0014-4827/79/060321-14$02.00/0

Experimental Cell Research 120 (1979) 321–334

THE USE OF FLUORESCEIN-CONJUGATED *BANDEIRAEA SIMPLICIFOLIA* B_4-ISOLECTIN AS A HISTOCHEMICAL REAGENT FOR THE DETECTION OF α-D-GALACTOPYRANOSYL GROUPS

Their Occurrence in Basement Membranes

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SUMMARY

The *Bandeiraea simplicifolia* B_4-isolectin, which combines specifically with α-D-galactopyranosyl groups, has been conjugated with fluorescein isothiocyanate and demonstrated to be a reliable histochemical probe for the detection of these groups in normal tissues of mouse, rabbit, rat and man. Specificity of binding of the fluorescein-conjugated *B. simplicifolia* B_4-isolectin to native cryostat tissue sections was demonstrated in two ways:

1. The hapten inhibitor methyl α-D-galactopyranoside prevented the binding of the lectin to tissues whereas the non-hapten methyl α-D-glucopyranoside did not.

2. Pretreatment of tissue sections with coffee bean α-galactoside abolished lectin binding whereas pretreatment with *A. niger* or *E. coli* β-galactosidase did not. The fluorescein-conjugated isolectin visualized α-D-galactopyranosyl groups in basement membranes and on the surface of certain epithelial cells of mouse, rat, rabbit, and on the surface of the TA3 murine mammary carcinoma. These studies suggest that the *B. simplicifolia* B_4-isolectin may be of great utility in studying the family of α-D-galactosyl-containing glycoconjugates of basement membranes in pathological states accompanied by basement membrane changes, such as diabetes mellitus, and in neoplasms that secrete basement membrane.

A lectin exhibiting binding specificity for α-D-galactopyranosyl groups was isolated by Hayes & Goldstein [1] from saline extracts of *Bandeiraea simplicifolia* seeds. Subsequently, Murphy & Goldstein [2] demonstrated that this lectin is actually a family of five isolectins. The tetrameric lectin is composed of combinations of two distinct subunits, each subunit possessing a unique carbohydrate-binding specificity. The A subunit binds both α-D-galactopyranosyl groups and 2-acetamido-2-deoxy-α-D-galactopyranosyl groups whereas the B subunit binds α-D-galactopyranosyl groups with rigid specificity [2].

Friberg et al. reported [3] that TA3-St murine mammary carcinoma cells are agglutinated at high titer by crude extracts of *B. simplicifolia* seeds. We have observed that the TA3-St cells and Ehrlich ascites tumor cells [4] are agglutinated by the purified BS $I\text{-}B_4$ isolectin, indicating that both carcinomas express α-D-galactosyl groups on their surface. The prominence on tumor cell surfaces of this linkage, reported to occur with low frequency in mammalian glycoconjugates [5–13], has prompted us to examine the distribution of α-D-galactopyranosyl groups in normal tissues of mouse, rabbit and man. Our studies demonstrate

Table 1. *Protocol for labelling cryostat sections of each tissue*

Tissue section	First treatment	Second treatment
1	PBS buffer	PBS buffer
2	–	FITC-B_4 (200 μg/ml)
3	–	FITC-B_4 (50 μg/ml)
4	–	FITC-B_4 (10 μg/ml)
5	–	FITC-B_4 (2 μg/ml)
6	–	FITC-B_4 (50 μg/ml) +10 mM Me α-D-Gal*p*
7	–	FITC-B_4 (50 μg/ml) +20 mM Me α-D-Glc*p*
8	Coffee bean α-galactosidase (1 U/ml)	FITC-B_4 (50 μg/ml)
9	*A. niger* β-galactosidase (2 U/ml)	FITC-B_4 (50 μg/ml)
10	Anti-NBM (50 μg/ml)	FITC-goat-anti-rabbit-IgG-antiserum
11	Non-immune IgG (50 μg/ml)	FITC-goat-anti-rabbit-IgG-antiserum
12	PBS buffer	FITC-goat-anti-rabbit-IgG-antiserum

Each treatment was for 30 min at 22°C in a dark, moist chamber, and was followed by three 3 min rinses in PBS buffer.

–, No treatment was applied.

that the BS I-B_4 isolectin, conjugated with fluorescein isothiocyanate (FITC-B_4), is a specific probe for α-D-galactopyranosyl groups (α-D-Gal*p*) in native cryostat sections, and that the lectin binds to basement membranes and certain epithelial cell types in tissues of mouse and rabbit, but not of man.

MATERIALS AND METHODS

The B_4-isolectin was isolated from *B. simplicifolia* seeds (Calbiochem) as described by Murphy & Goldstein [2].

Conjugation of BS I-B_4 isolectin with fluorescein isothiocyanate

A solution of BS I-B_4 isolectin (6–8 mg/ml) was dialyzed against PBS buffer (sodium phosphate, 0.01 M and sodium chloride, 0.15 M, pH 7.0) containing 0.1 mM $CaCl_2$. To 1.0 ml of the BS I-B_4 solution, 43 mg methyl α-D-galactopyranoside (Me α-D-Gal*p*, Pfanstiehl) was added, followed by 0.5 ml of 0.2 M Na_2HPO_4. The pH was adjusted to 9.5 with 0.1 M Na_3PO_4 and the volume brought to 2.0 ml with 0.15 M NaCl. The BS I-B_4 solution was dialyzed with stirring against a freshly prepared solution of 3.5 mg fluorescein isothiocyanate (Nutritional Biochemicals) and 430 mg Me α-D-Gal*p* in 20 ml 0.1 M sodium phosphate pH 9.5 for 2½ h at 22°C in the dark. The pH of the dialysate was monitored at 15 min intervals, and maintained at 9.5 with 0.1 M Na_3PO_4. The fluorescein-conjugated BS I-B_4 (FITC-B_4) was then dialyzed against 500 ml of cold PBS buffer containing 0.1 mM $CaCl_2$ and 2.5 mg/ml Me α-D-Gal*p* followed by three 500 ml changes of PBS buffer. FITC-B_4 was purified by affinity chromatography on a column (8×70 mm) of epichlorohydrin cross-linked guaran as described by Lönngren et al. [14]. Typically, less than 10% of the material applied to the column, measured by absorbance at 280 nm, washed through unbound whereas the remaining 90% bound material was eluted from the column with 2.5 mg/ml Me α-D-Gal*p*. FITC-B_4 bound to and eluted from the guaran column was dialyzed free of Me α-D-Gal*p* against PBS buffer containing 0.02% NaN_3, and stored in the cold for as long as 6 months without loss of activity. The ability of BS I-B_4 to agglutinate human type B erythrocytes was not affected by the fluorescein conjugation, which incorporated on the average 1.7 fluorescein molecules/molecule of lectin.

