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Invited Review

Reverse lectin histochemistry: Design and application of glycoligands for detection of cell and tissue lectins

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Summary. Plant and invertebrate lectins are valuable cyto- and histological tools for the localization of defined carbohydrate determinants. The well-documented ubiquitous occurrence of sugar receptors encourages functional considerations. Undoubtedly, analysis of the presence of vertebrate lectins in tissues and cells is required to answer the pertinent and tempting question on the physiological relevance of protein (lectin)-carbohydrate recognition in situ. Carrier-immobilized glycoligands, derived from custom-made chemical synthesis, enable the visualization of respective binding sites. Histochemically inert proteins or synthetic polymers with appropriate functional groups are suitable carrier molecules for essential incorporation of ligand and label. The resulting neoglycoconjugates can track down tissue receptors that are neither impaired by fixation procedures nor blocked by endogenous high-affinity ligands. Lectins, especially the receptors of the tissue under investigation (endogenous lectins), and appropriately tailored immobilized glycoligands or lectin-specific antibodies (when available) are complementary tools to test the attractive hypothesis that diverse, functionally relevant glycobiological processes within or between cells are operative. Concomitant evaluation of both sides of lectin histochemistry, namely lectins as tools and lectins as functionally important molecules in situ, will indubitably render desired progress amenable in our often still fragmentary understanding of the importance of tissue lectin and glycoconjugate expression and its regulation.

Key words: Lectin, Glycoprotein, Histochemistry, Malignancy.

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Introduction

Plant and invertebrate lectins enjoy a respectable popularity as tools to localize defined carbohydrate structures cyto- and histochemically. Alterations in the expression of such determinants have been mapped in detail during vertebrate embryogenesis, differentiation and malignant transformation (Mann, 1988; Muramatsu, 1988; Alhadeff, 1989; Hakomori, 1989; Bourrillon and Aubery, 1989; Kimber, 1990). These consequences of the regulation of certain glycosyltransferases inevitably prompt the question on the potential functional meaning of the observed structural changes. Having employed exogenous lectins as probes to detect defined carbohydrate sequence stretches, the assumption is obvious to hypothetically attribute fundamental relevance in a productive glycobiological interplay to endogenous lectins. The supposition to seriously endeavour to prove this idea is fulfilled. Ubiquitous expression of lectins including mammals has already been convincingly documented (Sharon and Lis, 1989; Gabius, 1991). Thus, it is reasonable to propose that the concomitant monitoring of both sides of a protein-carbohydrate interaction, namely the glycoconjugate structure as well as the sugar-binding protein, is essential to unequivocally provide evidence for any functional implications. In practical terms, adequate tools are required to enable assessment of sugar receptor expression.

Because lectins are defined by their carbohydrate-specific binding, mono- or oligosaccharides associate with them, if they match the topography of the binding site. Glycoligands that are attached to a labelled carrier offer the intriguing possibility of tracking down tissue binding sites in the quest to correlate observed functions like cell adhesion to certain types of molecular interaction (Gabius, 1988). Naturally-occurring

glycoproteins quite often display heterogeneity in their glycosylation pattern and can exhibit unwanted ligand properties in their protein part. In such cases that restrict the obtention of definite conclusions on the relation between binding and presence of a distinct ligand, the synthesis of conjugates, composed of clusters of a homogeneous sugar part and a rather inert carrier, offers a practicable alternative. Artificial modification of proteins accounts for the prefix «neo» in the technical term «neoglycoprotein» for these compounds (Lee and Lee, 1991). The chemical synthesis of carbohydrate ligand-exposing markers for histological purposes entitles one to allude to the meaning of histochemistry, taken literally in this approach.

Preparation of neoglycoconjugates

A prodigious variety of carbohydrate derivatives and reaction pathways has been carefully elaborated to meet demands for an impeccable design of a neoglycoprotein, being attentive to parameters such as coupling density, nature of spacer and of modification on the sugar and protein parts (Stowell and Lee, 1980; Aplin and Wriston, 1981; Ohsumi et al., 1988; Lee and Lee, 1991). Starting with commercially available p-aminophenyl glycosides, the two steps of a glycosylation reaction of bovine serum albumin as carrier are exemplarily illustrated to make the ease of such a procedure obvious (Fig. 1). Structures of four conjugates with differences in linker group or attachment point are given in Fig. 2. Their capacity to visualize ß-galactoside-specific binding sites in breast cancer sections has been comparatively analyzed (Gabius et al., 1990a). Neoglycoproteins, prepared with 2-imino-2-methoxy-ethyl 1-thioglycosides by amidination or with 1,2-diethoxycyclobutene-3,4-dione as spacer, have also proven suitable for histochemical lectin detection (Lee et al., 1976; Harms et al., 1990; Tietze et al., 1991). Since any alteration of the limited set of functional groups on the protein's surface will change its properties, such as the solubility or the net charge, the inevitable impact on the surface characteristics can generate interfering side effects (Jansen et al., 1991). This situation is an incentive to use synthetic polymers from the chemical drawing-board as carriers.

