 (5) | 425.2±45.1 (6) | 420.0±165.3 (4) |
| | D | 984.0±224.0c (5) | 637.1±239.0a (5) | 642.0±253.5 (5) | 752.5±175.5a (5) | 458.2±76.5 (6) | 345.1±115.6 (6) | 350.1±75.3 (4) | 435.0±85.5 (4) |
| Kidney | S | 639.2±189.1a (6) | 787.1±186.4*,c (5) | 768.2±131.0*,c (5) | 392.5±142.3 (5) | 405.7±49.5 (5) | 333.3±126.3 (6) | 297.5±21.9 (6) | 363.7±82.3 (4) |
| | D | 782.5±271.3 (6) | 837.5±280.5b (5) | 612.5±115.3*,b (4) | 1240.0±60.5 (4) | 502.2±93.1** (5) | 355.0±74.2** (6) | 335.1±56.9** (4) | 932.1±210.2 (4) |
| Liver | S | 1161.0±330.5b (8) | 750.5±90.8c (5) | 691.2±242.0* (8) | 1203.6±265.3c (8) | 441.3±80.5* (6) | 429.3±80.3* (5) | 448.3±113.0* (5) | 247.8±54.0 (5) |
| | D | 941.9±147.4c (5) | 849.3±253.3b (6) | 811.0±171.2b (4) | 596.0±239.3a (5) | 623.0±99.5** (7) | 362.5±129.5 (5) | 315.2±116.7 (4) | 330.0±65.8 (5) |

S: salt extract; D: detergent extract; *: $0.05 \geq p > 0.01$; **: $0.01 \geq p > 0.001$; ***: $p < 0.001$: significant difference of result for fetal stages relative to that of the adult organ; a: $0.05 \geq p > 0.01$; b: $0.01 \geq p > 0.001$, c: $p \leq 0.001$: significant difference of result for galectin-1 relative to that for the plant agglutinin. Number in brackets denotes the number of individual animals for each developmental stage.

**Table 4.** Determination of the content of lectin-binding glycoproteins in salt and detergent extracts of fetal and adult bovine tissues, expressed as ng model ligand (asialofetuin)/mg protein.

| TISSUE TYPE | EXTRACT TYPE | GALECTIN-1 Developmental stage | | | Viscum album AGGLUTININ Developmental stage | | |
|---|---|---|---|---|---|---|---|
| | | F1 | F3 | A | F1 | F3 | A |
| Lung | S | 681.2±229.0*,b (7) | 484.0±68.1* (4) | 1151.2±449.5 (6) | 293.1±82.2*** (7) | 462.0±76.3** (5) | 762.5±175.6 (4) |
| | D | 1157.3±273.4 (8) | 715.0±230.1*** (8) | 1162.1±133.5 (8) | 615.4±103.2*** (7) | 542.2±73.2*** (4) | 1487.5±296.1 (4) |
| Spleen | S | 775.0±271.3*,a (6) | 405.5±30.1b (6) | 530.5±51.2a (5) | 520.0±28.3* (6) | 332.0±112.1 (6) | 383.8±119.5 (5) |
| | D | 671.2±124.6 (6) | 775.2±165.3* (6) | 506.2±208.7 (5) | 682.0±205.8 (6) | 718.2±332.5 (6) | 707.5±236.6 (5) |
| Colon | S | 698.0±92.6*** (6) | 482.5±34.0 (4) | 375.5±103.7b (6) | 574.1±124.6*** (5) | 552.5±89.6*** (4) | 136.7±37.8 (4) |
| | D | 668.0±62.2***,c (6) | 1080.0±130.0*,b (6) | 1510.0±562.5b (6) | 1077.5±113.2*** (4) | 706.7±90.7*** (4) | 283.4±21.5 (4) |
| Intestine | S | 713.3±131.4 (6) | 406.0±69.5 (5) | 442.2±80.3 (6) | 763.7±163.1** (6) | 790.1±200.5** (5) | 260.0±45.8 (4) |
| | D | 635.0±90.5*** (6) | 822.5±282.5*** (5) | 1734.5±230.3c (5) | 805.2±117.5** (6) | 756.1±187.9** (5) | 428.0±55.5 (5) |
| Pancreas | S | 765.3±35.4**,b (5) | 503.3±143.6a (5) | 306.7±204.2 (5) | 485.0±148.5** (5) | 236.7±40.4 (4) | 240.0±66.3 (5) |
| | D | 1050.5±49.8b (5) | 828.3±317.5b (5) | 839.5±395.4b (5) | 450.5±215.7* (4) | 230.0±39.2 (4) | 179.3±30.7 (5) |
| Thymus | S | 872.5±241.3*,a (6) | 504.0±87.1b (7) | 575.5±138.6b (5) | 531.5±128.2 (6) | 332.5±45.7 (5) | 218.8±28.5 (4) |
| | D | 778.3±178.5 (6) | 705.8±311.3 (7) | 436.3±133.3 (5) | 695.4±435.4*** (7) | 491.2±188.1 (4) | 285.0±37.0 (4) |

S: salt extract; D: detergent extract; *: $0.05 \geq p > 0.01$; **: $0.01 \geq p > 0.001$; ***: $p \leq 0.001$: significant difference of result for fetal stages relative to that of the adult organ; a: $0.05 \geq p > 0.01$; b: $0.01 \geq p > 0.001$; c: $p \leq 0.001$: significant difference of result for galectin-1 relative to that for the plant agglutinin. Number in brackets denotes the number of individual animals for each developmental stage.

its ligand-binding capacity and could thus be employed as a tool to determine the extent of accessible glycoprotein ligands *in vitro*. The presence of 0.1M lactose during tissue homogenization or treatment of extracts with denaturing agents did not markedly increase this parameter for heart, liver, kidney and lung extracts, as noted for the yield of lectin from several rabbit organs (Harrison et al., 1984). Lectin depletion of extracts by immunoaffinity chromatography with resin-immobilized antibodies likewise did not affect the response in the assay for heart extract, excluding a marked masking effect in this case. Since lectin-reactive determinants were measured with focus on protein-carbohydrate interactions, the calculation was exclusively based on the extent of sugar-inhibitable lectin binding in the assay. Under these conditions, the relation of lectin-reactive glycoproteins and lectin expression in ng lectin or model ligand per mg protein ranged from approximately 1-10. It was obvious that the increase in lectin content of heart and liver was not associated with a similar alteration in the level of lectin-reactive glycoepitopes, whereas a correlation of these two parameters was detectable in kidney (Table 3). Quantitative display of the respective ß-galactosides was in the same order of magnitude, with variations between fractions, for other organs tested. An indication for a phenomenological co-regulation was evident for lung,

colon and intestine (Table 4).