Labelling native cryostat tissue sections with FITC-B_4

Tissues were rapidly removed and trimmed with a razor blade to a block no larger than 1 cm^3. The tissue blocks were immersed in a drop of Tissue-Tek OCT embedding medium on a brass cryostat chuck and were quick-frozen in a stream of CO_2 (Tissue-Tek CO_2 freezing attachment). Alternatively, very small tissue samples were placed into a no. 00 gelatin capsule, covered with OCT embedding medium, and quick-frozen in a dry ice-hexane bath as described by Klann [15].

Cryostat sections were cut at 6 μm and dried on warm, acetone-washed microscope slides. A minimum of 12 sections was prepared for each tissue examined. The sections were dried under a stream of warm air (hair dryer) for 10 min and washed twice in PBS buffer. Each section was incubated under a drop (15–40 μl depending on the size of the section) of the appropriate reagent for 30 min at 22°C in a moist, dark chamber. Following each incubation, unbound lectin or antibody was washed away with three 3 min rinses in PBS buffer. The labeling protocol for the 12 tissue sections is summarized in table 1. The first section was treated with PBS buffer only, and served to evaluate autofluorescence of the tissue. Sections 2–5 were treated with varying concentrations of FITC-B_4 from 2–200 μg/ml. Two different controls were performed to verify that the FITC-B_4 binds specifically to α-D-galactosyl groups in tissue sections. For the first control, tissue sections 6 and 7 were incubated with FITC-B_4 containing either the hapten inhibitor Me α-D-Gal*p* (10

Table 2. *The ability of coffee bean α-galactosidase and A. niger β-galactosidase to hydrolyze various p-nitrophenyl glycosides*

Nitrophenyl substrate (source)		Coffee bean α-galactosidase	*A. niger* β-galactosidase
α-D-Gal	(Cyclo Chemicals)	100	0.16
α-D-Gal	+14 mM galactose	11	–
α-D-Gal	+14 mM glucose	98	–
β-D-Gal	(Cyclo Chemicals)	0.03	100
α-D-GalNac	(Koch-Light)	0.01>	0.24
β-D-GalNAc	(Cyclo Chemicals)	0.01>	0.03
β-D-GlcNAc	(Koch-Light)	0.03	0.04
α-D-Man	(Calbiochem)	0.01>	0.01>
β-D-Man	(Sigma)	0.01>	0.01>
α-L-Fuc	(Sigma)	0.01>	0.01>
α-D-Glc	(Koch-Light)	0.01>	0.01>

Rates of *p*-nitrophenol liberation relative to best substrate. Glycosidase assays were performed as described in Methods. Sufficient glycosidase was assayed so that a relative activity of 0.1 would produce an absorbance of approx. 0.1 at 400 nm.

mM) or twice the concentration (20 mM) of the non-inhibitory glycoside, methyl α-D-glucopyranoside (Me α-D-Glc*p*). For the second control, sections 8 and 9 were pre-treated either with coffee bean α-galactosidase (Boehringer-Mannheim, 1 U/ml in McIlvaine's phosphate-citrate buffer, pH 5.1) or with *Aspergillus niger* β-galactosidase (Calbiochem, 2 U/ml in McIlvaine's phosphate-citrate buffer, pH 5.1). With some tissues, *E. coli* β-galactosidase (Boehringer Mannheim, 3 U/ml in PBS buffer) was used in addition to *A. niger* β-galactosidase. Following glycosidase pretreatment, the tissue sections were washed with three 3 min rinses in PBS buffer, and incubated under a drop of FITC-B_4 (50 μg/ml) in the usual manner. The final three sections were used to evaluate the ability of rabbit antibody against the basement membrane of murine parietal yolk sac carcinoma [16] (a generous gift of Dr A. Martinez-Hernandez, Hahnemann Medical College and Dr G. B. Pierce, University of Colorado) to bind to tissue sections. Section 10 was treated with rabbit anti-neoplastic basement membrane IgG (anti-NBM, 50 μg/ml in PBS with 1 mg/ml Pentex 5× crystallized bovine serum albumin); section 11 was treated with rabbit non-immune IgG (50 μg/ml in PBS with 1 mg/ml bovine serum albumin); section 12 was treated with 1 mg/ml bovine serum albumin in PBS buffer. After three 3 min rinses in PBS buffer, the sections were treated with FITC-goat anti-rabbit IgG antiserum (Meloy, 40-fold dilution in PBS buffer).

Fluorescence microscopy

After the treated sections were washed free of unbound reagent, sections were examined within 3 h on a Leitz Diavert microscope equipped with incident light Ploemopak and Leitz filter system I (for FITC), and illuminated with a 50 W mercury lamp. The microscope was kindly provided by Dr Bernard Agranoff of this University. Photomicrographs were initially taken with Kodak High Speed Ektachrome (EHB 135-Tungsten) and processed by Kodak to ASA 320. In later experiments Kodak Ektachrome 200 (ED 135-Daylight) was used and processed by Kodak to ASA 400.

The binding of FITC-B_4 to isolated glomerular basement membrane

Bovine glomerular basement membrane, rat skin collagen (both from Dr R. G. Spiro, Harvard Medical School), and type II collagen from the Swarm rat chondrosarcoma (from Dr V. Hascall, NIH) were suspended at a concentration of 2 mg/ml in 0.15 M sodium chloride employing gentle Dounce homogenization. Aliquots (100 μl) of each suspension were incubated for 30 min at 22°C with 0.5 ml FITC-B_4 (100 μg/ml in PBS buffer) containing either 5 mM Me α-D-Glc*p* or 5 mM Me α-D-Gal*p*. Particulate material was pelleted by centrifugation and washed free of unbound FITC-B_4 by two 1.0 ml washes of PBS buffer. The washed pellets were photographed under long wave UV illumination.

The glomerular basement membrane suspension (100 μl) was pre-treated with coffee bean α-galactosidase (50 μl, 1 U/ml) or with *A. niger* β-galactosidase (50 μl, 2 U/ml) in McIlvaine's phosphate-citrate buffer, pH 5.1 for 2 h at 22°C. The glycosidase-treated basement membrane was washed by centrifugation with two 1.0 ml portions of PBS buffer, and labelled with FITC-B_4 as previously described.