Besides proteins, soluble artificial polymers offer functional groups for subsequent carbohydrate and label incorporation. Commercially available poly-L-lysine has been glycosylated with p-(carboxymethyl)phenyl glycosides via an amide linkage and the remaining cationic groups are then removed by glyconoylation (Derrien et al., 1989). These polymers are promising candidates for drug-targeting attempts (Midoux et al., 1990). Soluble acrylamide-based polymers for lectin detection have been prepared, following two strategies: copolymerization of acrylamide and a glycoderivative, yielding poly(glycosyloxyallylaminoallylacrylamide) copolymers; or attachment of carbohydrate derivatives to pure or activated polyacrylic acid (Bovin et al., 1992; Chadli et al., 1992). Without the inherent restrictions of limited presence of certain functional groups on the surface of a carrier protein the polymer can be purposefully shaped, until adequate ligand properties, solubility and net charge are established. Acrylic acid or an activated ester like p-nitrophenylacrylate are polymerized and 3-aminopropylglycosides can be conjugated to the polymer (Fig. 3). Artificial glycopolymers of this type bind specifically to fixed blood cells (Abramenko et al., 1992). Comparison with the properties of neoglycoproteins has revealed a consistent and reliable staining of lung cancer sections with such a neoglycoconjugate, underscoring the

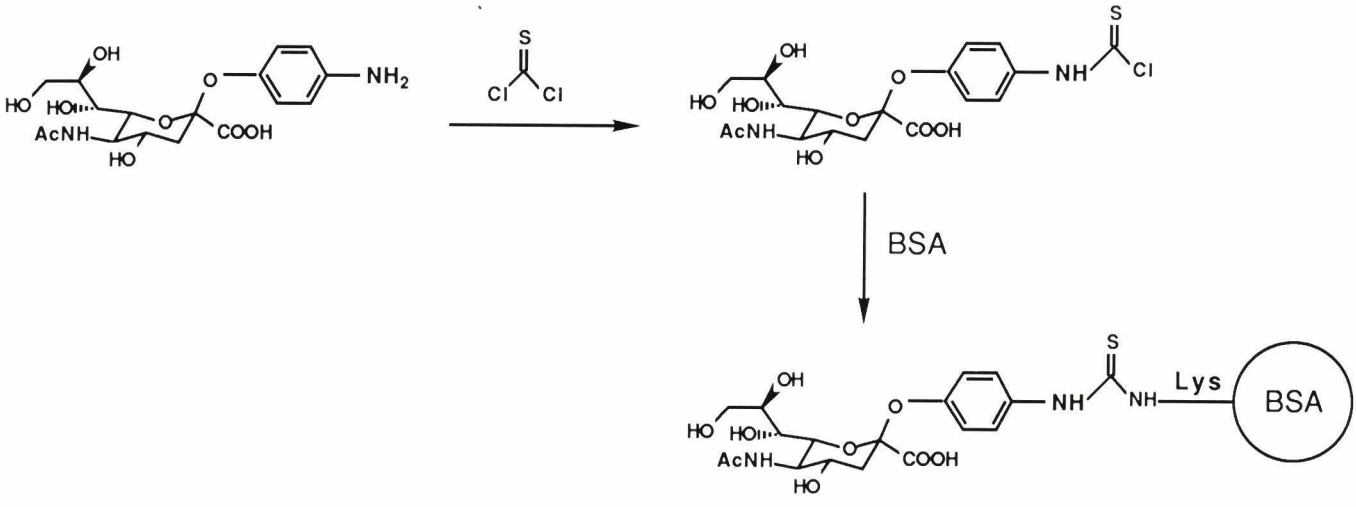


Fig. 1. Schematic illustration of a reaction pathway that leads to the synthesis of glycosylated bovine serum albumin (BSA). The p-aminophenyl derivative of a sugar compound (N-acetylneuraminic acid) is converted to the p-isothiocyanatophenyl glycoside by reaction with thiophosgene. The product is coupled to the carrier protein primarily via lysine residues.

competitiveness of artificial polymers in relation to neoglycoproteins (Table 1).

So far, the conjugation step and the nature of the carrier backbone have been focused upon. Evidently, the complexity of the carbohydrate part has to be deliberately dealt with. The synthetic steps for the production of the ß-isomer of the Thomsen-Friedenreich-antigen, a disaccharide, are outlined in Fig. 4. This procedure has been instrumental in detecting specific binding sites for the disaccharide in cells and tissue sections (Gabius et al., 1990b). Interlocked chemical and histological efforts are thus a step to understand the importance of glycobiological recognition. Advances in the chemo-enzymatic synthesis of oligosaccharides, combining astute chemistry with the highly specific action of glycosyltransferases, is certain to be of considerable benefit to gain access to ligands of any desired structural complexity, attuned to the tissue lectins (Kunz, 1987; Toone et al., 1989; Ichikawa et al., 1992). Starting from disaccharides, these currently developing protocols will allow custom-made preparation of glycoligands of a complexity that could so far only be achieved by isolation of naturally occurring glycopeptides. The steps from the glycoprotein to the glycopeptide-containing neoglycoprotein are sketched in Fig. 5. Homo- or heterobifunctional linking agents like bis(sulphosuccinimidyl)suberate facilitate the conjugation of the glycopeptide to the carrier (Lee et al., 1989; Gabius et al., 1991a). Similarly, activated lysogangliosides (sphingosine N-alkyl (sulphosuccinimidyl)