ß-Galactosides are a common terminal part of oligosaccharide chains of glycoproteins and constitute a group of isomeric structures. Since galactose-binding lectins can exhibit differences in selectivity, application of a plant lectin with similar, not identical ligand-binding properties is helpful to infer the degree of variability of expression within this family. Despite an inevitable overlap this comparison can indicate whether distinct alterations can occur in the presentation of ß-galactosides with no or minor reactivity to galectin-1. With few exceptions the monitoring of the extent of plant agglutinin-reactive glycoproteins revealed preferential or quantitatively similar binding of the endogenous lectin to the extract preparations under these experimental conditions (Tables 3, 4). Especially the elevated amount of this fraction in adult stages can indicate an adaptation between galactoside-containing glycoprotein expression and the specificity of the endogenous receptor relative to a plant agglutinin with nominally identical monosaccharide specificity.

The solid-phase assay enables one to obtain quantitative data on glycoprotein presentation with respect to a distinct lectin. Since several possibilities can theoretically explain the results on the level of individual molecules, the nature of lectin-reactive glycoprotein(s) was assessed by lectin blotting. Visualization of
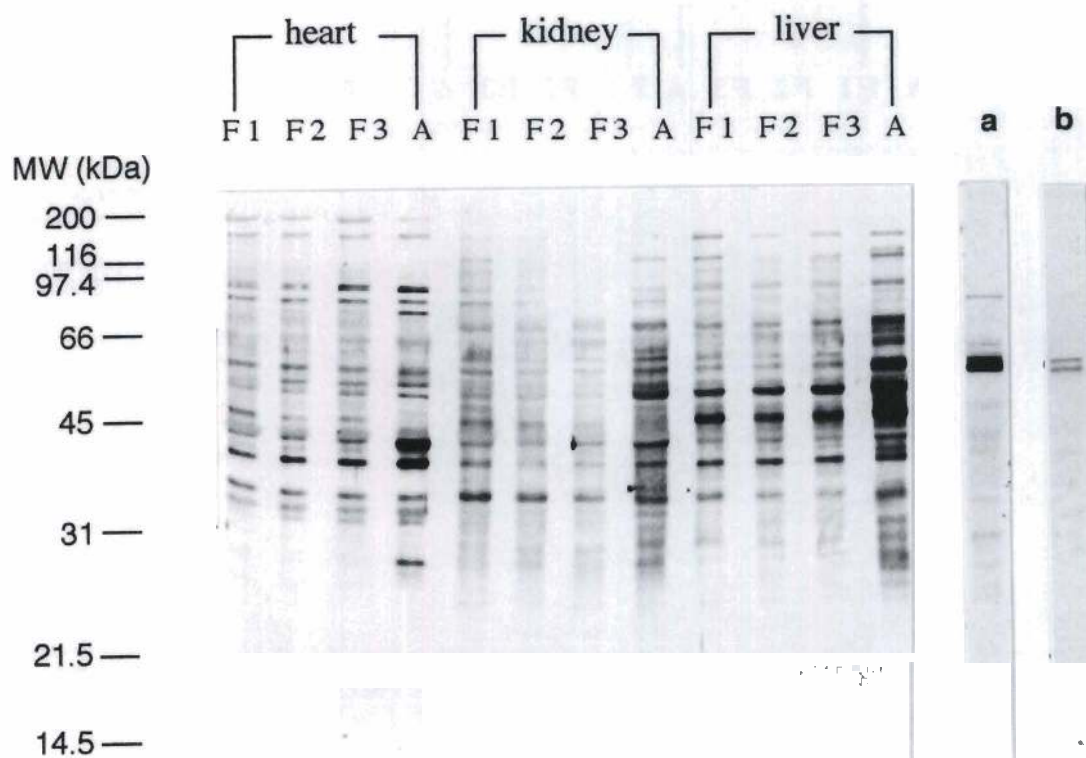


**Fig. 2.** Detection of galectin-1-binding glyocoproteins from salt extracts of fetal and adult bovine heart, kidney and liver. Protein aliquots of identical quantity (5 µg per lane) are separated by gel electrophoresis under denaturing and reductive conditions, electroblotted onto nitrocellulose and visualized by successive incubation steps with biotinylated galectin-1 and signal-generating reagents. The effect of the presence of 0.3M lactose and 0.25 mg/ml asialofetuin to block carbohydrate-dependent binding is documented for extracts from adult liver (lane a). Staining of proteins by the streptavidin/alkaline phosphatase conjugate in the absence of the biotinylated probe is shown for an aliquot of this specimen (lane b). The blot is calibrated with colored standard molecular weight markers.

carbohydrate-dependent binding was ensured by incubation of the labeled lectin in the presence of glyco-inhibitors, as likewise performed in the solid-phase assay in the microtiter plate wells. Except for a doublet with apparent reactivity to kit reagent, presence of haptenic glycocompounds drastically reduced lectin binding to the target molecules, exemplarily shown in Fig. 2. Based on this experimental system with nitrocellulose-immobilized lectin-reactive glycoproteins, major alterations in the ligand profile during development appeared to be quantitative for each organ type (Fig. 2). The disparity of ligand display on the level of glyco-proteins between the mammalian and plant lectin corroborated the results of the microtiter plate assay (Fig. 3).

