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Use of *Phaseolus vulgaris* leukoagglutinating lectin in histochemical and blotting techniques: a comparison of digoxigenin- and biotin-labelled lectins

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Abstract. An increase in the number of β 1,6 branches of the trimannosyl core of asparagine-linked oligosaccharides has been shown to be directly correlated with the metastatic potential of cultured tumour cells. The *Phaseolus vulgaris* leukoagglutinating lectin (PHA-L) binds to β 1,6 branches of tri- and tetra-antennary oligosaccharides. We have applied digoxigenin- and biotin-conjugated PHA-L to establish a non-radioactive detection system for β 1,6 branches, which can be used in lectin blotting as well as light and electron microscopic cytochemistry. For this purpose the HCT116 human colon carcinoma cell line and colon carcinoma tissue were investigated. Digoxigenin-conjugated PHA-L in conjunction with alkaline phosphatase-conjugated anti-digoxigenin antibodies was superior to biotin-conjugated PHA-L in lectin blotting with respect to sensitivity and specificity. Similarly, the digoxigenin conjugated PHA-L in conjunction with gold-labelled anti-digoxigenin antibodies resulted in more intense specific staining and lower background compared to biotin-conjugated PHA-L visualized with a streptavidin immunogold complex. The specificity of lectin binding in blotting and cytochemical studies was demonstrated by the absence of staining when the lectin was omitted or preabsorbed with glycoprotein, and following pretreatment of the cellular homogenates or tissue sections by *N*-glycosidase F. Our results demonstrate that digoxigenin-conjugated PHA-L provides high sensitivity and specificity for histochemical and blotting techniques and is amenable for quantification. The technique should have applications in tumour research.

Introduction

Alterations in the composition and structure of cell surface glycoconjugates have been found to occur following malignant transformation of cells (Feizi 1985; Fukuda 1985; Hakomori 1989; Dennis 1992). Various basic forms of alterations can be distinguished which comprise incomplete synthesis of oligosaccharide side chains, enhanced synthesis of a structure normally present, occurrence of oligosaccharidic structures not found in the parental normal cell types, and re-expression of glycoconjugates exclusively found during organogenesis in the fetus. Many of these observed changes have become useful as histochemical markers in diagnostic pathology although their functional significance remains to be elucidated. In a few cases, however, the functional significance of the altered glycoconjugate expression for the growth behaviour of the tumour cells could be established (Yogeeswaran and Salk 1981; Fogel et al. 1983; Irimura et al. 1986; Dennis et al. 1987, 1989; Roth et al. 1988a, b; Morgenthaler et al. 1990; Scheidegger et al. 1994).

The transformation-related increase in the size of asparagine-linked oligosaccharides seems, in part, to be due to an increase in the number of GlcNAc β1,6 Man α1,6 branches on the trimannosyl core (Yamashita et al. 1984), the so-called β 1,6 branching. In virus and oncogene-transformed cell lines, the increased β 1,6 branching has been shown to be correlated with elevated levels of the *N*-acetylglucosaminyltransferase V involved in the synthesis of this structure (Yamashita et al. 1985; Yousefi et al. 1991). Studies on animal and human cell lines have revealed that the increase in the number of β 1,6 branches is directly correlated with the metastatic potential (Dennis et al. 1987, 1989a, b; Dennis and Laferté 1989; Laferté and Dennis 1989; Rosenwald et al. 1989; Yagel et al. 1989; Fernandes et al. 1991; Yousefi et al. 1991). Despite this demonstrated correlation, certain normal cell types have been shown by histochemical techniques to possess a high number of histochemically detectable β 1,6 branches (Dennis and Laferté 1989; Dennis 1992).

Currently two reagents exist for the detection of β 1,6 branches. Firstly, an antiserum raised against complete Freund's adjuvants containing *Mycobacterium smegmatis* (Chechik et al. 1986), which was shown to bind with highest affinity to five to six GlcNAc residues linked to mannose (Chechik and Brockhausen 1988). By immuno-

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histochemistry, this antiserum stained neoplastic and sclerodermal chicken fibroblasts but rarely stained normal quiescent chicken fibroblasts (Chechik and Fernandes 1989). Secondly, the generally available *Phaseolus vulgaris* leukoagglutinating lectin (PHA-L), which binds to β 1,6 branches of tri- and tetra-antennary oligosaccharides (Cummings and Kornfeld 1982; Hammarström et al. 1982; Green and Baenziger 1987). Further glycosylation resulting in the addition of polylactosamine and α 2,3-linked sialic acid does not impair PHA-L binding whereas the occurrence of α 2,6-linked sialic acid at certain antennae reduces lectin binding (Bierhuizen et al. 1988). Dennis and colleagues have extensively used PHA-L in conjunction with a specific antiserum and an indirect streptavidin-biotin-peroxidase detection system for histochemical investigations on normal and transformed cells and tissues (Dennis et al. 1987; Dennis and Laferté 1989; Fernandes et al. 1991; Yousefi et al. 1991). For the detection of the PHA-L reactive glycoproteins by blotting techniques they used directly ^{125} I -labelled lectin (Dennis et al. 1987) or an indirect immunoenzyme technique (Dennis and Laferté 1989).

In the present study we have tested digoxigenin (dig)- and biotin-labelled PHA-L in order to establish a non-radioactive detection system, which can be used in lectin blotting as well as light and electron microscopic investigations. Alkaline phosphatase-conjugated anti-dig antibodies and streptavidin were used as revealing reagents in lectin blotting techniques. Gold-labelled anti-dig antibodies (Sata et al. 1990a) and a streptavidin-biotin immunogold technique (Roth et al. 1992a, b) were applied for visualization by light and electron microscopy. The results demonstrated that dig-labelled PHA-L is superior to biotin-labelled lectin for blotting and for microscopy since it provides higher specificity and greater intensity of staining.