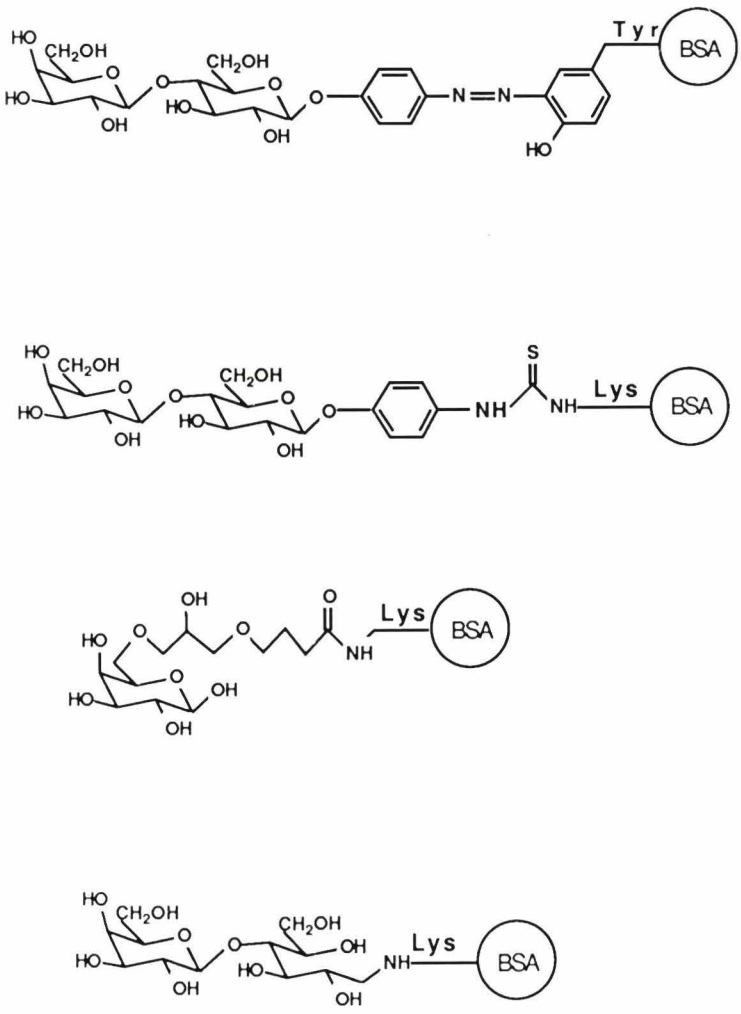
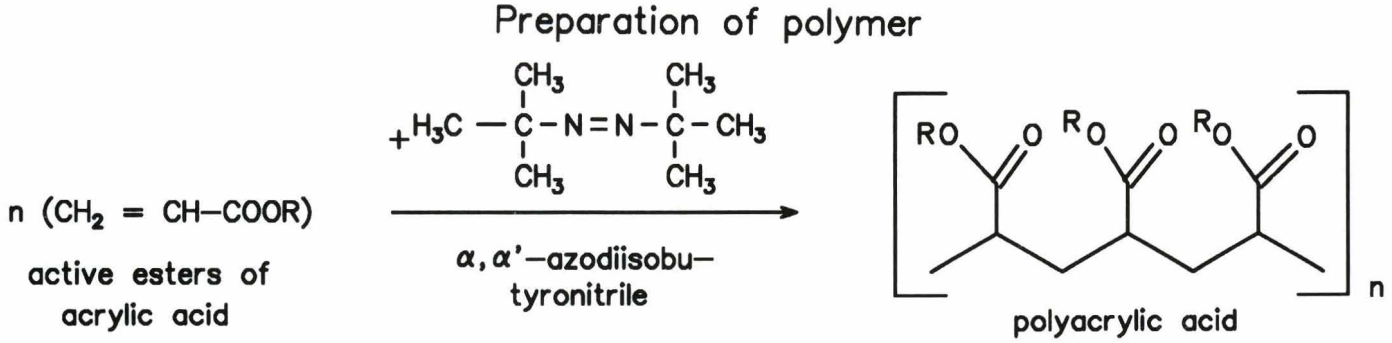
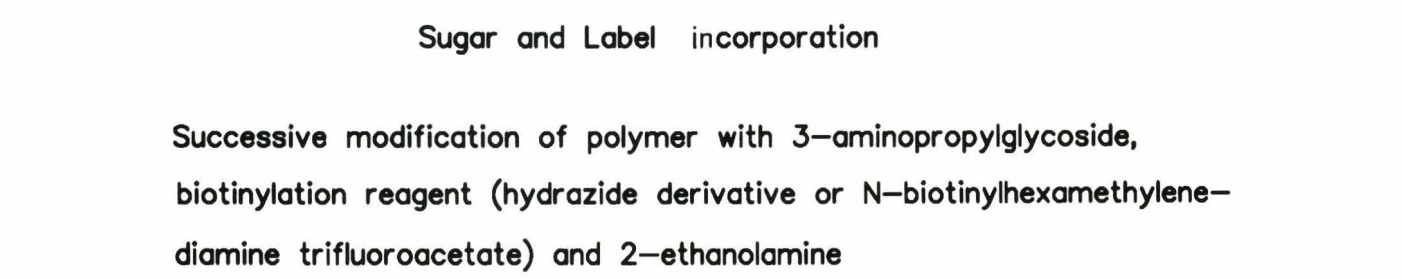


Fig. 2. Schematic illustration of structures of the linking region between the sugar and the protein part of neoglycoproteins; e.g., lactosylated or galactosylated bovine serum albumin, derived from different synthetic pathways. The diazo derivative of p-aminophenyl lactoside couples primarily to tyrosine residues of the carrier protein (top) and p-isothiocyanato glycoside attaches to lysine residues, as shown in Fig. 1. Besides aromatic linker groups, aliphatic spacers can be incorporated, for example by using the galactose derivative of (2,3-epoxypropane)-4-oxybutyric acid that reacts with lysine residues in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Oligosaccharides can be conjugated to NH_2-groups of the protein by reductive amination in the presence of sodium cyanoborohydride, opening the ring structure at the attachment point.