In addition to glycoproteins galectin-1 can also bind to glycolipids. The extent of this interaction is assayable *in vitro* under similar conditions, as used for the glycoprotein ligand detection. A standard extraction procedure was performed to determine the extent of expression of lectin-reactive (sugar-inhibitable) neutral glycolipids and gangliosides separately. Surface immobilization restricts accessibility to lipid-linked sugar structures which need to be more extended than one lactose unit. Indeed, lactosylceramide as potential ligand was not reactive in the solid-phase assay. The results revealed that *in vitro* binding to both fractions

could occur in the case of galectin-1 (Fig. 4). The comparison of these data with the regulation of lectin expression showed that the lectin-reactive neutral glycolipid fraction in heart and liver as well as the ganglioside fraction in liver appear to be upregulated in line with lectin expression. Relative to the endogenous lectin the extent of reactivity to the plant agglutinin was clearly reduced, possibly reflecting the rather poor tolerance of the α2,3-linked sialic acid extension by this lectin (Fig. 5). As already noticed for galectin-1 reactivity to glycoproteins, expressed with respect to a model glycoprotein ligand, the display of ß-galactosides on a lipid backbone in adult organs appeared to be comparatively enhanced for the endogenous lectin relative to the exogenous probe. As ceramide portions with short oligosaccharide chains are not likely to serve as main interaction sites in a bulky glycocalyx, the assay conditions with similar accessibility requirements will probably not mask major membrane-embedded ligands. However, the tissue processing for the biochemical assays will definitely preclude to ascribe a certain cellular localization to the lectin and its *in vitro* ligands. Therefore, it is necessary to supplement the quantitative determination of the level of expression with results of respective histochemical studies.

For immunohistochemistry, optimal conditions were set by examining the quality and quantity of signal
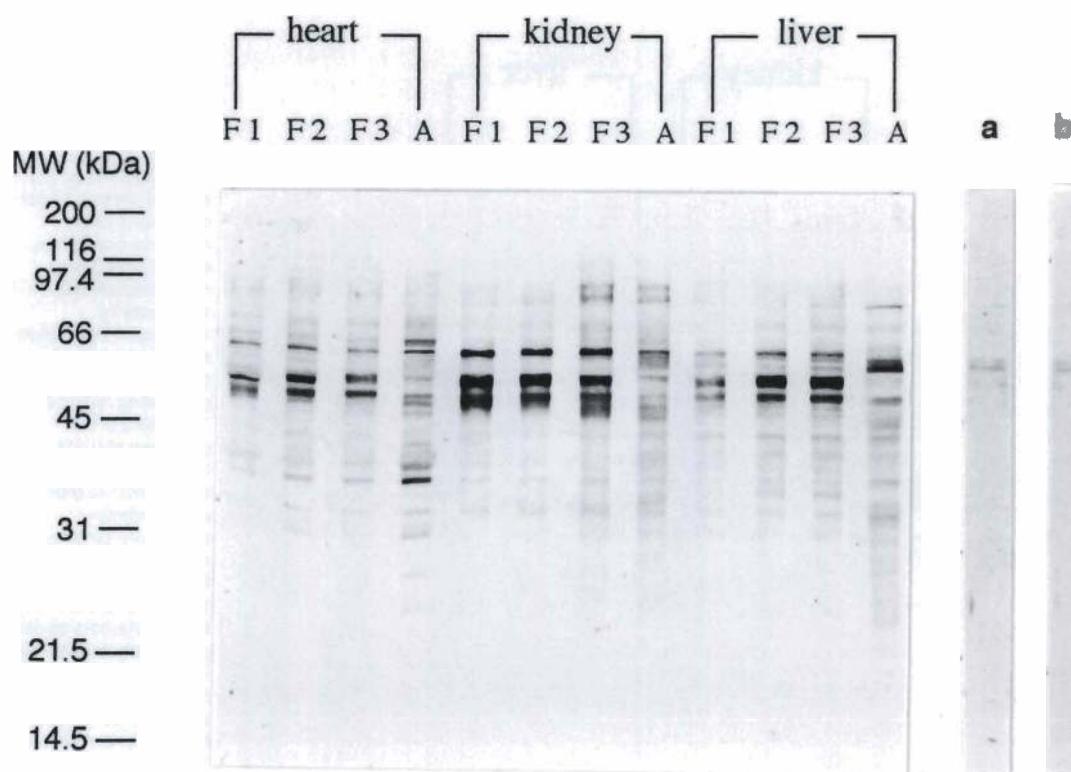


**Fig. 3.** Detection of mistletoe lectin-binding glycoproteins from salt extracts of fetal and adult bovine heart, kidney and liver. Protein aliquots of identical quantity (5 µg per lane) are separated by gel electrophoresis under denaturing and reductive conditions, electroblotted onto nitrocellulose and visualized by successive incubation steps with biotinylated mistletoe lectin and signal-generating reagents. The effect of the presence of 0.3M lactose and 0.25 mg/ml asialofetuin to block carbohydrate-dependent binding is documented for extracts from adult liver (lane a). Staining of proteins by the streptavidin/alkaline phosphatase-conjugate in the absence of the biotinylated lectin is shown for an aliquot of this specimen (lane b). The blot is calibrated with colored standard molecular weight markers.

development in dependence of fixative, probe concentration, and blocking conditions. It was thus performed in sections after methanol/glacial acetic acid fixation, and lectin histochemistry proved reliable after application of Bouin's fixative. Whereas fixation in paraformaldehyde or methanol/glacial acetic acid had no influence on the result for the mistletoe lectin, altered stain development and increased background was observed with the endogenous lectin, corroborating the notion for a differential ligand display. As shown in Fig. 6, the generation of signal was strictly dependent on the presence of the antigen or on the protein-carbohydrate interaction. Preadsorption of the immunoglobulin with purified lectin or presence of haptenic glycoinhibitors reduced the staining intensity to background values. As already noted, solid-phase assays had confirmed the assumption that ligand-bound galectin was still reactive with the antibody, excluding spatial or conformational impairment of antibody binding by a glycoligand. Notably, the staining patterns of three individual organ samples of each stage were nearly indistinguishable, revealing no indication for marked interindividual variability.