Materials and methods

Reagents and gold labelling

Dig-conjugated PHA-L, polyclonal sheep anti-dig antibodies, alkaline phosphatase-conjugated polyclonal sheep anti-dig Fab' fragments, streptavidin-alkaline phosphatase conjugate, recombinant *N*-glycosidase F, *Vibrio cholerae* sialidase and nitroblue tetrazolium/X-phosphate were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). Biotinylated PHA-L was obtained from Boehringer Mannheim Biochemica, Sigma Chemicals Co., Ltd. (St Louis, Mo., USA) and Vector Laboratories (Burlingame, Calif., USA). Affinity-purified rabbit anti-horseradish peroxidase (HRP) antibody and streptavidin-HRP conjugate were from Jackson Immuno Research Laboratories, (West Grove, Pa., USA). Monodisperse colloidal gold with a diameter of 8 nm was prepared according to Slot and Geuze (1985). Sheep anti-dig and rabbit anti-HRP antibodies were complexed to 8 nm gold particles as described by Sata et al. (1990a) and Gee et al. (1991), respectively. Bovine thyroglobulin, bovine serum albumin and poly-L-lysine (mol.wt. 150 000) were obtained from Sigma Chemical Co., Ltd. (St Louis, Mo., USA). Silver acetate, hydroquinone and glutaraldehyde (25%, vacuum distilled) were from Fluka (Buchs, Switzerland), and RPMI 1640 medium, fetal calf serum and Hanks balanced salt solution (HBSS) from Gibco (Life Technologies Ltd., Paisley, UK).

Cell culture and tissue processing

HCT116, a human colon carcinoma cell line, was obtained from Prof. R. Brossmer (University of Heidelberg) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Exponentially growing cells were rinsed with HBSS and fixed in 2% paraformaldehyde-0.1% glutaraldehyde in HBSS (pH 7.4) for 30 min at 4°C. The fixation was stopped by ice-cold 50 m*M* NH_4Cl in PBS (10 m*M* phosphate buffer, pH 7.4, 0.15 *M* NaCl) for 30 min to amidinate free aldehyde groups. The cells were mechanically removed from the petri dishes and enclosed in 2% bacto-agar (Difco Laboratories, Detroit, Mich., USA) in PBS. Samples were embedded in paraffin according to a routine protocol or in Lowicryl K4M at low temperature as previously described (Roth et al. 1981; Roth 1989). Human colon carcinoma tissue was obtained at surgical intervention, then fixed in phosphate-buffered formaldehyde and embedded in paraffin.

Cytochemical labelling

For light microscopical labelling, paraffin sections (~5 μm-thick) and semithin Lowicryl K4M sections (1 μm-thick) of HCT116 cells and colon carcinoma tissues were cut and placed on poly-*L*-lysine-activated glass slides (Lucocq and Roth 1985 a, b). Paraffin sections were dewaxed in two changes of xylene (10 min each) and rehydrated through a series of graded ethanol and placed in PBS. The sections (paraffin and Lowicryl K4M) were conditioned with PBS (pH 7.4) containing 1% bovine serum albumin, 0.05% Tween 20 and 0.05% Triton X-100 (buffer 1) for 10 min at room temperature. The sections were then incubated with either dig-PHA-L (10 μg/ml in buffer 1) or biotin-PHA-L (10 μg/ml in buffer 1) for 1 h at room temperature followed by two rinses in PBS (5 min each). Sections incubated with dig-PHA-L were covered with gold-labelled anti-dig antibodies (diluted with buffer 1 to give an $OD_{525\,nm}$ of 0.05) for 1 h at room temperature. Sections incubated with biotin-PHA-L were covered with a preformed soluble complex of streptavidin-HRP conjugate (1.25 μg/ml in buffer 1) and anti-HRP-gold ($OD_{525\,nm}$ of 0.1) for 1 h at room temperature (Roth et al. 1992b). Following this incubation step, sections were rinsed in PBS twice for 5 min each, fixed with 1% glutaraldehyde in PBS for 20 min, then briefly rinsed in PBS and several changes of double distilled water (5–10 min) and air dried. Signal amplification by a photochemical silver reaction was carried out as previously described (Roth 1989; Roth et al. 1992a). Paraffin sections were stained with nuclear fast red as required.

For electron microscopical labelling, ultrathin sections of Lowicryl K4M embedded HCT116 cells were placed on Parlodion/carbon coated 150-mesh nickel grids. Grids with the thin sections were conditioned for 10 min at room temperature on droplets of PBS (pH 7.4) containing 1% BSA, 0.01% Tween 20 and 0.01% Triton X-100 (buffer 2). The incubation steps with dig- and biotin-PHA-L, gold-labelled anti-dig antibody, soluble complex of streptavidin-HRP and anti-HRP-gold were performed as described above for light microscopy in buffer 2 except that the lectins were used at a concentration of 30 μg/ml. After the incubation steps, grids with the attached thin sections were rinsed with PBS (6 × 3 min) and double distilled water, air-dried and counterstained with uranyl and lead acetate (Roth 1989). Thin sections were examined with a EM 910 Zeiss electron microscope.

Quantification of gold particle labelling

Photographs were taken at the original magnification of × 20 000 from Lowicryl K4M thin sections incubated with either dig- or biotin-PHA-L followed by the respective gold-labelled secondary reagents or incubated with the gold-labelled secondary reagents alone. The density of gold particles per length in μm of free and lateral plasma membrane was evaluated on photographic prints from at least 50 different cells.