Glycosidase assay

The appropriate *p*-nitrophenyl glycoside substrate (100 μl, 5 mM in H_2O) was added to a 100 μl aliquot of glycosidase diluted in McIlvaine's phosphate-citrate buffer, pH 5.1, containing 1 mg/ml bovine serum albumin. Reaction was terminated with the addition of 1.0 ml of 0.5 M Na_2CO_3 and the liberated *p*-nitrophenol

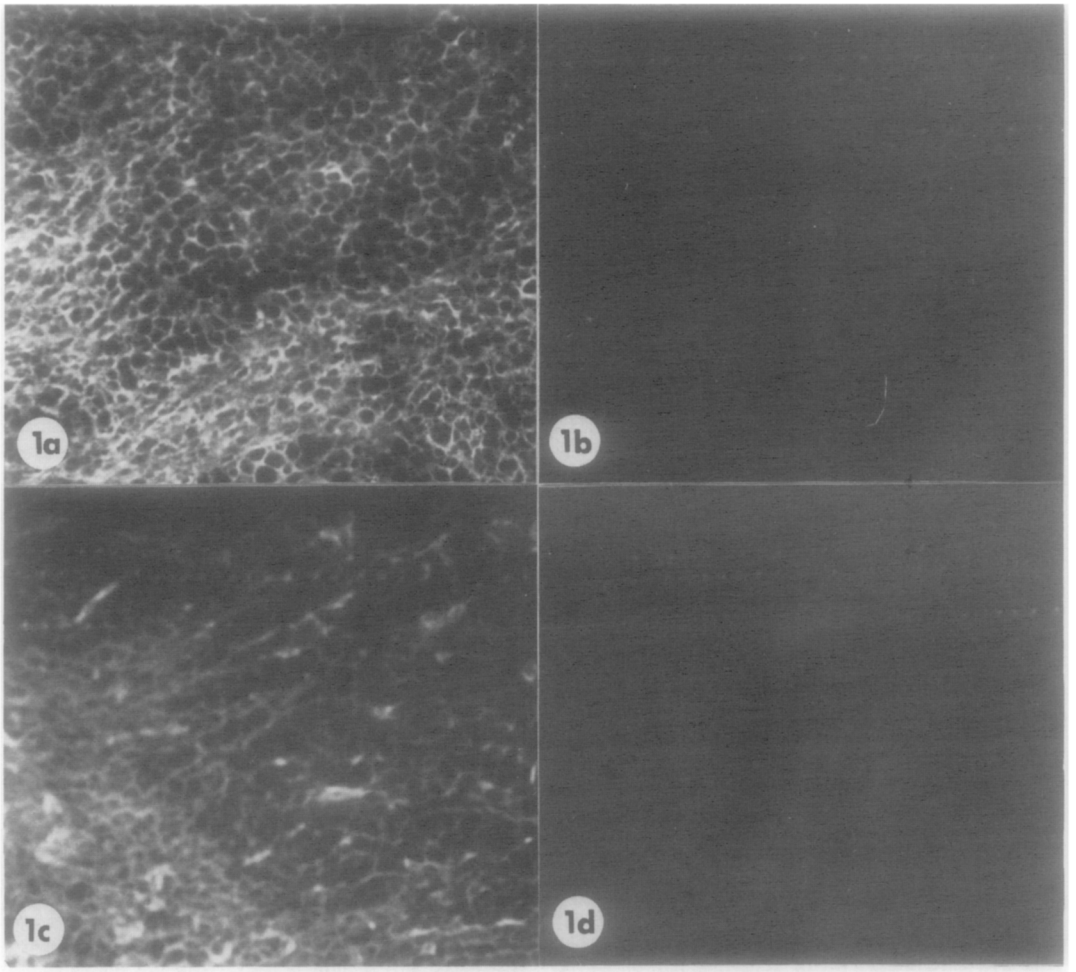

Fig. 1. TA3-St solid tumor: (*a*) labelled with FITC-B_4, 50 μg/ml, ×160, 30 sec exposure; (*b*) same as (*a*), but pre-treated with coffee bean α-galactosidase; (*c*) labelled with anti-NBM, 50 μg/ml, ×160, 30 sec exposure; (*d*) same as (*c*), but non-immune IgG was substituted for anti-NBM.

was measured spectrophotometrically at 400 nm. Controls lacking enzyme were performed. One unit of glycosidase activity corresponds to 1 μmole *p*-nitrophenol liberated per min at 22°C.

RESULTS

The specificity of FITC-B_4 binding to native cryostat tissue sections

In all tissue sections examined, maximum FITC-B_4 binding occurred at a lectin concentration of 50 μg/ml or higher. No fluorescence was observed in tissue treated with 2 μg/ml FITC-B_4 while those sections exposed to 10 μg/ml FITC-B_4 exhibited intermediate fluorescence intensity.

The binding of FITC-B_4 to tissue sections was prevented by the hapten inhibitor Me α-D-Gal*p* (10 mM) but not by Me α-D-Glc*p* (20 mM) nor by Me α-D-GalNAc*p* (10 mM).

The binding of FITC-B_4 to tissue sections was abolished by pretreatment with coffee bean α-galactosidase but unaffected by identical pretreatment with either *A. niger*