Since the sample processing in histochemistry can affect ligand presentation and, consequently, staining intensity qualitatively and quantitatively, cryostat and paraffin sections of liver, heart and kidney at each stage were compared. A notable difference was only observed in a single case, namely adult liver. In these sections, primarily nuclei of hepatocytes, and only in distinct regions endothelial cells, were reactive with the three probes in cryostat sections compared to endothelial cells in paraffin-embedded sections (Fig. 7a,b). Remarkably, the staining pattern of the cryostat sections of the adult organ resembled that of paraffin-embedded sections for

the fetal stages. In general, methanol- and xylene-extractable glycoconjugates did not appear to be main ligands for the lectins in histochemical localization. To demonstrate whether the endogenous lectin could block reactive sites *in situ*, incubation with an excess of ß-galactosides was carried out. In line with the biochemical results, exposure of cryostat sections of adult kidney to 0.2M lactose and 0.65 mg/ml asialofetuin, which served as efficient inhibitors of lectin binding in solid-phase assays and on the surface of paraffin-embedded tissue sections, either without or after fixation with 2% paraformaldehyde, led to no increase in the extent of binding sites for galectin-1. The staining pattern, i.e. positive glomeruli in adult kidney, was not altered.

The histochemical analysis revealed a non-uniform pattern of intensity levels for the three selected organs. As already indicated, the three developmental stages of liver were characterized by preferential nuclear staining of hepatocytes, the ligand abundance for galectin-1 being comparatively small in relation to the other two probes (Fig. 7c, e, f), whereas cytoplasmic endothelial cell staining predominated in the adult organ with no change of the relative intensity distribution in inter-marker comparison (Fig. 7b, d). In contrast to liver a quantitative change for a marker was apparent in fetal stages of kidney. This alteration mainly concerned the intensity, not the principal distribution, i.e. positivity of glomeruli and the interstitium between tubuli for the galectin, and glomerular and tubular positivity for the lectin ligands (Figs. 6c, e, 8d-f, 9a-d). Galectin-1 ligand presentation obviously increased from F1 to F2 with following gradual decrease of intensity in F3 and the adult stage
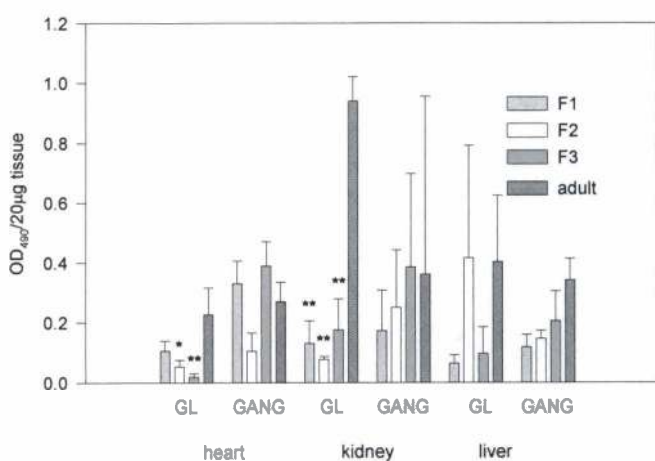


Fig. 4. Extent of expression of galectin-1-binding neutral glycolipids (GL) and gangliosides (GANG) in a solid-phase assay of tissue samples from fetal and adult bovine heart, kidney and liver. The given OD-value for each developmental stage is normalized to the response of an aliquot of each fraction which is equivalent of 20 µg tissue (wet weight). Statistical difference of the OD-values for each developmental stage relative to that of the respective adult organs is given as *: 0.05≥p>0.01; and ** : 0.01 ≥p>0.001.
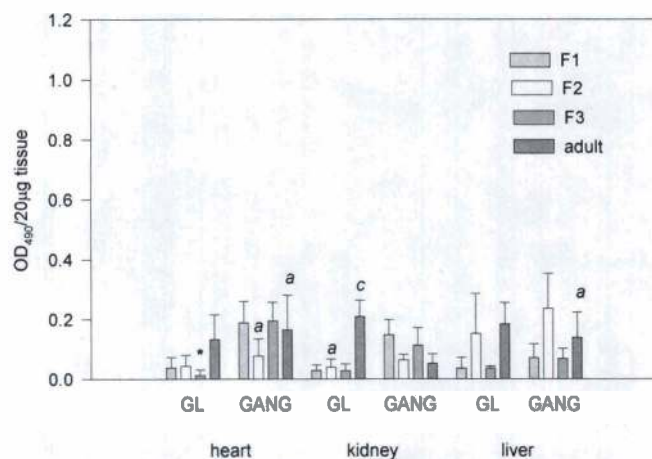