Lectin blotting

HCT116 cell samples were homogenized on ice in PBS containing 1% Triton X-100, 1 m*M* phenyl methyl sulphonyl fluoride (PMSF) and 1% aprotinin. After centrifugation (10 000 × g for 5 min), the supernatant (2.5–6 mg protein/ml as determined by the Bradford assay) was denatured by boiling in Laemmli buffer for 5 min. The samples (40 µg per lane) were electrophoretically resolved in 3–10% gradient SDS-polyacrylamide gels and transferred to nitrocellulose essentially according to the method of Towbin et al. (1979) but using a semidry blotting apparatus. The nitrocellulose strips were blocked for 1 h with TBS (0.1 *M TRIS*-HCl, pH 7.4, 0.15 *M* NaCl) containing 1% defatted milk powder and 0.05% Tween 20. Subsequently the strips were incubated at room temperature with either dig- or biotin-PHA-L (0.2 µ g/ml in TBS containing 0.05% Tween 20). PHA-L binding to proteins on the nitrocellulose was detected using either alkaline phosphatase-conjugated polyclonal sheep anti-dig Fab' fragments or streptavidin-alkaline phosphatase conjugate (both diluted 1:5000 in TBS containing 0.05% Tween 20) for 1 h at room temperature followed by the nitroblue tetrazolium/X-phosphate reaction according to the manufacturer's recommendations.

In order to suppress endogenous streptavidin-binding material, blocked nitrocellulose strips were incubated with streptavidin (0.013–0.33 mg /ml TBS) followed by D-biotin (0.04–1 mg/ml TBS) before the biotin-PHA-L incubation steps.

Spot blots

Serial dilutions of thyroglobulin and HCT116 cell homogenate (0.5 µl) were spotted onto nitrocellulose strips and air-dried. Nitrocellulose strips were blocked with buffer 1 containing 1% defatted milk followed by the incubation steps described above for lectin labelling for light microscopy.

Controls

The specificity of the lectin blotting and histochemical staining was checked by *N*-glycosidase F pretreatment of the protein samples or sections, by omission of the dig- or biotin-PHA-L, and by preabsorption of the PHA-L conjugates with thyroglobulin prior to use.

Enzymatic removal of asparagine-linked oligosaccharides by *N*-glycosidase F (Plummer et al. 1984; Tarentino et al. 1985; Lemp et al. 1990) was performed as follows. To SDS- and heat denatured protein samples (5 µl containing 40 µg protein) 1.6 units of recombinant *N*-glycosidase F in 35 µl of incubation buffer [100 mmol/l sodium phosphate, pH 7.4; 25 mmol/l EDTA, 1.5% Nonidet P40, (v/v)] were added and incubated for 17 h at 37°C. Dewaxed and rehydrated sections were pre-washed with incubation buffer for 3 min and incubated with *N*-glycosidase F (2 units in 200 µl incubation buffer) for 17 h at 37°C. The sections were subsequently washed with the incubation buffer (3 min) and PBS followed by PHA-L staining as described above. In glycoprotein inhibition tests, dig- or biotin-PHA-L was incubated with 9 µ*M* bovine thyroglobulin in PBS for 2 h at room temperature prior to use.

In order to evaluate the influence of sialic acids on the staining intensity with PHA-L, sialic acid residues were removed using *V. cholerae* sialidase. For lectin blotting, the samples were treated with 0.5 units/ml of sialidase in acetate buffer (50 m*M* sodium acetate, 4 m*M* calcium chloride, 0.01% BSA, 0.05% Tween 20, pH 5.5) for 17 h at 37°C. Dewaxed and rehydrated sections were washed in acetate buffer for 3 min and incubated with 1 unit/ml of sialidase solution for 6 h at 37°C. After washing in acetate buffer (3 min) and PBS, the sections were incubated for PHA-L staining as described above. In other experiments protein samples and sections were treated with acetate buffer alone.

Results

PHA-L reactive glycoproteins in HCT116 colon carcinoma cells

By lectin blotting using dig-PHA-L a single broad band at approximately 140 kDa was detectable (Fig. 1, lane 1). This reactivity was abolished when the dig-PHA-L was either omitted (Fig. 1, lane 5) or preincubated with thyroglobulin (Fig. 2, lane 4). Furthermore, pretreatment of the samples with *N*-glycosidase F resulted in the absence of the PHA-L reactive band (Fig. 2, lane 3). The use of biotin-PHA-L from different commercial sources revealed the same band detected with dig-PHA-L (Fig. 1, lanes 2–4). However, additional faint bands of higher molecular weight and many strongly positive bands with molecular weights of less than 140 kDa were observed (Fig. 1, lanes 2–4). These bands were still visible under the different conditions of the specificity controls (Fig. 1, lane 6; Fig. 2, lanes 7 and 8), which resulted in the absence of the 140 kDa band. Preincubation of the nitrocellulose strips with excess amounts of streptavidin and biotin did not prevent the occurrence of the numerous unspecific bands (data not shown).

Sialidase pretreatment of the homogenates did not increase the intensity of the PHA-L reactivity; however the electrophoretic mobility of the reactive glycoprotein(s)

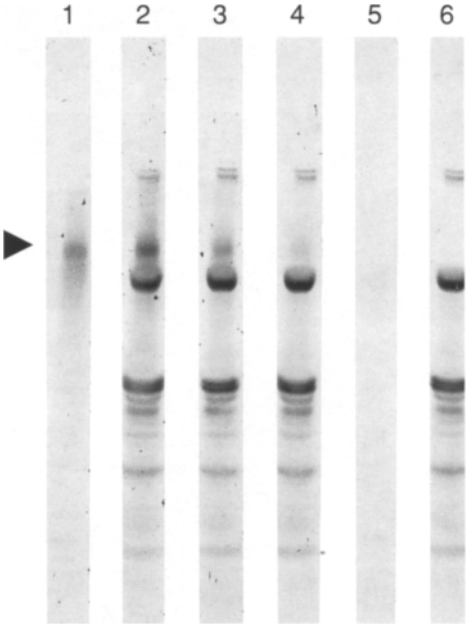

Fig. 1. Detection of *Phaseolus vulgaris* leukoagglutinating lectin (PHA-L) reactive glycoproteins of HCT116 colon carcinoma cells by lectin blotting. Cellular homogenates were resolved electrophoretically in 3–10% gradient polyacrylamide gels and transferred to nitrocellulose. *Lane 1.* Incubation with digoxigenin (dig)-labelled PHA-L and alkaline phosphatase-conjugated dig antibodies; *lanes 2–4*, incubation with biotin-PHA-L from Boehringer, Sigma and Vector, respectively, and alkaline phosphatase-conjugated streptavidin; *lane 5*, omission of dig-PHA-L; *lane 6*, omission of biotin-PHA-L. The *arrowhead* marks the position of the 140-kDa band to which specific PHA-L binding occurred. The additional bands seen in *lanes 2–4* and *6* are not due to specific lectin binding