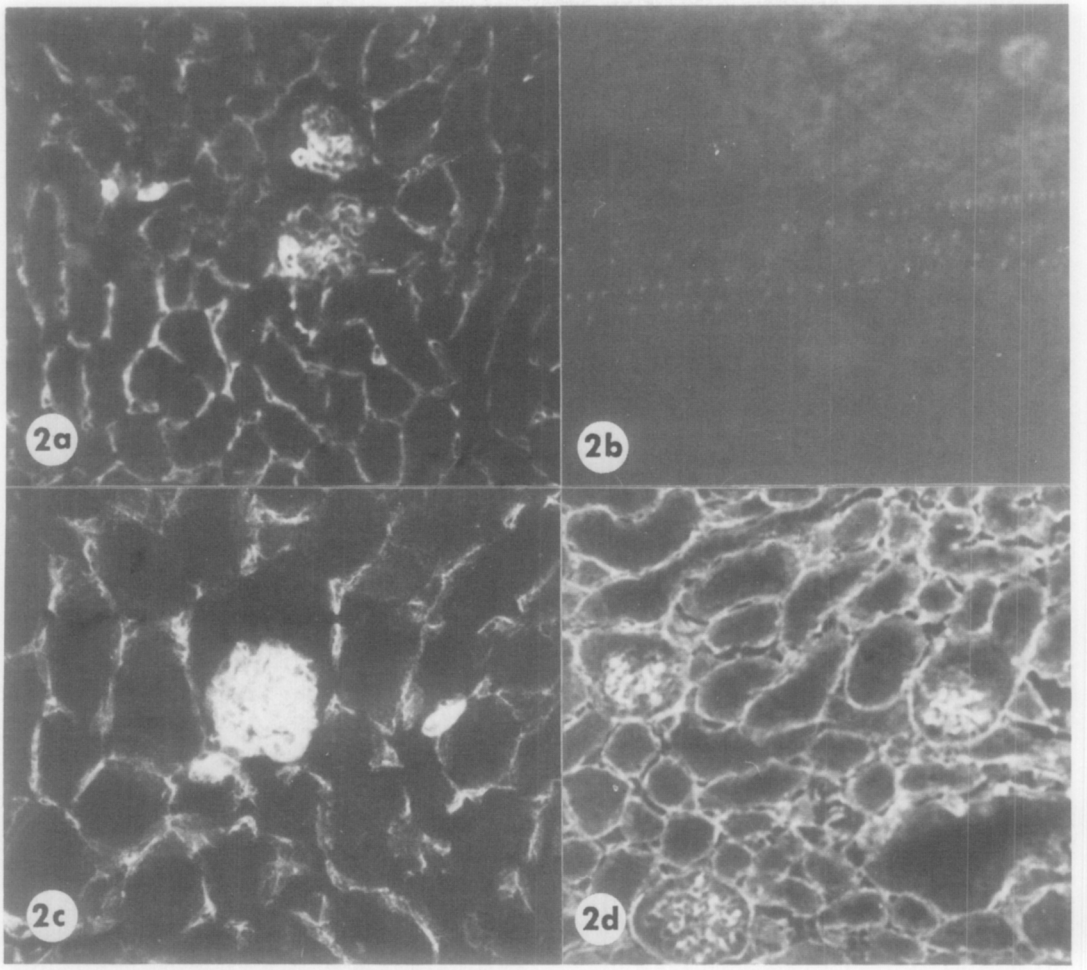

Fig. 2. Mouse kidney cortex: (*a*) labelled with FITC-B$_4$, 50 μg/ml, ×160, 90 sec exposure; (*b*) same as (*a*), but pre-treated with coffee bean α-galactosidase; (*c*) same as (*a*), ×250; (*d*) labelled with anti-NBM, 50 μg/ml, ×160, 60 sec exposure.

or *E. coli* β-galactosidases. The following observations substantiate that the effect of the coffee bean α-galactosidase was due to α-D-galactoside hydrolysis rather than to the action of contaminating protease or glycosidase: (1) No protease activity was detectable in the commercial preparation of α-galactosidase employing either Azocoll or Azocasein as substrate; (2) the α-galactosidase preparation was essentially free of other glycosidase activities as measured with *p*-nitrophenyl glycoside substrates (table 2); (3) the action of α-galactosidase on tissue sections was prevented by adding D-galactose (14 mM), but not D-glucose (14 mM) to the enzyme. As shown in table 2, D-galactose is a specific product inhibitor of coffee bean α-galactosidase.

Locus of FITC-B$_4$ binding in mouse tissue sections

TA3 mammary carcinoma. FITC-B$_4$ bound specifically to tumor cells of both the TA3-St and TA3-Ha sublines. Sections of TA3-

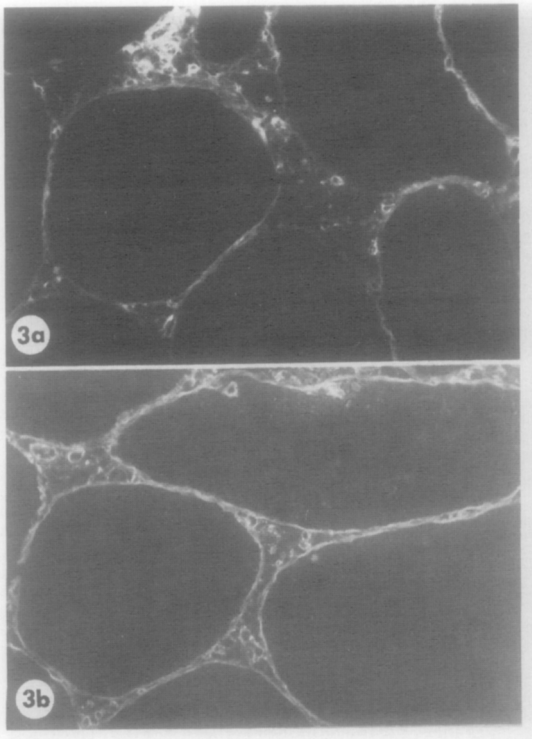

Fig. 3. Mouse testis: (*a*) labelled with FITC-B_4, 50 μg/ml, ×160, 60 sec exposure; (*b*) labelled with anti-NBM, 50 μg/ml, ×160, 60 sec exposure.

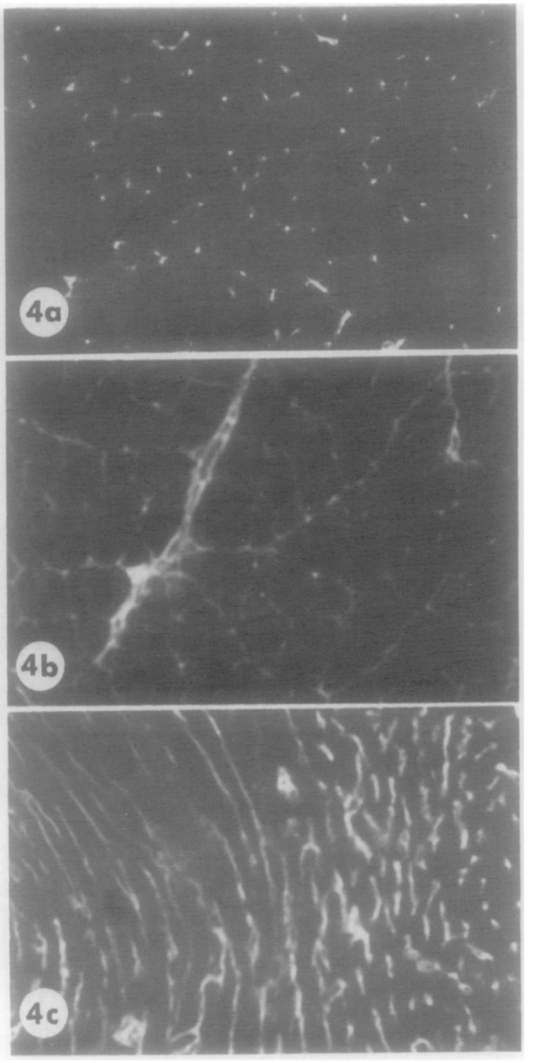

Fig. 4. Mouse muscle: (*a*) skeletal muscle labelled with FITC-B_4, 50 μg/ml, ×160, 120 sec exposure; (*b*) skeletal muscle labelled with anti-NBM, 50 μg/ml, ×160, 45 sec exposure; (*c*) heart labelled with FITC-B_4, 50 μg/ml, ×250, 45 sec exposure.