Fig. 5. Extent of expression of mistletoe lectin-binding neutral glycolipids (GL) and gangliosides (GANG) in a solid-phase assay of tissue samples from fetal and adult bovine heart, kidney and liver. The given OD-value for each developmental stage is normalized to the response of an aliquot of each fraction which is equivalent of 20 µg tissue (wet weight). Statistical difference of the OD-values for each developmental stage relative to that of the respective adult organs is given as *: 0.05 ≥p>0.01; and **: 0.01 ≥p> 0.001. Concerning the comparison of the two lectins statistical significance of the difference is denoted by a: 0.05≥p>0.01; and c: p ≤ 0.001.
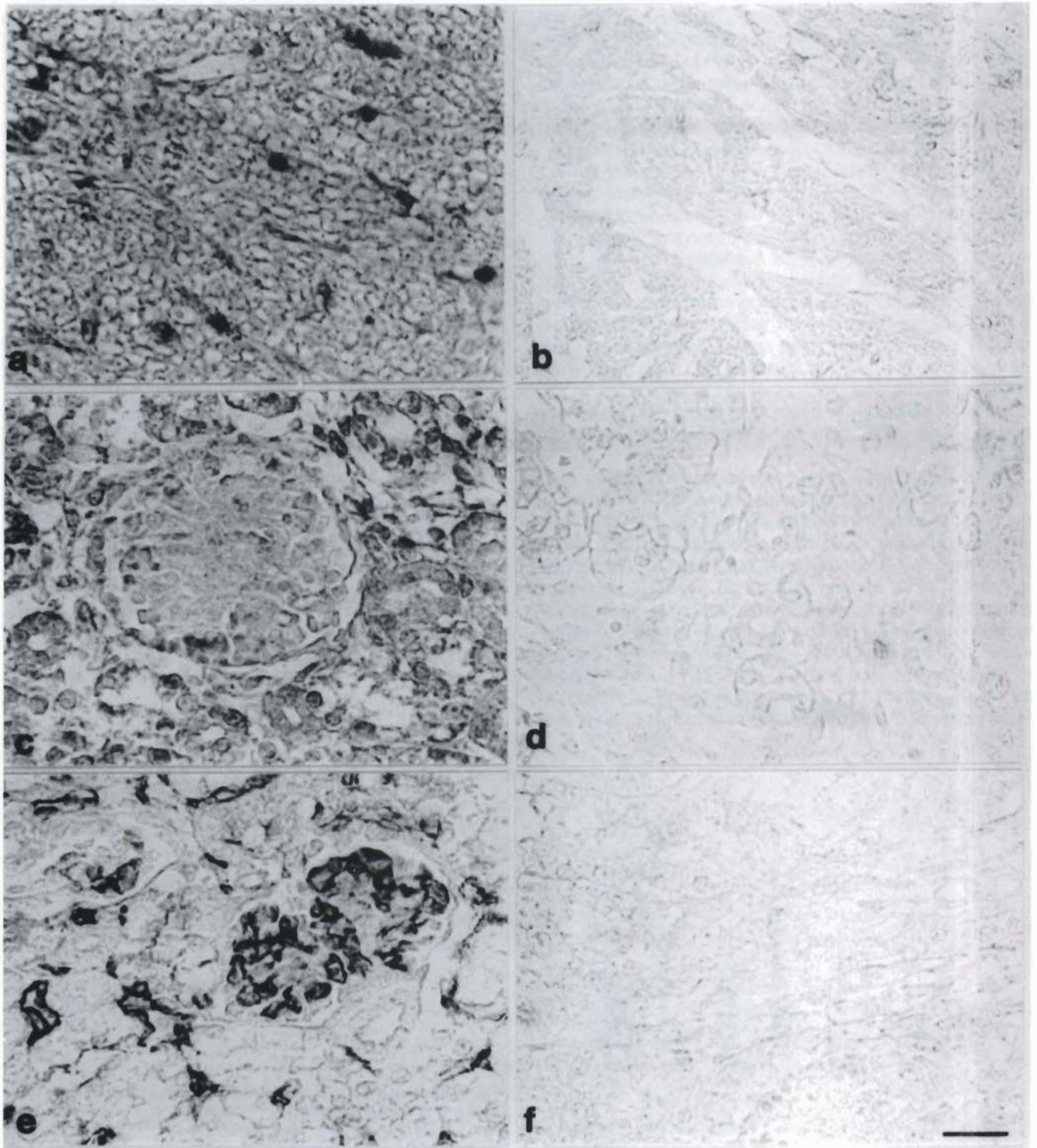
**Fig. 6.** Histochemical specificity controls of the applied antibody and the lectins. Localization of galectin-1 by the polyclonal antibody without (a) and with prior incubation of the antibody with an excess of purified antigen (b) in sections of fetal heart (F3), of galectin-1-specific binding sites after incubation of the labeled probe in the absence (c) and in the presence of glycoinhibitors to block carbohydrate-mediated binding (d) for sections of fetal kidney (F3), and of mistletoe lectin-specific binding sites (e) in sections of fetal kidney (F3). To exclude any binding of kit reagents the incubation step with the labeled probe is omitted in the otherwise identical processing (f). Bar: 250μm.