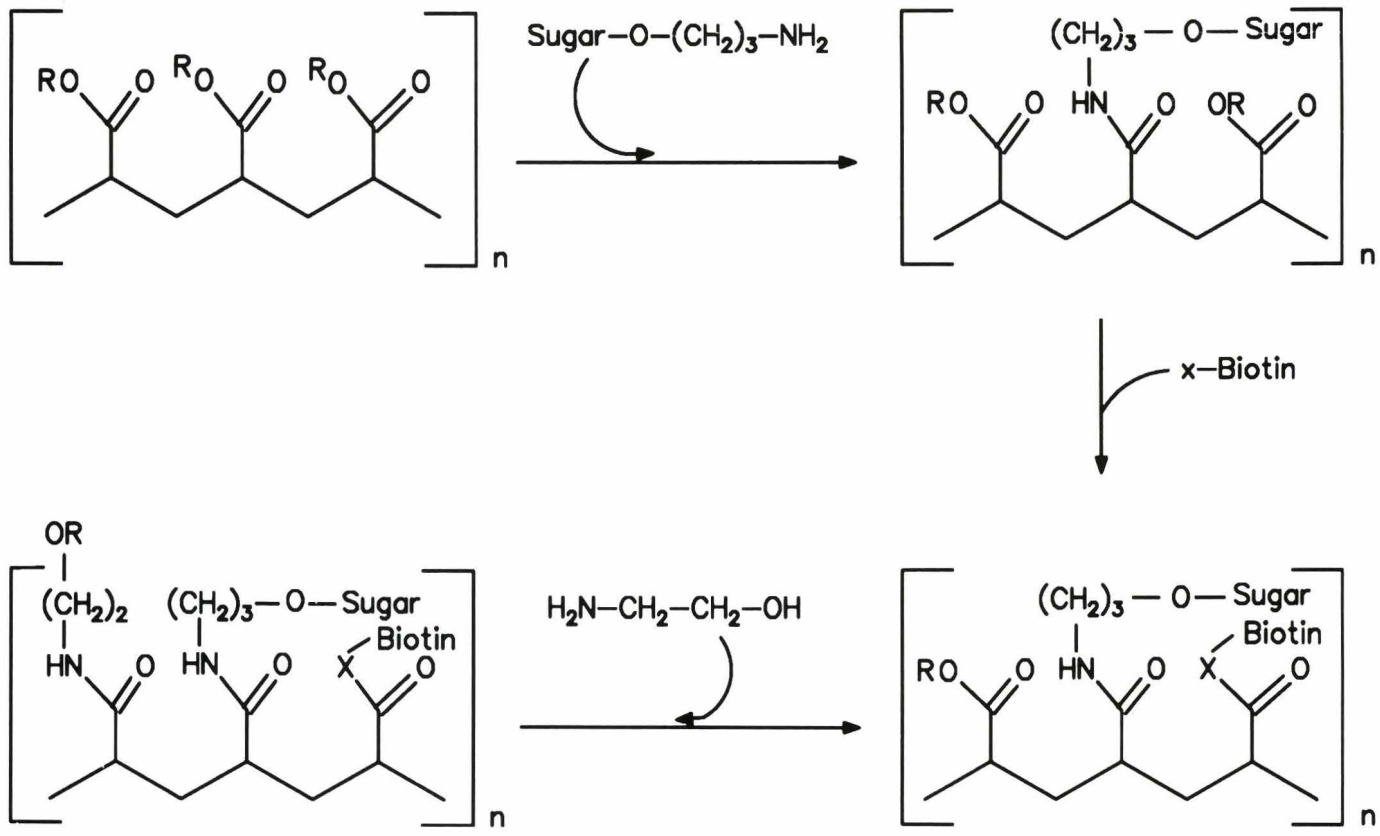


Fig. 3. Schematic illustration of the synthesis of biotinylated, carbohydrate-containing artificial polymers. Acrylic acid, especially activated esters such as p-nitrophenylacrylate, is polymerized in the presence of α, α'-azoisobutyronitrile to yield the polymer, whose functional groups can be modified by incorporation of 3-aminopropyl glycosides and any suitable biotin derivative. Residual active sites are blocked with ethanolamine.

Table 1. Extent of binding of two types of lectin-seeking probes, namely neoglycoproteins (ngp) and artificial polymer (ap) with covalently linked fucose (top) or α-N-acetylgalactosamine (bottom) moieties, to serial sections of 15 cases of primary human lung carcinoma[a]. The binding efficiency is grouped into the categories: strong; weak and no binding.

ngp	ap		
	strong	weak	no
strong	10	1	0
weak	0	0	0
no	0	0	4

ngp	ap		
	strong	weak	no
strong	4	0	0
weak	1	4	0
no	0	1	5

[a]: the following individual cases were analyzed with 20 µg/ml probe and ABC reagents: 9 cases of adenocarcinoma; 3 cases of epidermal carcinoma; 2 cases of large cell anaplastic carcinoma; and 1 case of a carcinoid.

ester derivatives) are coupled to carriers, yielding «neoganglioproteins» (Tiemeyer et al., 1989).

The prepared conjugates can be labelled by any convenient standard procedure, for example by fluorescent dyes, biotin derivatives, iodination, adsorption on colloidal gold granules or covalent attachment to modified latex minibeads (Kieda et al., 1979; Kolb-Bachofen, 1989; Matsuoka and Tavassoli, 1989; Gabius and Bardosi, 1991). When enzymes are glycosylated without impairing their activity, no further step is required to obtain effective probes due to the presence of a natural label; the enzymatic activity (Gabius et al., 1989). Having hereby acquired access to the glycoligand-bearing markers, the histological monitoring of expression of binding sites is feasible. The detection of such receptors in the tissue or on cells by neoglycoconjugates is termed «reverse lectin histochemistry» with reference to common lectin histochemistry, employing the lectin as a glycoconjugate-tracing device.