St solid tumor labelled with FITC-B_4 (fig. 1*a*) exhibited intense, uniform fluorescence surrounding each tumor cell. FITC-B_4 binding was prevented in the section pre-treated with coffee bean α-galactosidase (fig. 1*b*), demonstrating the small amount of non-specific binding of FITC-B_4 to the tumor tissue. The TA3-Ha solid tumor behaved identically to the TA3-St solid tumor. In comparison to normal mouse tissues examined, the fluorescence intensity of the tumors was significantly greater.

TA3-St ascites cells incubated in suspension with 50 μg/ml FITC-B_4 at 4°C, were strongly agglutinated and exhibited intense fluorescence on their surface. The TA3-Ha ascites tumor cells, although equally capable of binding FITC-B_4, were not agglutinated by the lectin. When allowed to warm to 25°C on the microscope slide for 15 min, the uniform cellular fluorescence gave way to patch formation in most TA3-Ha cells, and cap formation in a few cells.

Kidney cortex. Peritubular and glomerular fluorescence (fig. 2*a*, *c*) was observed in

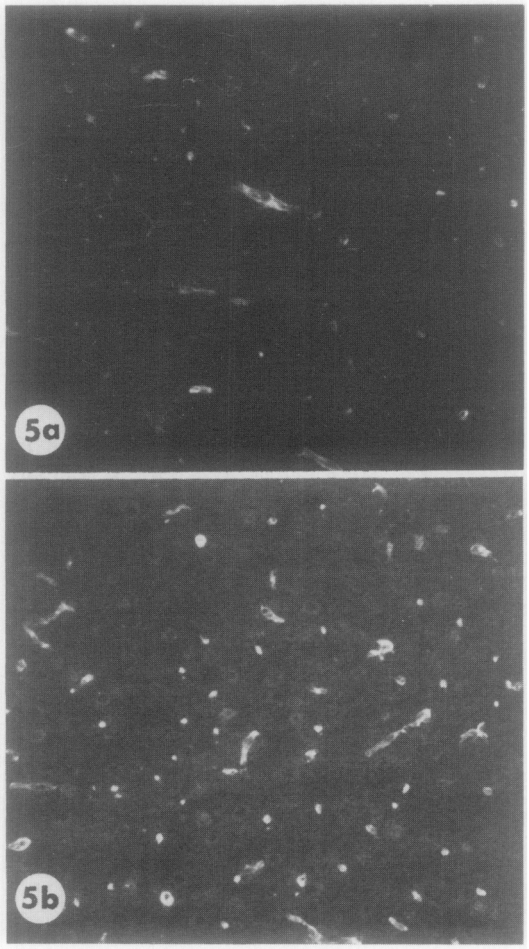

Fig. 5. Mouse midbrain: (*a*) labelled with FITC-B_4, 50 μg/ml, ×160, 60 sec exposure: (*b*) labelled with anti-NBM, 50 μg/ml, ×160, 60 sec exposure.

sections of kidney cortex labelled with FITC-B_4. The specificity of FITC-B_4 binding to kidney sections is exemplified by the ability of coffee bean α-galactosidase to abolish lectin binding (fig. 2*b*). The pattern of fluorescence suggests that FITC-B_4 binds to peritubular and glomerular basement membrane. As a basis of comparison, basement membranes of mouse kidney cortex were visualized with a second probe, antibody to murine parietal yolk sac carcinoma basement membrane (anti-NBM). Midgely & Pierce demonstrated that this antibody binds to epithelial, capillary and muscle basement membranes of normal mouse tissues [17]. The anti-NBM-labelled kidney cortex exhibited peritubular and glomerular fluorescence (fig. 2*d*) similar to that observed with FITC-B_4. The specificity of anti-NBM binding was shown by the absence of fluorescence when non-immune rabbit IgG was substituted for anti-NBM IgG in the staining protocol. Anti-NBM binding differed from FITC-B_4 binding in three respects: (1) anti-NBM visualized a continuous peritubular basement membrane, whereas FITC-B_4 revealed an interrupted peritubular pattern; (2) anti-NBM bound to the basement membrane under the parietal layer of Bowman's capsule, whereas FITC-B_4 did not; and (3) anti-NBM produced a weak, diffuse fluorescence in the tubular epithelia whereas FITC-B_4 did not.

Testis. Sections of testis labelled with FITC-B_4 exhibited fluorescence in peritubular basement membrane and capillaries of the angular interstices (fig. 3*a*). Anti-NBM produced an identical pattern of fluorescence (fig. 3*b*).

Skeletal and cardiac muscle. In both types of muscle, FITC-B_4 bound exclusively to capillaries (fig. 4*a, c*). In contrast, the anti-NBM bound the connective tissue endomysium (fig. 4*b*) surrounding each muscle fiber.

Brain. As in muscle, capillaries were the only structures visualized by FITC-B_4 (fig. 5*a*). This observation was made in several brain regions, e.g. cortex, hippocampus, corpus striatum, and midbrain. An identical result was obtained with anti-NBM (fig. 5*b*).

Liver. Neither FITC-B_4 nor anti-NBM bound to mouse liver sections.