(Fig. 9a-d). In contrast, the staining with the plant agglutinin remained rather constant (Figs. 6e, 8d). Similarly, galectin-1 expression did not change markedly in the course of development (Fig. 8f). However, a limited, albeit detectable degree of heterogeneity was seen in adult specimens with areas of low intensity or lack of signal. Concerning sections of bovine heart, no drastic change in expression of galectin-1- and plant
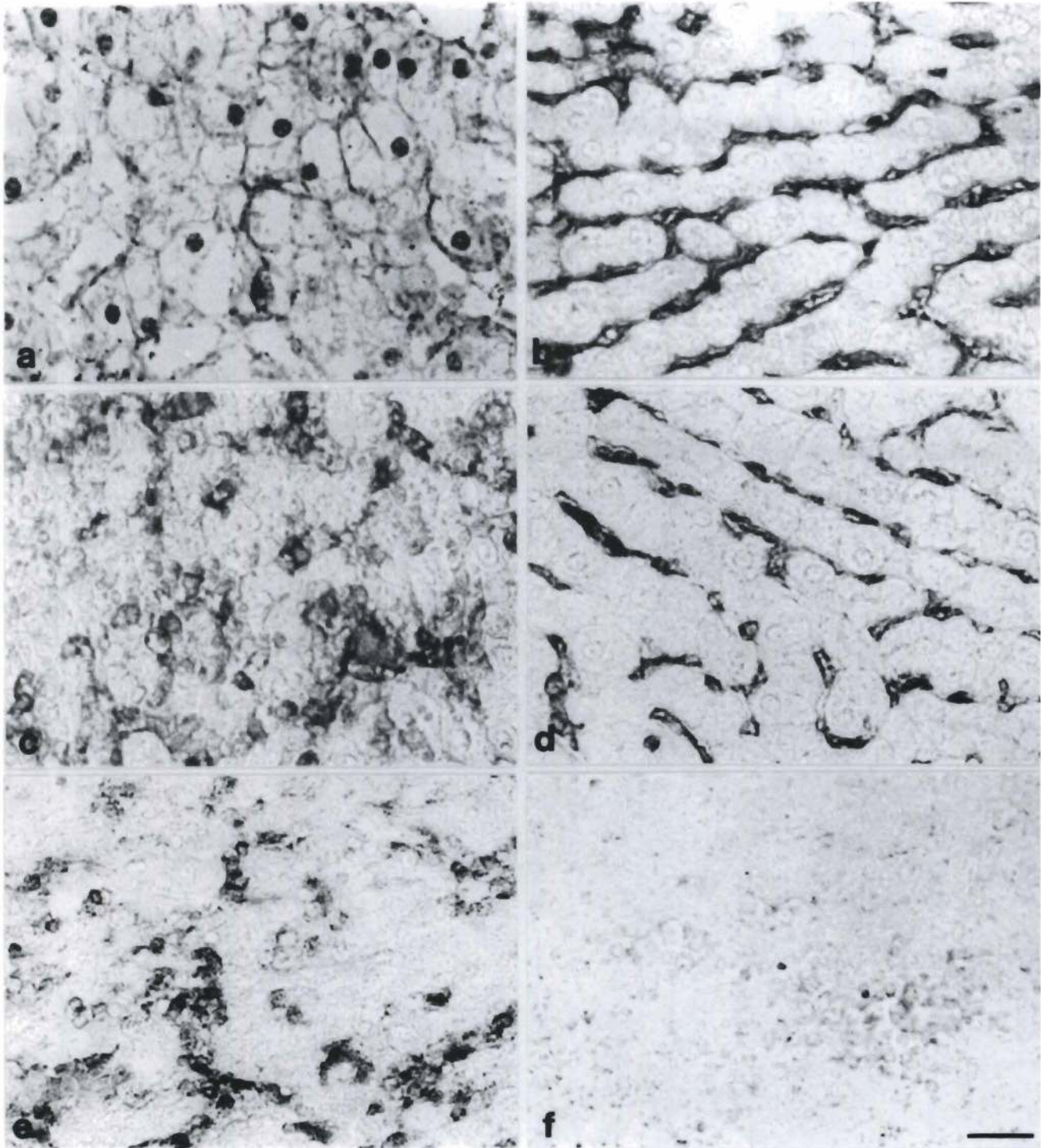


**Fig. 7.** Immunohistochemical localization of bovine galectin-1 in cryostat **(a)** and paraffin-embedded **(b)** sections of adult liver and visualization of mistletoe lectin-specific binding sites in paraffin-embedded sections of fetal (F2, **c**) and adult liver **(d)**. Immunohistochemical localization of galectin-1 **(e)** and lectinhistochemical visualization of galectin-1-specific binding sites **(f)** in sections of fetal liver (F1). Bar: 250μm.

agglutinin-specific sites was discerned. Galectin-1 was mainly seen in myocytes including nuclei, while plant agglutinin-reactive glycoconjugates were visible in the connective tissue between the myocytes (Figs. 6a, 8a, 9e, f). A considerable overlap of localization for the endogenous lectin and lectin-reactive glycocompounds was detectable by application of biotinylated galectin-1 (Fig. 8b, c). Relative to the immunohistochemical response
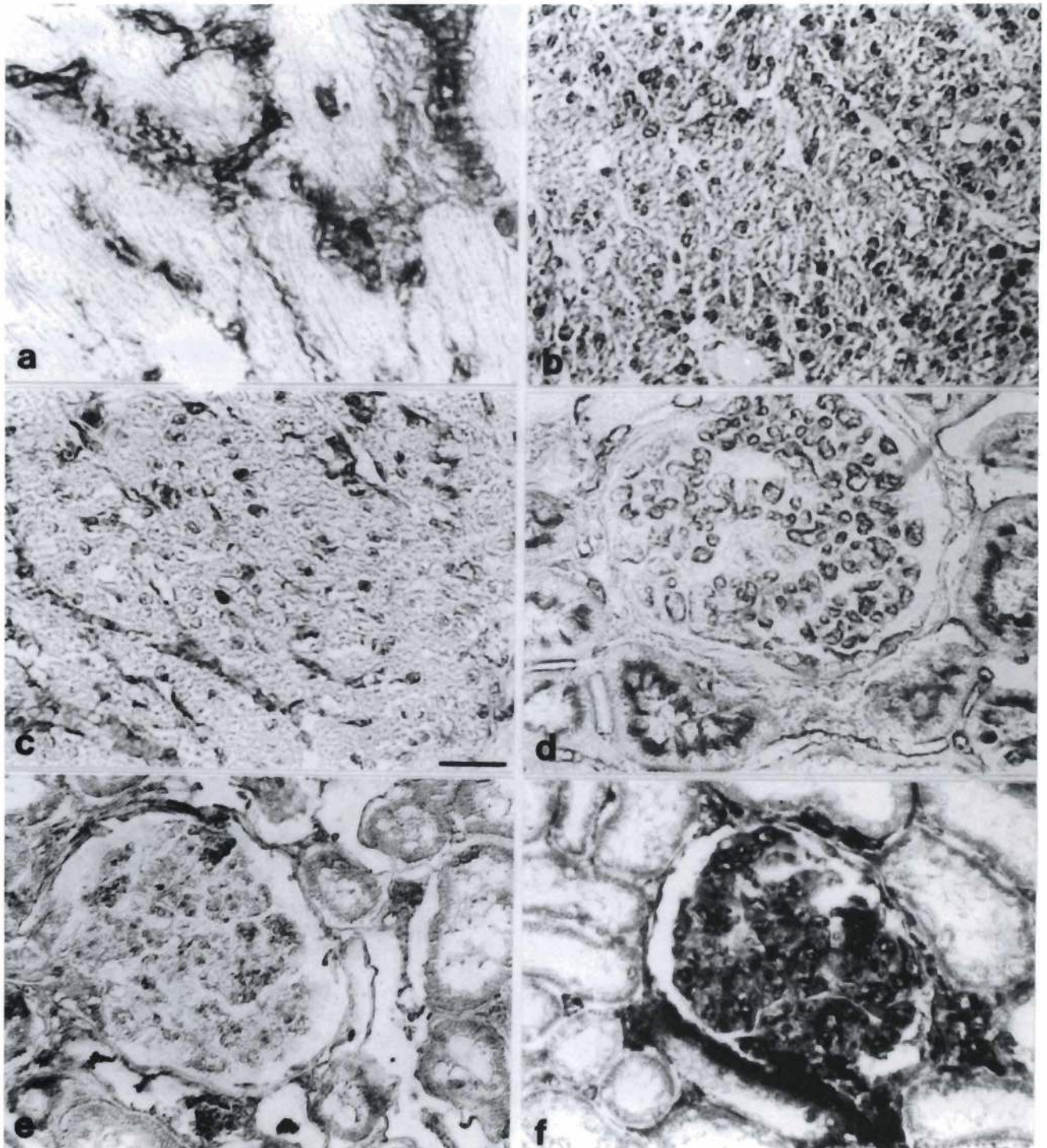