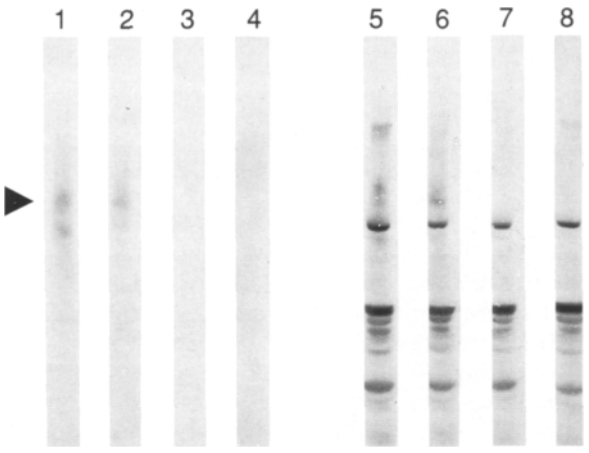

Fig. 2. Same material and procedures as in Fig. 1. *Lane 1*, dig-PHA-L; *lane 2*, sialidase pretreatment; dig-PHA-L; *lane 3*, *N*-glycosidase F pretreatment; dig-PHA-L; *lane 4*, preabsorption of dig-PHA-L with thyroglobulin; *lane 5*, biotin-PHA-L; *lane 6*, sialidase pretreatment; biotin-PHA-L; *lane 7*, *N*-glycosidase F pretreatment; biotin-PHA-L; *lane 8*, preabsorption of biotin-PHA-L with thyroglobulin. The *arrowhead* marks the position of the 140 kDa band

was increased due to the removal of sialic acid (Fig. 2, lanes 2 and 6). The amount of a given glycoprotein and HCT116 homogenate was positively correlated to the intensity of lectin staining in spot blots (not shown).

PHA-L staining in HCT116 colon carcinoma cells

When semithin sections of Lowicryl K4M embedded HCT116 cells were stained for PHA-L binding sites, cell surface and intracellular labelling were observed with the use of dig-PHA-L (Fig. 3a). The intensity of the cell surface-associated staining was variable, with cells exhibiting strong, moderate and weak staining. No labelling was detectable when the dig-PHA-L was omitted (Fig. 3b). Cell surface and intracellular staining were also found when biotin-PHA-L was applied although at a much lower intensity compared to the staining reaction obtained with the dig-PHA-L (not shown). No staining was observed when the biotin-PHA-L was omitted in the incubation protocol (not shown).