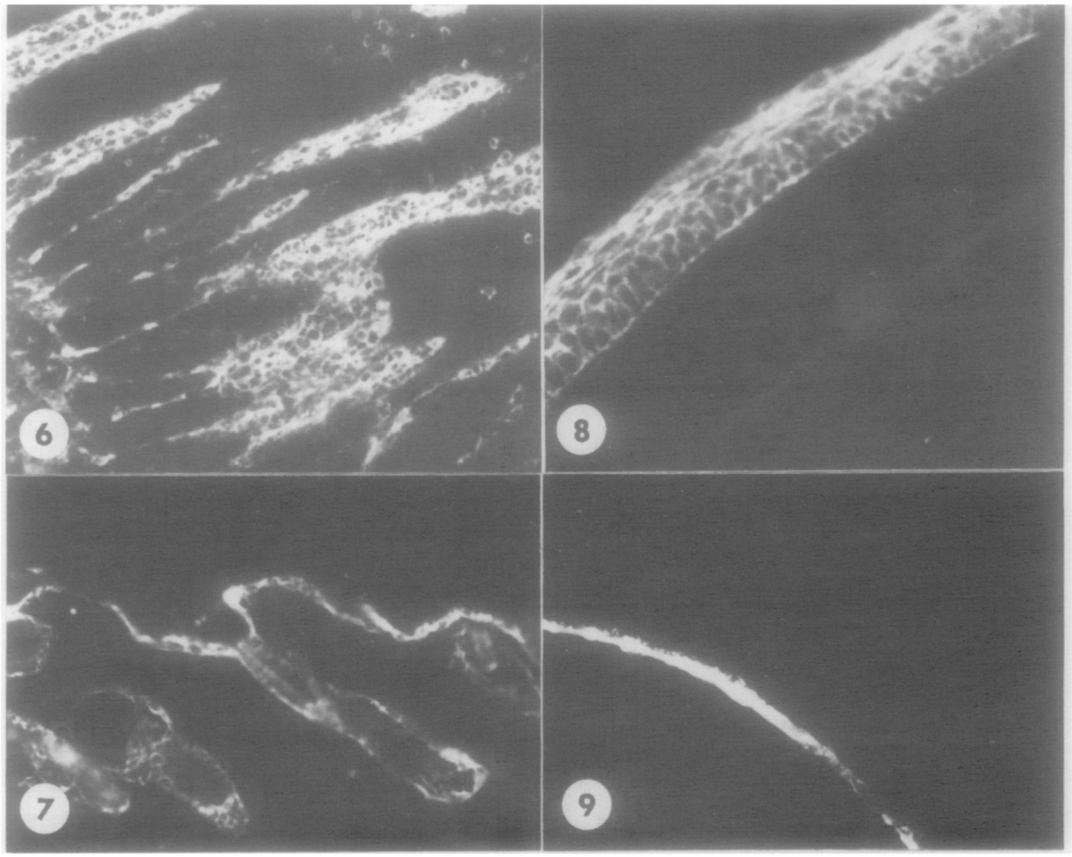

Fig. 6. Mouse duodenum labelled with FITC-B_4, 50 μg/ml, ×160, 30 sec exposure.

Fig. 7. Mouse skin labelled with FITC-B_4, 50 μg/ml, ×250, 60 sec exposure.

Fig. 8. Mouse cornea labelled with FITC-B_4, 50 μg/ml, ×250, 45 sec exposure.

Fig. 9. Mouse lens capsule labelled with FITC-B_4, 50 μg/ml, ×250, 45 sec exposure. The lens capsule has been torn away from the lens. A layer of epithelial cells remains attached to the capsule.

Duodenum. Both FITC-B_4 (fig. 6) and anti-NBM labelled the capillary network of the lamina propria.

Skin. The cells of the stratum basale and the continuation of this cell layer in the external root sheath of the hair follicles, bound FITC-B_4 on the cell surface (fig. 7). This is the first of three examples, along with the epithelia of lens and cornea, of normal cell types that bind FITC-B_4.

Cornea. The predominant locus of FITC-B_4 binding in cornea was the intercellular substance of the corneal epithelium (fig. 8). Bowman's membrane and Descemet's membrane, basement membranes subtending, respectively, the corneal epithelium and the corneal endothelium, exhibited a weak but definite capacity to bind FITC-B_4. The connective tissue of the cornea, rich in collagen and proteoglycan, did not bind FITC-B_4.

Lens. The lens capsule was rendered intensely fluorescent by FITC-B_4. The lens epithelium also bound FITC-B_4 (fig. 9).

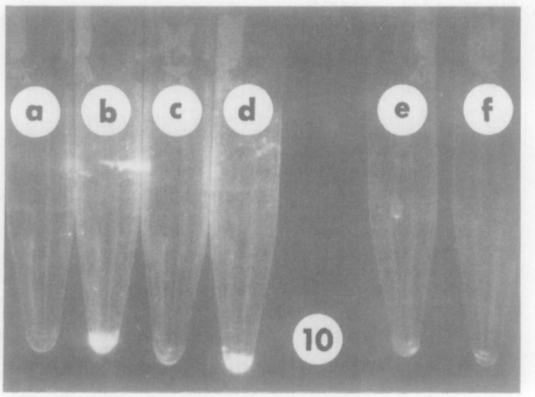

Fig. 10. The absorption of FITC-B$_4$ (100 μg/ml) by suspensions of bovine glomerular basement membrane (*a–d*), Type I collagen from rat skin (*e*) and Type II collagen from the Swarm rat chondrosarcoma (*f*). See Methods for experimental details. Basement membrane was incubated with FITC-B$_4$ containing either (*a*) 5 mM Me α-D-Gal*p* or (*b*) 5 mM Me α-D-Glc*p*. Basement membrane was pre-treated either with (*c*) coffee bean α-galactosidase or (*d*) *A. niger* β-galactosidase prior to incubation with FITC-B$_4$. FITC-B$_4$ bound to the washed pellets is revealed by long wave UV illumination.

The locus of FITC-B$_4$ binding in normal tissues of rabbit, rat, and man

Rabbit. The following tissues were labelled with FITC-B$_4$ identically to the corresponding tissue in mouse: kidney, skeletal and cardiac muscle, brain, duodenum, skin. In contrast to mouse liver, however, an intense uniform fluorescence of rabbit liver sections was observed. This fluorescence was only partially inhibited by Me α-D-Gal*p* and was unaffected by α-galactosidase pretreatment. This is the only significant example of non-specific FITC-B$_4$ binding we have encountered.

Rat. Both kidney and skin were labelled with FITC-B$_4$ with results identical to that obtained in mouse tissue.

Man. The human tissues we have studied did not bind FITC-B$_4$. Brain (autopsy), kidney (biopsy), foreskin and mammary carcinoma have been tested.

The interaction of FITC-B$_4$ with isolated bovine glomerular basement membrane

Glomerular basement membrane has been isolated and characterized by Spiro and coworkers [18–20]. Although this substance is insoluble in PBS buffer [21], FITC-B$_4$ was specifically absorbed to a suspension of glomerular basement membrane, but not to a suspension of either Type I collagen from rat skin or Type II collagen from the Swarm rat chondrosarcoma (fig. 10).