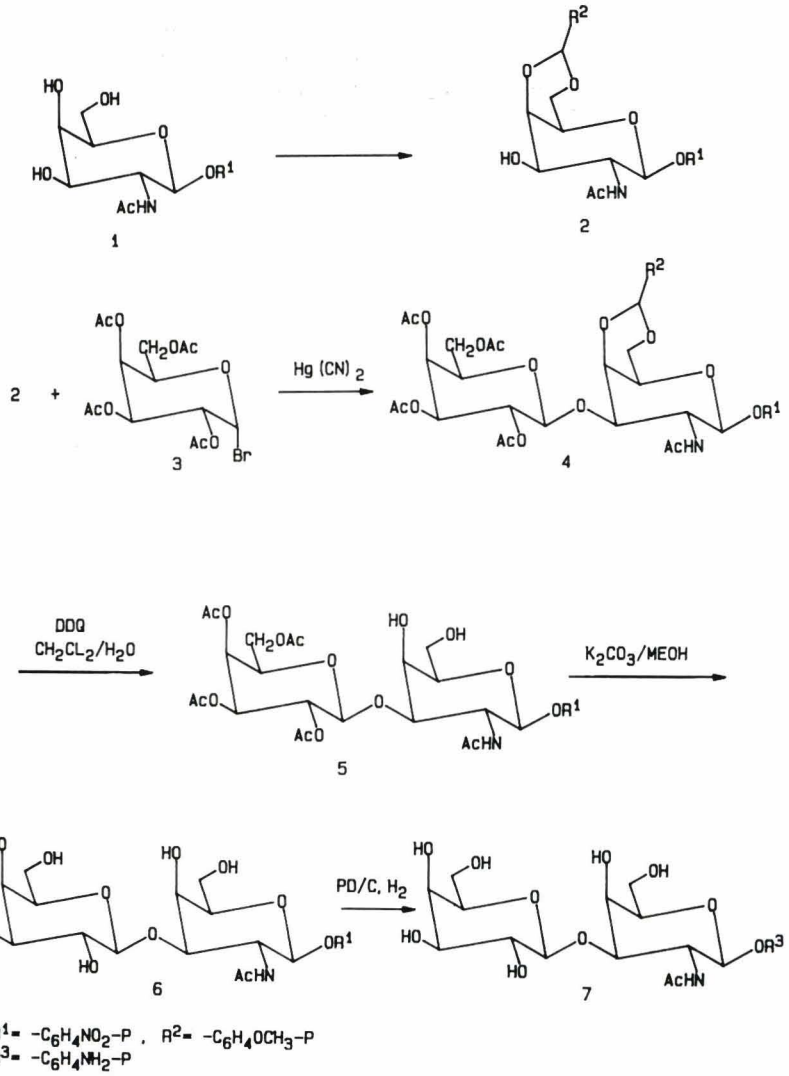


Fig. 4. Schematic illustration of the synthesis of the ß-isomer of the Thomsen-Friedenreich-antigen, p-aminophenyl 2-acetamido-2-deoxy-3-O-ß-D-galactopyranosyl-ß-D-galactopyranoside (compound 7). Starting with protection of the 4'-OH and 6'-OH-groups by reaction of the p-nitrophenyl derivative of 2-acetamido-2-deoxy-ß-D-galactopyranoside with p-methoxybenzaldehyde in the presence of zinc chloride, conjugation of 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide (compound 3) with the protected monosaccharide (compound 2) in the presence of mercuric cyanide was performed, the protective p-methoxybenzylidene group was then removed from the disaccharide (compound 4) with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), resulting in compound 5. Deacetylation and catalytic reduction of the NO_2-group by hydrogenation in the presence of palladium on charcoal completed the series of steps.