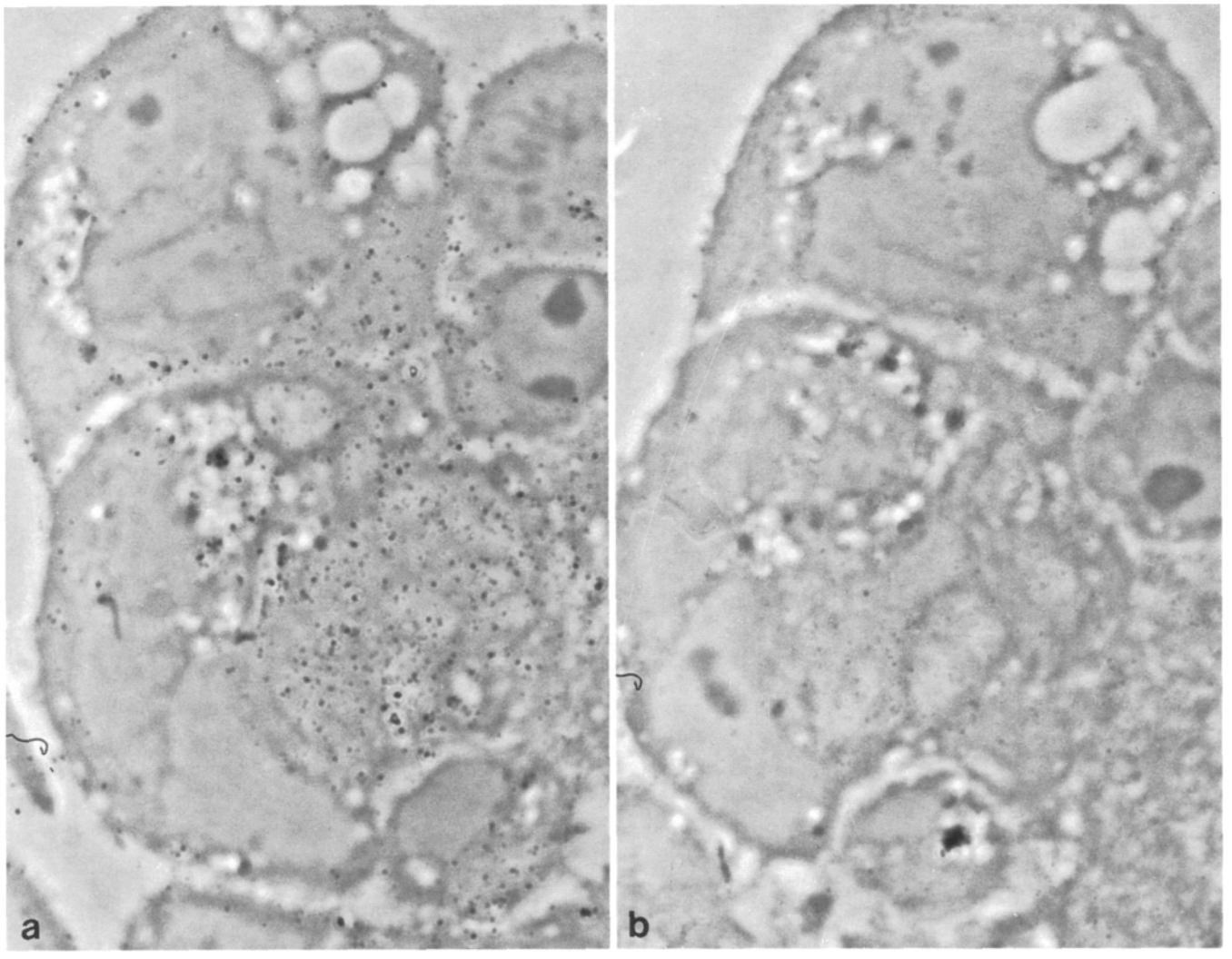

Fig. 3a,b. Adjacent semithin sections of Lowicryl K4M embedded HCT116 colon carcinoma cells incubated with dig-PHA-L followed by gold-labelled dig antibodies and silver intensification. **a** Specific lectin labelling is present along the cell surface and in the cytoplasm. The intensity of labelling varies from cell to cell. **b** Omission of the dig-PHA-L results in absence of cellular staining. **a–b** ×1750

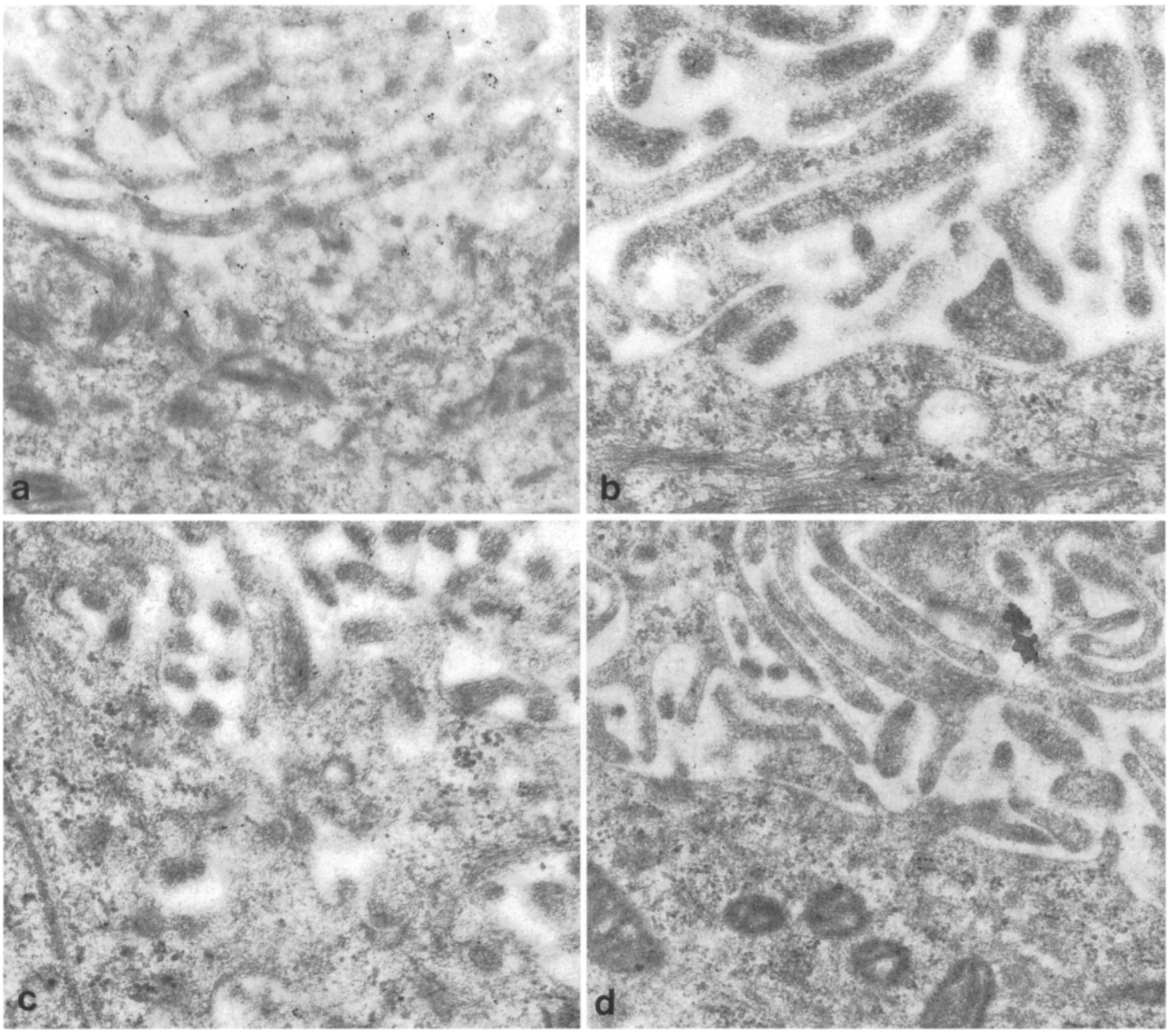

Fig. 4a–d. Ultrathin sections of Lowicryl K4M embedded HCT116 colon carcinoma cells. **a** Numerous gold particles are present along the plasma membrane and the microvilli following incubation with dig-PHA-L and gold-labelled dig antibodies. **b** Control incubation in which the dig-PHA-L was omitted. **c** Only a few gold particles are present along the plasma membrane following incubation with biotin-PHA-L and streptavidin immunogold complex. **d** Omission of biotin-PHA-L. **a** × 29 500; **b** × 40 000; **c** × 42 750; **d** × 28 500

The incubation of ultrathin sections of Lowicryl K4M embedded HCT116 cells resulted in labelling by gold particles at the outer surface of the plasma membrane. The labelling intensity was higher with dig-PHA-L compared to biotin-PHA-L (Fig. 4a and c Table 1). As already observed in semithin Lowicryl K4M sections, the intensity of cell surface labelling showed variations from cell to cell. Intracellular labelling was detectable over limited, bona fide *trans* regions of the Golgi apparatus cisternal stack, cytoplasmic vesicles and lysosomes but not over cisternae of the endoplasmic reticulum, nucleoplasm and mitochondria. Both cell surface and intracellular labelling by gold particles were very low when the dig-PHA-L and the biotin-PHA-L incubation steps were omitted (Fig. 4 b and d, Table 1).