DISCUSSION

The ability of plant lectins to bind specific saccharides has been widely exploited in the study of glycoconjugates of mammalian cells and tissues [22–26]. Of particular importance have been the investigations of Roth & Thoss [23–25] who established fluorescein-conjugated lectins as histochemical reagents. Similarly, we have now employed, the fluorescein-conjugated BS I-B$_4$ isolectin, specific for terminal α-D-galactopyranosyl groups, to study the occurrence and distribution of this uncom-

Table 3. *Distribution of α-D-galactopyranosyl groups in mouse tissues*

Tissue	Structures labelled by FITC-B$_4$
Kidney	Capillaries, glomeruli, peritubular basement membrane
Testis	Capillaries, peritubular basement membrane
Brain	Capillaries
Heart, skeletal muscle	Capillaries
Skin	Capillaries, basement membrane, basal epithelial cells
Eye	
Lens	Lens capsule, lens epithelium
Cornea	Bowman's membrane, Descemet's membrane, corneal epithelium
Liver	(None)
Spleen	Reticular cells

mon carbohydrate unit in tissues of mouse, rabbit, rat and man. The distribution of FITC-B_4 bound to native, cryostat sections of mouse tissues, summarized in table 3, supports the hypothesis that α-D-galactopyranosyl groups occur in epithelial and endothelial basement membranes as well as on the surface of certain epithelial cells.

FITC-B_4 binds to endothelial basement membranes in murine tissues

We have studied endothelial basement membranes of blood vessels (brain, kidney, testis, duodenum, muscle and skin), Descemet's membrane of the cornea, the connective tissue endomysium of skeletal and heart muscle, and the reticular network of spleen. As has been demonstrated by Pierce and coworkers using anti-NBM, these structures share a common endothelial basement membrane antigen [16, 17]. The following observations suggest that endothelial basement membranes are capable of reacting with FITC-B_4:

(1) Both FITC-B_4 and anti-NBM label in a histologically identical fashion blood vessels (of brain, testis, kidney, heart and skeletal muscle, duodenum) and the reticular network of spleen, but neither reagent binds to liver sections. The connective tissue endomysium of muscle, which binds anti-NBM but not FITC-B_4, is the only exception.

(2) FITC-B_4 binds to Descemet's membrane. In collaboration with Dr John Lillie (University of Michigan), we have tested the ability of bovine corneal endothelium, grown in culture, to bind FITC-B_4. These cultured cells secrete a layer of basement membrane biochemically and morphologically similar to Descemet's membrane [27]. We found that FITC-B_4 does not bind to the endothelial cells, but does bind specifically to the basement membrane secreted by the cells.

FITC-B_4 binds to epithelial basement membranes in murine tissues

The epithelial basement membranes we have studied include the peritubular basement membrane of kidney cortex and testis, the basement membrane of skin, Bowman's membrane of cornea and the lens capsule. The following observations suggest that epithelial basement membranes are capable of reacting with FITC-B_4:

(1) Both FITC-B_4 and anti-NBM label peritubular basement membrane of kidney and testis in a similar fashion. An exception in kidney is the basement membrane of the parietal layer of Bowman's capsule which binds anti-NBM but not FITC-B_4.

(2) The lens capsule, of sufficient thickness to be unequivocally identified with the light microscope, avidly binds FITC-B_4.

Specificity of FITC-B_4 binding

To equate fluorescence in tissue sections treated with FITC-B_4 with the occurrence of α-D-galactopyranosyl groups requires a rigorous demonstration of the specificity of FITC-B_4 binding. The first criterion of specific binding, that the lectin combines with the tissue only via its carbohydrate binding site, is supported by the ability of the monosaccharide hapten Me α-D-Gal*p* (but not Me α-D-GalNAc*p*) to completely prevent FITC-B_4 binding. Hapten inhibition of fluorescence is not sufficient, however, to identify the FITC-B_4-binding sites as α-D-galactopyranosyl groups. Two important objections remain: (1) A non-specific lectin–tissue interaction could be inhibited by the appropriate sugar hapten if, as a result of saccharide binding, a conformational change in the lectin distorted the non-specific binding site. Such a phenomenon has

been suggested for concanavalin A [28]. (2) Carbohydrate binding specificity of any lectin is relative. In the case of FITC-B_4, even though its affinity for the monosaccharide Me α-D-Gal*p* is 100-fold higher than for Me β-D-Gal*p* [2], the possibility that the lectin interacts with β-galactosyl groups in tissues is real, especially given optimum orientation and concentration of β-galactosyl groups. These objections are answered by the second criterion of specificity. Fluorescence is abolished by pretreatment of tissue with coffee bean α-galactosidase, a glycosidase capable of specifically hydrolyzing the α-galactosyl groups to which FITC-B_4 binds. That an inhibitor (galactose) of the α-galactosidase prevents its action on kidney sections substantiates that its effect is not due to a protease or glycosidase contaminant. These two criteria, met in all the tissues examined except rabbit liver, justify the identification of α-D-Gal*p* groups in tissue sections by the FITC-B_4 fluorescence.

The TA3 tumor

The BS I-B_4 isolectin at 50 μg/ml agglutinates ascites cells of the TA3-St subline but not of the TA3-Ha subline, confirming the observation by Friberg et al. [3] with a crude extract of *B. simplicifolia* seeds. Both TA3 sublines, however, express α-D-Gal*p* groups on their surface as shown by the ability of FITC-B_4 to bind identically to each. That FITC-B_4 binds, but does not agglutinate, TA3-Ha ascites cells is probably related to the presence of the high molecular weight mucin epiglycannin on the surface of these cells, which has been shown to interfere with the agglutination of these cells by other lectins [29, 30].

The solid tumors produced by both TA3 sublines avidly bind FITC-B_4. The fluorescence is localized to the cell surface or perhaps to intercellular substance. Because FITC-B_4 and anti-NBM bind to identical structures in almost all normal mouse tissues tested, the ability of anti-NBM to bind to the TA3 tumor was examined. In both sublines, the ascites and solid tumor cells bound anti-NBM. (The St solid tumor labelled with anti-NBM is shown in fig. 1*b*.) We therefore add the TA3 carcinomas to the growing list, originated by Dr G. B. Pierce, of murine tumors expressing basement membrane-like substituents on their cell surface [16, 31, 32]. These observations extend the correlation between the occurrence of α-D-galactosyl groups and of basement membrane in murine tissues.