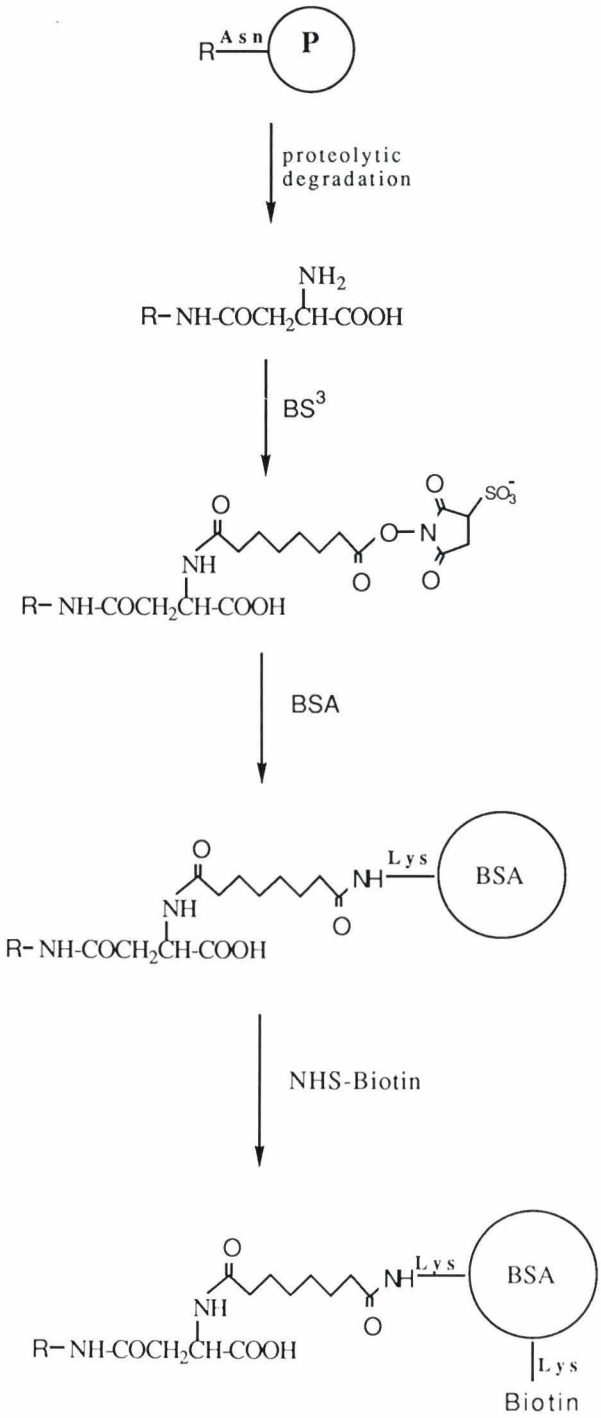


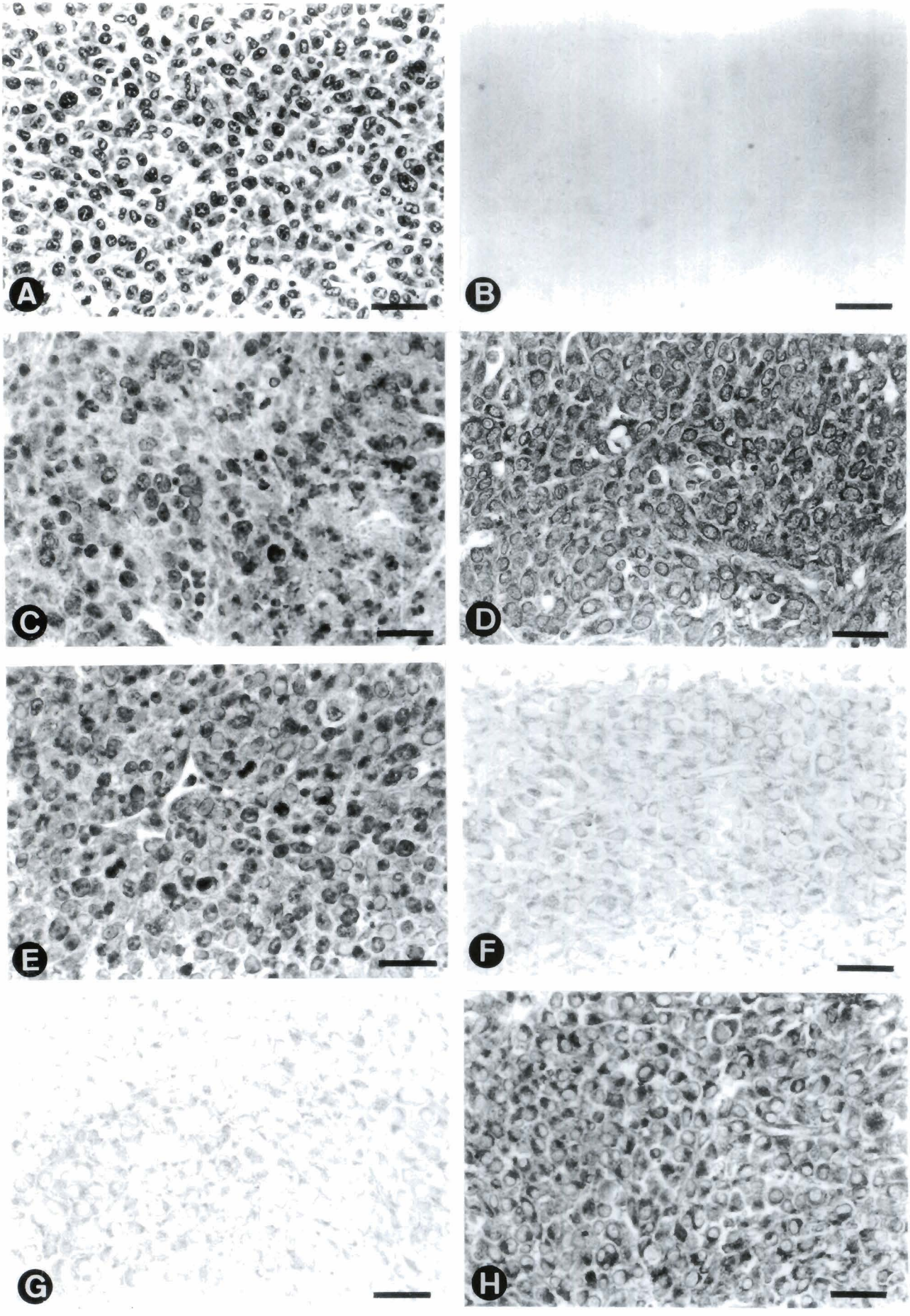
Fig. 5. Schematic illustration of the chemical glycosylation of bovine serum albumin with a selected glycopeptide. This glycopeptide has first been isolated after proteolytic degradation of the glycoprotein, then activated with a homobifunctional crosslinker like bis(sulphosuccinimidyl)suberate and attached to lysine groups of bovine serum albumin. The glycosylated carrier can further be labelled; e.g., with biotinyl-N-hydroxysuccinimide ester.

Reverse lectin histochemistry

Frozen or fixed sections of mammalian tissue have been studied with neoglycoproteins to determine the level of specific binding, which is neither impaired by processing nor blocked by high-affinity ligands, among them are samples for brain, placenta, muscle, thyroid gland or spleen (Alquier et al., 1988; Harms et al., 1990; Gabius and Bardosi, 1991). One focus of research is the assessment of the presence of binding sites for a panel of markers in tumors to aid tumor classification and to discern any possible prognostic marker (Gabius and Gabius, 1991). Indeed, non-uniform neoglycoprotein binding among tumor subtypes justifies further investigation, differences among primary lung cancer types and between mesothelioma and metastatic carcinoma serving as an instructive example (Kayser et al., 1989, 1992). Grade-dependent alterations have also been noted; e.g., in bladder lesions (Gabius et al., 1992). With respect to metastasis formation, where it is attractive to assume a role of lectins in the homing process, the influence of the microenvironment on lectin expression must not be overlooked to avoid misinterpretations. This premonitory comment is substantiated by model studies on tumors, grown at different sites in the animal (Glaves et al., 1989, 1991; Vidal-Vanaclocha et al., 1990). An exemplary illustration of colon tumors, grown subcutaneously and processed glycohistochemically is given in Fig. 6. Besides standard control reactions a further proof of the specificity of the neoglycoprotein binding can be provided, when antibodies to predominant lectins are available.