In paraffin sections of HCT116 cells, dig-PHA-L yielded a clearly detectable staining reaction along the cell surface and in the cytoplasm. (Fig. 5a). The latter was characterized by the presence of positive granules of varying sizes. The use of biotin-PHA-L resulted in a weaker level of cellular staining (Fig. 5c). Omission of either dig-PHA-L (Fig. 5b) or biotin-PHA-L (Fig. 5d) resulted in the absence of cellular staining. Moreover pretreatment of tissue sections with *N*-glycosidase F (Fig. 5e) and preincubation of PHA-L with thyroglobulin (Fig. 5 f) abolished the staining with dig-PHA-L. Sialidase pretreatment of the sections had no detectable influence on the intensity of PHA-L staining (not shown).

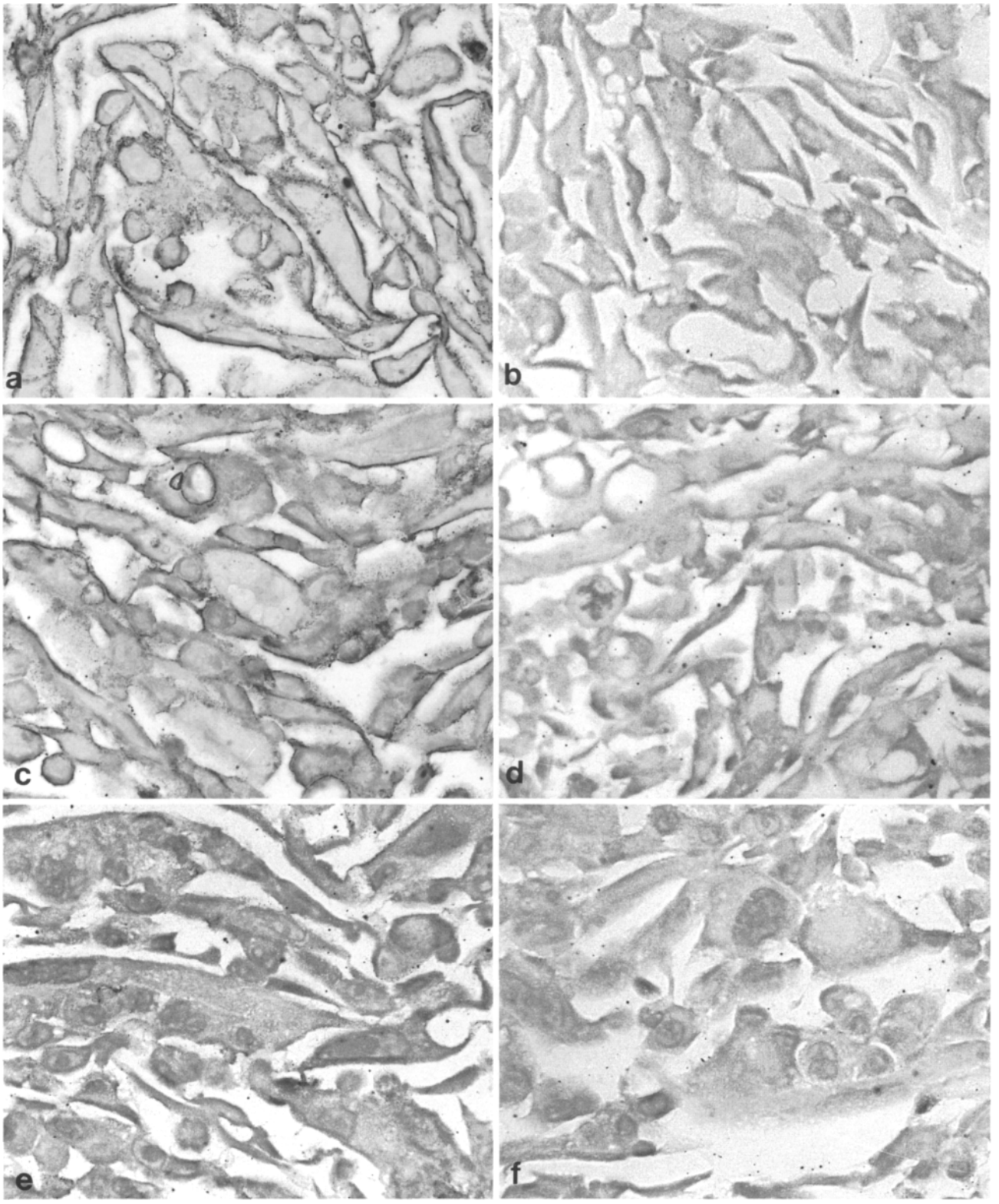

Fig. 5a–f. Sections of paraffin embedded HCT116 colon carcinoma cells; silver intensification of the gold particle labelling and counterstaining with nuclear fast red. **a** Intense cell surface staining and some intracellular labelling is observed following incubation with dig-PHA-L and gold-labelled dig antibodies. **b** Control incubation in which dig-PHA-L was omitted. **c** Intensity of cell surface staining is generally weaker using biotin-PHA-L and streptavidin immunogold complex (cp.to Fig. 5a). **d** Omission of biotin-PHA-L. **e** Pretreatment with *N*-glycosidase F followed by incubation with dig-PHA-L and gold-labelled dig antibodies. **f** Preincubation of dig-PHA-L with thyroglobulin. **a–c, e, f** × 750; **d** × 400