Fig. 8. Localization of **mistletoe lectin-specific** binding sites (a, d), of galectin-1-specific binding sites (b, e) and of galectin-1 (c, f) in sections of fetal heart (F3; a, b, c) and adult kidney (d, e, f). Bar: 250 µm (a-c), or 30 µm (d-f).

reactivity to the endogenous lectin was only barely detectable in the early fetal stage and increased markedly thereafter without a change in the distribution profile.

## Discussion

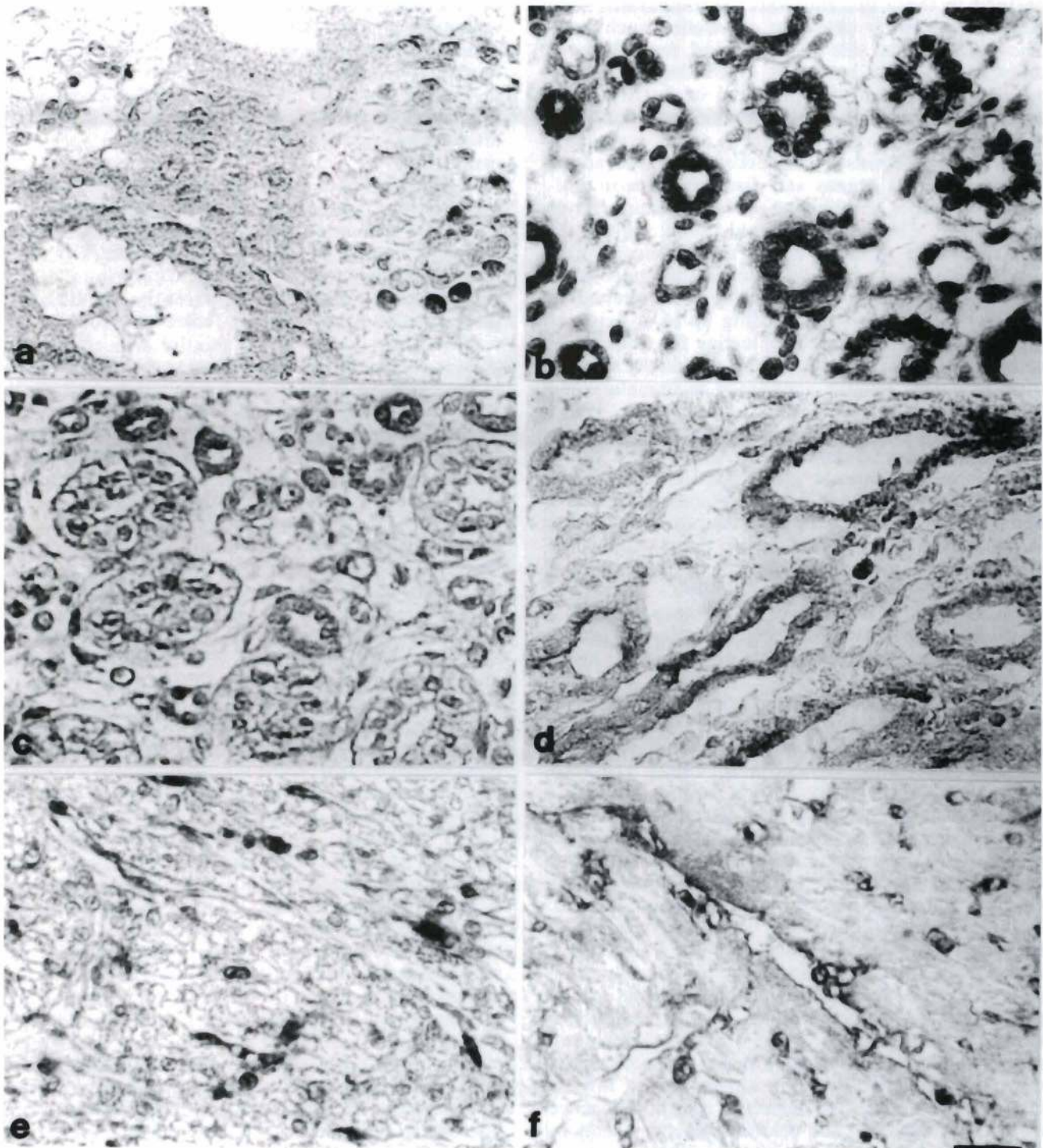The enzymatic machinery of glycosylation generates a well-described array of diverse structures with



**Fig. 9.** Localization of galectin-1-specific binding sites in sections of the three different developmental stages (F1-F3; **a-c**) of kidney and of the adult organ **(d)** and immunohistochemical visualization of galectin-1 in sections of fetal (F1; **e**) and adult heart **(f)**. Bar: 250 μm.