Contrasting BS I-B_4 isolectin with Ricinus communis agglutinin

The remarkable ability of FITC-B_4 to bind exclusively to basement membranes and certain epithelial cells is accentuated by the studies of Roth [25] and Etzler [33, 34] employing fluorescein-labelled *Ricinus communis* agglutinin (FITC-RCA_{120}). Whereas both lectins label the basement membranes of kidney and capillaries of brain, muscle, and duodenum, FITC-RCA_{120} in addition labels structures with which FITC-B_4 does not react: connective tissues of liver and muscle, epithelial cells of the kidney tubules, and the brush borders of kidney tubules and duodenal epithelium. Since both RCA_{120} [35] and BS I-B_4 [2] combine with α-D-Gal*p* groups, it is not surprising that they react with a common subset of histological structures. The primary binding specificity of RCA_{120} is, however, for β-D-Gal*p* groups [35] which occur with high frequency in a variety of mammalian glycoproteins [36]. The interaction of FITC-RCA_{120} with this common saccharide is reflected by its ability to bind to a much more inclusive set of histological structures.

Bretton & Bariety, labelling rat kidney with peroxidase-conjugated RCA_{120}, found lectin-binding sites in the lamina densa of the glomerular basement membrane, in that portion of the pedicell membrane in contact with the basement membrane, and in the mesangial matrix [37]. Similar ultrastructural studies with the BS I-B_4 isolectin would be of interest to determine which, if any, of the RCA_{120} binding sites are α-D-Gal*p* groups.

Nature of the glycoconjugates bearing α-D-Galp groups

Glycolipid. The glycosphingolipids digalactosyl ceramide and trihexosyl ceramide (CTH) possess α-D-Gal*p* groups [8, 42] and occur in murine and human kidney [38–42]. These molecules are potential binding sites for FITC-B_4. The following arguments dissuade us, however, that these binding sites contribute significantly to the fluorescence profiles observed: (1) Chloroform-methanol extraction of cryostat sections of mouse kidney and brain has no effect on the fluorescence pattern; (2) human kidney [39, 40] with a level of CTH comparable to mouse kidney [38], does not bind FITC-B_4: (3) cell types known to contain these glycolipids (BHK-21/C13 [43], human P_1 erythrocytes [44, 51]) do not bind FITC-B_4. The failure of a cell or tissue to bind FITC-B_4 therefore does not prove the absence of α-D-galactosyl groups. Possible explanations for the apparent crypticity of these glycosphingolipids could be: (1) FITC-B_4 combines with these molecules, but they are present in amounts too low to detect by this technique; or (2) the glycolipids are masked by protein or mucopolysaccharide of the glycocalyx [46, 47].

Plasma protein. In light of the studies of Westberg & Michael demonstrating the binding of plasma proteins to glomerular basement membrane [48], it may be argued that the intense reaction of capillaries with FITC-B_4 could be due to the adsorption of a BS I-B_4-reactive plasma protein to capillary endothelium or basement membrane. Our initial observations suggest that mouse plasma does contain small amounts of BS I-B_4-reactive substances. The fact that avascular basement membranes (Descemet's membrane and the lens capsule, and Descemet's membrane in culture) react strongly with FITC-B_4, whereas highly vascularized liver does not, argues against, but does not rule out, this hypothesis.

Basement membrane glycoprotein. Although it has long been known that basement membranes are rich in carbohydrate, the carbohydrate structure of basement membranes is still an unsettled question. Spiro has shown in the case of bovine glomerular basement membrane that half of the hexose occurs as the collagen disaccharide [19] (α-D-Glc*p*-(1→2)-β-D-Gal*p*-(1→*O*) hydroxylysine) [20]. The remainder of the hexose occurs as a polydisperse heterosaccharide [19]. Since FITC-B_4 binds specifically to this preparation of glomerular basement membrane but does not interact with the collagen disaccharide (fig. 10), it is likely that the heterosaccharide moieties bear α-D-Gal*p* groups. Lui & Kalant isolated heterosaccharide glycopeptides from purified rat kidney glomerular basement membrane and demonstrated that on the basis of susceptibility to hydrolysis by coffee bean α-galactosidase 10% of the galactose occurs in α-linkage [49]. Our histological studies with FITC-B_4 confirm the occurrence of α-D-Gal*p* groups in glomerular basement membrane, and we speculate that the heterosaccharide groups constitute, at least in part, FITC-B_4-binding sites in basement membranes of mouse, rat, and rabbit.

In summary, we have established that the BS I-B_4 isolectin is a reliable histochemical probe for α-D-Gal*p* groups, and that these groups are found in basement membranes and certain epithelial cells of mouse, rat, rabbit, and in high amount on the surface of the TA3 murine mammary carcinoma. These studies suggest that the BS I-B_4 isolectin may be of great utility in studying the family of α-D-galactosyl-containing glycoconjugates of basement membranes in those pathological states accompanied by basement membrane changes, such as diabetes mellitus, and in neoplasms that secrete basement membrane. Characterization of the FITC-B_4-binding sites is currently the focus of our investigations.

This work was supported by grants (AM 10171 and CA 20424) from the NIH, USPHS. We are grateful to Dr G. B. Pierce (University of Colorado) and to Dr A. Martinez (Hahnemann Medical College) for their generous gift of the antibody to murine neoplastic basement membrane. We thank Dr R G. Spiro (Harvard Medical School) for kindly supplying bovine glomerular basement membrane and rat skin collagen, and Dr V. C. Hascall (NIH) for his gift of Type II collagen from the Swarm rat chondrosarcoma. We thank Dr M. J. Deegan (University of Pennsylvania) for his advice and helpful discussions. We gratefully acknowledge the help of the following people from the University of Michigan: Dr B. W. Agranoff for the use of the fluorescence microscope and Eva Feldman for her help with the microscopy; Dr T. V. Fischer for the use of the cryostat; Dr J. H. Lillie for supplying cultures of bovine corneal endothelium; Paul Fan and Dr P. Datta for supplying BHK21/C13 cells; and John Judd for supplying human P_1 erythrocytes; Kay Brabac for helpful discussion and Allen Eckhardt for suggesting the use of the guaran column to purify FITC-B_4. A preliminary report of this work was presented at the ASBC/AAI Meetings, June, 1978, Atlanta, Ga.

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Received September 29, 1978
Accepted November 20, 1978