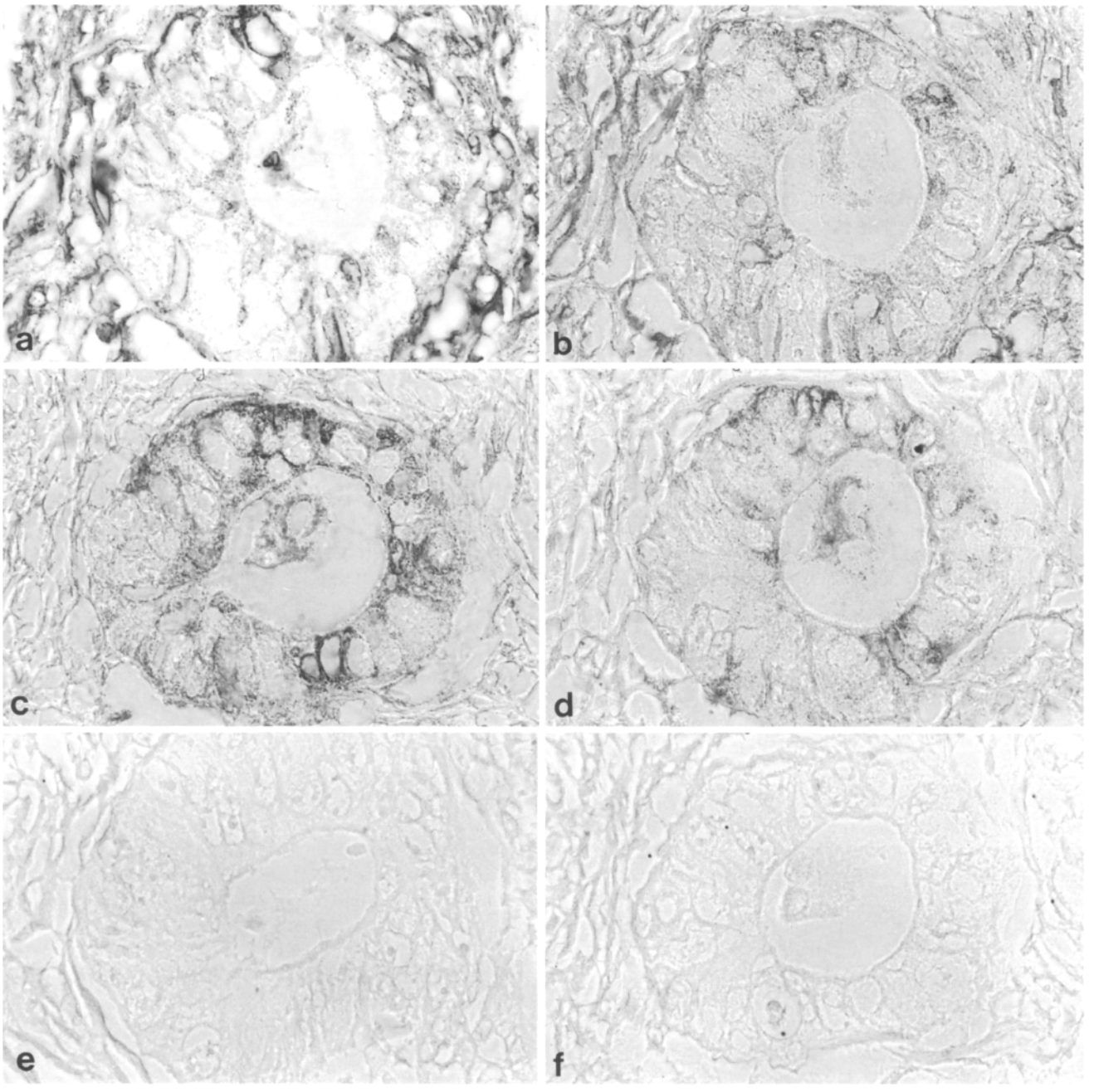

Fig. 6a–f. Consecutive sections of paraffin embedded human colon carcinoma; silver intensification of the gold particle labelling. A cross-sectioned gland-like structure with surrounding interstitial tissue is shown. **a** Incubation with dig-PHA-L and gold-labelled dig antibodies. **b–d** Incubation with biotin-PHA-L from (**b**) Boehringer. (**c**) Incubation with biotin-PHA-L from Sigma and (**d**) Vector. **e** Omission of dig-PHA-L or **f** of biotin-PHA-L. **a–f** × 850

PHA-L staining in paraffin sections of colon carcinoma

Serial sections of archival paraffin blocks of human colon carcinomas were processed for direct comparison of the results of the different staining protocols. The use of dig-PHA-L in conjunction with gold-labelled anti-dig antibodies and silver intensification resulted in an intense staining reaction on the surface and in the cytoplasm of the colon carcinoma cells and various cells of the stroma (Fig. 6a). Carcinoma cells in single gland-like structures exhibited varying degrees of cell surface-associated and intracellular staining (Fig. 6 a). The use of biotin-PHA-L also resulted in positive staining in the same glands but usually of lower intensity (Fig. 6b–d). Carcinoma cells weakly positive with dig-PHA-L were not positive with biotin-PHA-L upon examination of consecutive sections. Omission of the PHA-L resulted in the absence of the staining reaction (Fig. 6e and f).

Table 1. Comparison of labelling intensities in HCT116 colon carcinoma cells obtained with digoxigenin-labelled *Phaseolus vulgaris* leukoagglutinating lectin (dig-PHA-L) and biotin-PHA-L

Plasma membrane	dig-PHA-L	biotin-PHA-L	Controls[a]	
			dig	biotin
Free	1.82±0.14[b]	0.46±0.06	0.04±0.01	0.11±0.02
Lateral	1.75±0.15	0.67±0.09	0.04±0.02	0.13±0.02

[a] Controls consisted of omission of the respective lectin conjugate
[b] Number of gold particles per length in μm of plasma membrane. Values are based on the examination of micrographs from at least 50 different cells

Discussion

Changes in cellular glycoconjugates occurring during malignant transformation of cells may be of relevance for diagnostic and prognostic purposes. The increased number of β 1,6 branches in asparagine-linked glycoproteins seems to be directly correlated with the metastatic potential of tumour cells (Dennis et al. 1987, 1989b; Dennis 1992). Therefore, techniques to detect and quantitate such changes at the cellular and subcellular levels and to identify the molecule(s) involved would be desirable.