Concomitant monitoring of breast cancer sections with lactosylated neoglycoprotein and an antibody to the predominant endogenous ß-galactoside-specific lectin has made clear that both types of tool label the cells with qualitatively and often quantitatively similar efficiency, any additional synthetic alteration of the subterminal sugar in the ligand part accounting for quantitative changes (Gabius et al., 1986, 1991b). This similarity between glyco- and immunohistochemical results and the impact of synthetic details on the performance of the probe underscores the specific molecular interplay between carbohydrate ligand and the lectin. Such a combined study on rat cerebellum likewise reveals a significant extent of accordance for two types of lectin (Kuchler et al., 1990). The observed differences can be

Fig. 6. Light micrographs of ethanol-fixed, paraffin-embedded mouse colon 26 carcinoma sections. The tumors were grown subcutaneously. Specific binding sites were visualized for biotinylated heparin (C) and fucoidan (D), biotinylated rhamnosylated albumin (E), ß-N-acetylglucosaminylated albumin (F), α-fucosylated albumin (G) and the glycoprotein asialotransferrin (H) by application of ABC reagents and the chromogen 3,3'-diaminobenzidine. No counterstaining was performed. Matching serial sections were either stained with standard haematoxylin and eosin (A) or incubated with labelled, carbohydrate-free albumin as probe (B) for control purpose. Bar: 20 µm; x 400


A
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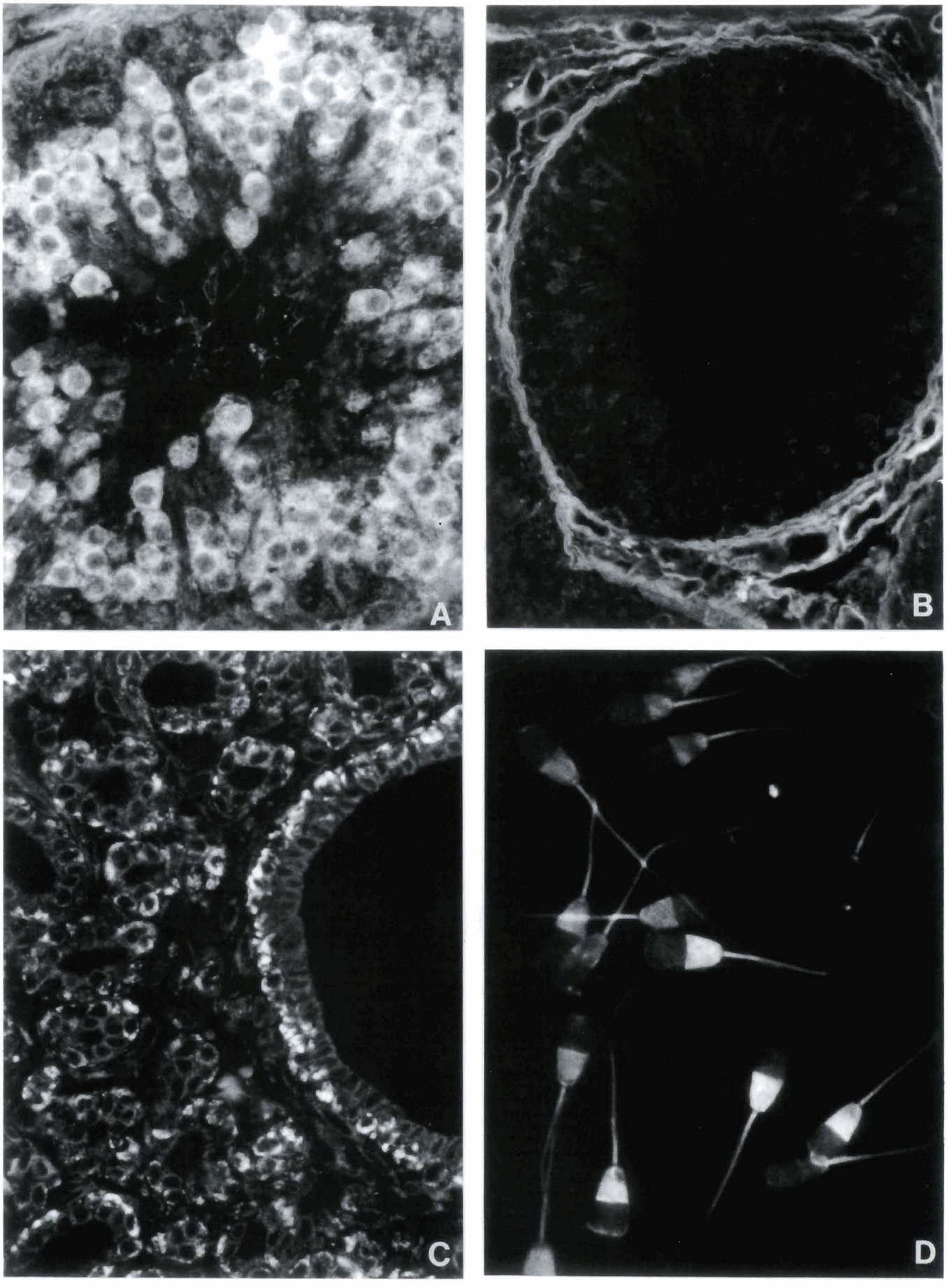
A
B
C
D

Fig. 7. Visualization of specific carbohydrate-binding sites for fluorescent (neo)glycoproteins, namely asialolactoferrin-FTC (A), maltosylated albumin-FTC (B), asialotransferrin-FTC (C) and lactosylated albumin-FTC (D), on sections of bovine testis, revealing distinct fluorescence of the round spermatids in the germinal epithelium (A) or at the basal membrane of tubuli seminiferi contorti and in the interstitial stroma (B), on a section of Dunning R 3327 prostatic carcinoma, showing staining in the basal cells of the tumor epithelium (C) and of bovine spermatozoa from cauda epididymis which had been fixed with buffered $HgCl_2$ (D). Scale of magnification: A-B, x 520; C, x 512; and D, x 160

attributed to fixation and inaccessibility of the binding sites, which especially appears to be the case for cell membrane lectins in cerebellum (Kuchler et al., 1992). Ultrastructural localization of cytoplasmic and nuclear lectins does not appear to be affected to the same extent, comprehensive studies being indispensable to reach a clear-cut conclusion (Facy et al., 1990; Kuchler et al., 1992). To monitor glycoprotein binding, uptake and transcytosis by lectins, perfused organs have been exposed to neoglycoproteins to visualize endocytic lectin-dependent processes (Breitfeld et al., 1985; Kempka and Kolb-Bachofen, 1988). Since mammalian tissues exhibit no unique features, any samples that fit into the standard criteria for histological protocols can undergo this procedure in search of lectins; for example, specimens from lower vertebrates (Danguy et al., 1991).