different levels of abundance (Brockhausen and Schachter, 1997; Kopitz, 1997; Sharon and Lis, 1997). Functional analysis for a carbohydrate epitope is indubitably made easier, when its expression is spatially and/or temporarily restricted. However, presumedly operative protein-carbohydrate recognition is not confined to these determinants, encompassing also epitopes with fairly abundant expression such as ß-galactosides. On the side of the receptor, multiplicity of lectins can likewise be found, precluding a straight-forward solution to the problem of defining the functional significance of individual lectins of this category, e.g. galectins. Whereas avian organisms express two galectins with marked level alterations between different organs and dissimilarities in cell-binding properties and binding site architecture (Beyer and Barondes, 1982; Schneller et al., 1995; Solís et al., 1996), mammals appear to harbor a predominant lectin in most adult organs. In addition to this galectin-1, various related members of this family can be present in certain cell or organ types, adding up to at least six reported ß-galactoside-binding lectins for example in human spleen (Sharma et al., 1992). As initially seen for rat organs, their relative abundance in immature and adult tissues can vary significantly (Cerra et al., 1985). Regulation of glycoligand expression has so far not been monitored in prenatal development. Although it would certainly be presumptuous to expect immediate and definitive answers under these circumstances, it is a reasonable step to at first focus on the nearly ubiquitous galectin-1 with special emphasis on the assessment of qualitative and quantitative aspects of expression of lectin-reactive glycoproteins and glycolipids *in vitro* and *in situ*. Organ specimens from early, morphologically defined fetal stages have been selected in comparison to adult organs, particular efforts being devoted to heart, kidney and liver.

The ELISA results are in agreement with RNA blot analysis of murine embryogenesis, corroborating the maintenance of a certain level of transcript presence after 12.5 days post coitum (Poirier et al., 1992). The adult organs nearly invariably have a larger amount of galectin-1 than the specimens from the respective organs during early fetal development. When *in vitro* conditions are used with presentation of oligosaccharide chains from matrix surface-immobilized glycoconjugates, the extent of lectin-reactive epitopes can be quantitated. These experiments document a similar increase in display of lectin-binding epitopes only for a few organs. However, it would not be justified to automatically claim that a lack of correlation of expression of lectin and its ligands is proven. As thoughtfully discussed for selectins (Varki, 1994), differences in presentation and conformation can cause reactive (inert) glycoconjugates *in vitro* to potentially lose (gain) activity *in vivo*. In view of the nearly identical distribution of galectin-1-reactive binding sites in frozen and paraffin-embedded sections and the solid-phase assays with glycolipids, it seems probable that lipid-linked neutral sugar chains, which are extractable during histochemical tissue processing, may

not be major galectin-1 ligands. The exemplary application of the plant agglutinin with identical nominal monosaccharide specificity and known fine-specificity differences for oligosaccharides to galectin-1 reveals clear quantitative and qualitative dissimilarities. This result intimates that the application of a plant lectin must be interpreted cautiously beyond its apparent sugar-inhibitable binding. Plant lectin binding can thus only be assumed to resemble the respective profile of a tissue lectin, if rigorous specificity controls have been performed. Similarly noteworthy is the observation that the pattern of lectin-binding glycoproteins is primarily affected quantitatively with differences in inter-organ comparison.

Cell models have shown that the lectin after synthesis on free ribosomes can be diffusely distributed in the cytoplasm with constitutive externalization without or with appearance of vesiculated structures (Cooper and Barondes, 1990; Cho and Cummings, 1995). Intracellular presence is apparently not linked to predominantly tight complex formation. However, our results clearly document that reactive sites are not only present extracellularly. Binding to carbohydrate ligands in the oxidative extracellular environment stabilizes the lectin and prevents inactivation of sugar recognition by internal disulfide bond formation (Tracey et al., 1992). In this form, galectin-1 has been reported to trigger strong mitogenic activity of 3T3 cells without the involvement of carbohydrate ligands (Yamaoka et al., 1996). Since the binding site(s) for inactivated galectin-1 is presently unknown and since the lectin has been described to interact with other cellular proteins *in vitro* via protein-protein interactions (Joubert et al., 1992), we decided to focus on the analysis of carbohydrate-dependent binding. In addition to intracellular sites it is also seen extracellularly. It is thus tempting to draw an analogy to the proteoglycan-dependent storage of cytokines in the extracellular matrix (Tanaka et al., 1993). Considering the importance of this special aspect in development the reports that galectin expression is dispensable in frog embryogenesis and in knock-out mice in embryonic and adult tissues must be taken into account (Poirier and Robertson, 1993; Marschal et al., 1994). If indeed an actual vital function would be impaired under these circumstances, a compensation by presence of a related protein will have to be facilitated. Not only presence, but also a similar spatial distribution and ligand recognition is then called for to prevent aberrant development. This reasoning highlights the importance of concomitant histochemical studies, although the effect of fixation on presence and presentation of the epitope under scrutiny should not be neglected. As commonly known from plant lectin studies, tissue processing can affect the binding pattern quantitatively and qualitatively, demanding guarded interpretation. Therefore, discrepancies between signal intensity in solid-phase assays and staining intensity are plausible.

An important finding of this study is that both lectin-reactive glycoprotein display, measured biochemically,

and pattern of localization, monitored by immuno- and lectinhistochemical methods, exhibit organ type-related alterations. Interestingly, human galectin-1 has been reported to recognize different glycoprotein sets in extracts of dermis and epidermis and to be localized in the nucleus, cytoplasm, the basement membrane and the extracellular matrix of different cell types in human skin (Akimoto et al., 1995). Further steps in elucidation of function(s) of galectin-1 will thus have to unravel the nature of the probable *in vivo* ligands. In this respect, the situation is evocative of the quest to describe the function(s) of plant lectins, which in the case of the leguminous agglutinins even share the basic folding pattern, namely the jelly-roll motif, with galectins (Sharon and Lis, 1990; Peumans and van Damme, 1995; Rini, 1995; Kijne, 1996; Rüdiger, 1997). It remains to be seen whether the structural relatedness may translate into comparable cellular roles of these two groups of evolutionarily remarkably conserved proteins.

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