In the present study we have used the lectin PHA-L to detect β 1,6 branches as performed by other investigators in previous studies (Dennis et al. 1987; Dennis and Laferté 1989; Fernandes et al. 1991). However instead of using an indirect streptavidin-biotin-immunoperoxidase visualization system, we applied immunogold techniques and silver intensification for light microscopy. Applying the immunogold techniques we also localized PHA-L binding sites for the first time by electron microscopy. Furthermore, we have compared dig-PHA-L and biotin-PHA-L with respect to the labelling efficiency and specificity on sections of cultured cells and tumours. Both dig-PHA-L and biotin-PHA-L in conjunction with alkaline phosphatase conjugated antibodies or streptavidin were tested for use in lectin blotting techniques. Our present results provide further evidence for the versatility of the steroid hapten digoxigenin in carbohydrate research (Haselbeck and Hösel 1990; Haselbeck et al. 1990, Sata et al. 1990a, b). Unlike the ubiquitous vitamin biotin, the steroid hapten digoxigenin is not present in animal cells and tissues and, therefore, is less likely to contribute to the background staining or false positive results.

When applied in blotting experiments to identify PHA-L reactive glycoproteins, a single positive band was consistently observed with the use of dig-PHA-L. The specificity of this reaction could be demonstrated in various controls including digestion of cell homogenate with *N*-glycosidase F to remove asparagine-linked oligosaccharides. When the biotin-PHA-L was applied in lectin blotting, a number of additional reactive bands were observed which remained reactive under the various control conditions. This non-specific binding of alkaline phosphatase-conjugated streptavidin could not be overcome by preincubation of the nitrocellulose (after transfer of the electrophoretically resolved proteins) with streptavidin and biotin. The reasons for the failure of this protocol as applied successfully to tissue sections (Wood and Warnke 1981; Banerjee and Pettit 1984) are not understood at present. In blotting techniques, other advantages provided by dig-PHA-L compared to ^{125}I-labelled PHA-L are the shorter turnaround time, the avoidance of radioactively labelled reagents and the long shelf life of digoxigenated reagents.

Both the dig- and biotin-conjugated PHA-L could be successfully applied for staining by light microscopy on paraffin and semithin Lowicryl K4 M sections. In contrast to the problems of background reactivity encountered in lectin blots, the biotin-PHA-L as detected with a streptavidin immunogold complex (Roth et al. 1992b) produced virtually no background staining in sections of cultured colon carcinoma cells and carcinoma tissues. Preincubation of the sections with streptavidin and biotin was not required. However at the electron microscope level, threefold higher non-specific binding was found of the streptavidin immunogold complex compared to the gold-labelled dig antibodies. In certain tumour cell lines the levels of endogenous biotin-containing enzymes have been shown to vary depending on their degree of tumorigenicity (Bramwell and Humm 1992). Accordingly, varying degrees of background levels may be found which may interfere with the quantitative evaluation of the staining results (see below). Furthermore we observed that the three tested commercial preparations of biotin-PHA-L resulted in weaker staining compared to dig-PHA-L. This was also evident when the intensity of gold particle labelling at the plasma membrane was quantified by electron microscopy. This finding is surprising in view of the anticipated higher amplification effect due to the use of the streptavidin immunogold complex used in conjunction with biotin-PHA-L. The reasons for this observation are not clear at present and could be manyfold. We observed that the digoxigenin-to-lectin molar ratio is important for optimal results and that a certain upper threshold exists after which the dig-lectin binding is drastically reduced (unpublished). It could not be determinded whether or not this phenomenon was of importance in our staining experiments with biotin-PHA-L.

The binding of PHA-L to glycoproteins has been shown to be abolished or reduced if addition of α2,6-linked sialic acid occurs either to the Gal β1,4 GlcNAc β1,2 Man α1,6 branch or the Gal β1,4 GlcNAc β1,6 Man α1,6 branch in oligosaccharides (Bierhuizen et al. 1988). Pretreatment of sections of HCT116 colon carcinoma cells with *V. cholerae* sialidase did not result in stronger lectin labelling. This finding is in agreement with

data showing that the observed 140 kDa PHA-L reactive band does not contain α 2,6-linked sialic acid as detected with the *Sambucus nigra* I lectin (Murayama T, Zuber C. and Roth J., in preparation). Therefore the higher electrophoretic mobility of the PHA-L reactive band observed after sialidase treatment must be due to sialic acid present in other ketosidic linkages. Similar to the observation for HCT116 colon carcinoma cells, sialidase pretreatment of colon carcinoma sections did not influence the intensity of lectin labelling. The reported carcinoma-associated occurrence of α 2,6-linked sialic acid in human colon carcinomas (Sata et al. 1991) does not interfere with the PHA-L staining since it is not present on the PHA-L reactive 140 kDa glycoprotein. In the spot blots, the intensity of the silver intensified gold particle signal was positively correlated to the amount of thyroglobulin and HCT116 colon carcinoma cell homogenate. Taken together, these results indicate that the observed differences in PHA-L staining are most probably due to differences in the amount of β 1,6 branches in the cultured colon carcinoma cells and the colon carcinoma. This is an important conclusion for the further application of the technique to study PHA-L binding patterns in human colon carcinoma tissue sections. Studies to estimate a possible correlation of the PHA-L staining pattern and the tumour stage as well as the growth behaviour of the carcinoma are in progress. In conclusion, we have shown that dig-PHA-L provides high sensitivity and specificity for histochemical and blotting techniques and is amenable to quantification. The technique should have many applications in tumour research and its potential use as an aid in the prognostic assessment of human tumours makes it particularly interesting.

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