Similar to binding sites in tissue sections, cell lectins can be visualized with neoglycoconjugates, as exemplarily shown in Fig. 7 for fluorescent labels on testicular tissue and spermatozoa, and in Fig. 8 for biotinylated neoglycoproteins and tumor cells. Since they can mediate recognition of other cells, establishing cell adhesion, their localization is a significant step in delineating the still often ill-defined mechanisms of specificity of cell interactions, as proposed for instance for an entomopathogenic fungus or the trichocyst tips of paramecia (Haacke-Bell and Plattner, 1987; Latge et al., 1988). Sperm-egg recognition and attachment of parasites like the coccidian or trypanosomatid protozoa *Toxoplasma gondii* or *Leishmania* throws light upon the two sides of the coin in lectin-mediated adhesion (Sinowatz et al., 1989; Robert et al., 1991; Schottelius, 1992, Schottelius and Gabius, 1992). Fluorescent labelling of protozoan parasites by neoglycoproteins proves their capability to welcome sugar moieties as ligands (Fig. 9). To turn this initial demonstration into more than a footnote in the collection of organisms with

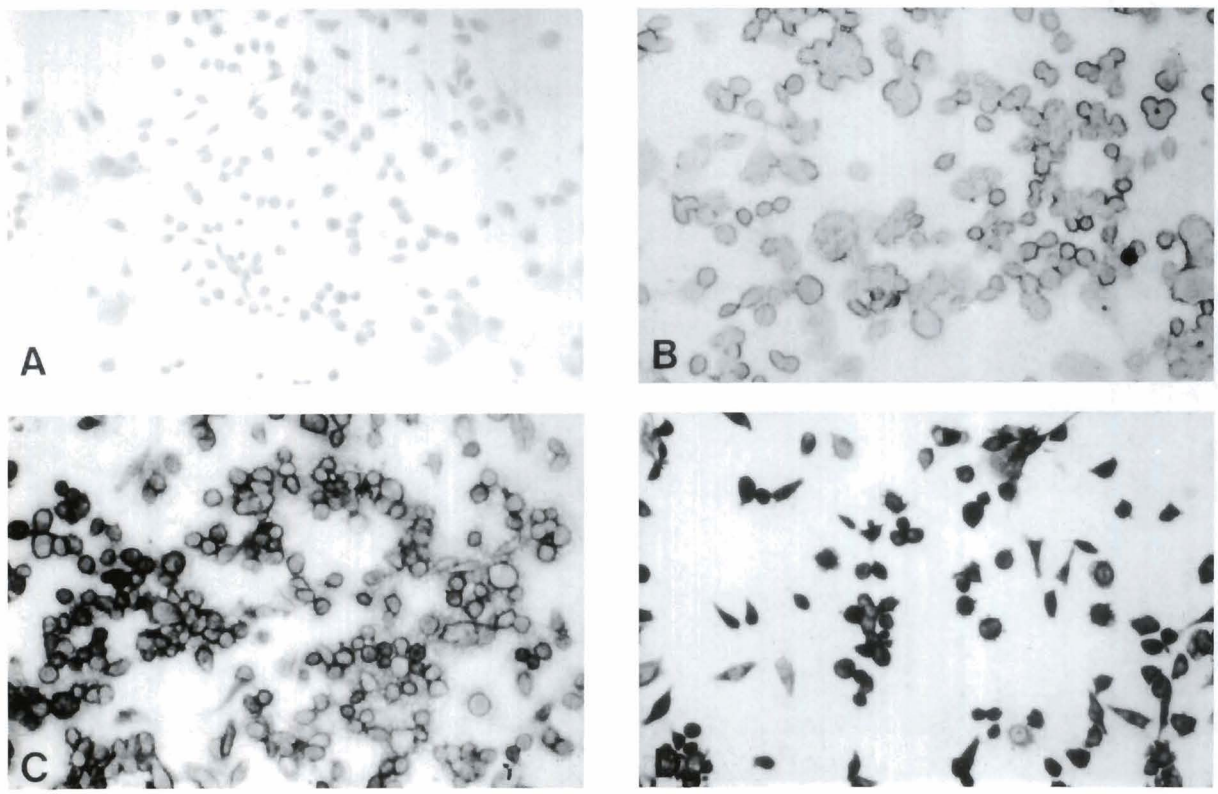

Fig. 8. Visualization of specific binding sites for biotinylated ß-N-acetylgalactosaminylated albumin (B), α-mannosylated albumin (C) and lactosylated albumin (D) on acetone/methanol/formalin-fixed murine breast carcinoma cells after application of ABC reagents and the chromogen 3,3'-diamino-benzidine. The control reactions included incubation with labelled, carbohydrate-free albumin and identical subsequent treatment for signal development (A). x 45

surface lectin expression, quantitative measurements, adhesion studies on immobilized glycoconjugates as a model and with physiological target cells as well as lectin purification are definitely necessary. Quantitative determinations of lectin expression on native cells can either be accomplished with neoglycoenzymes or with FACS scans (Figs. 10, 11). Notably, only the latter method will reliably give information on occurrence of subpopulations with differences in lectin expression. Electron microscopic identification of sugar receptors on the cell surface also supplies quantitative data (Matsuoka and Tavassoli, 1989). This information provides a clear guideline for the ensuing cell biological studies like interference of cell adhesion with

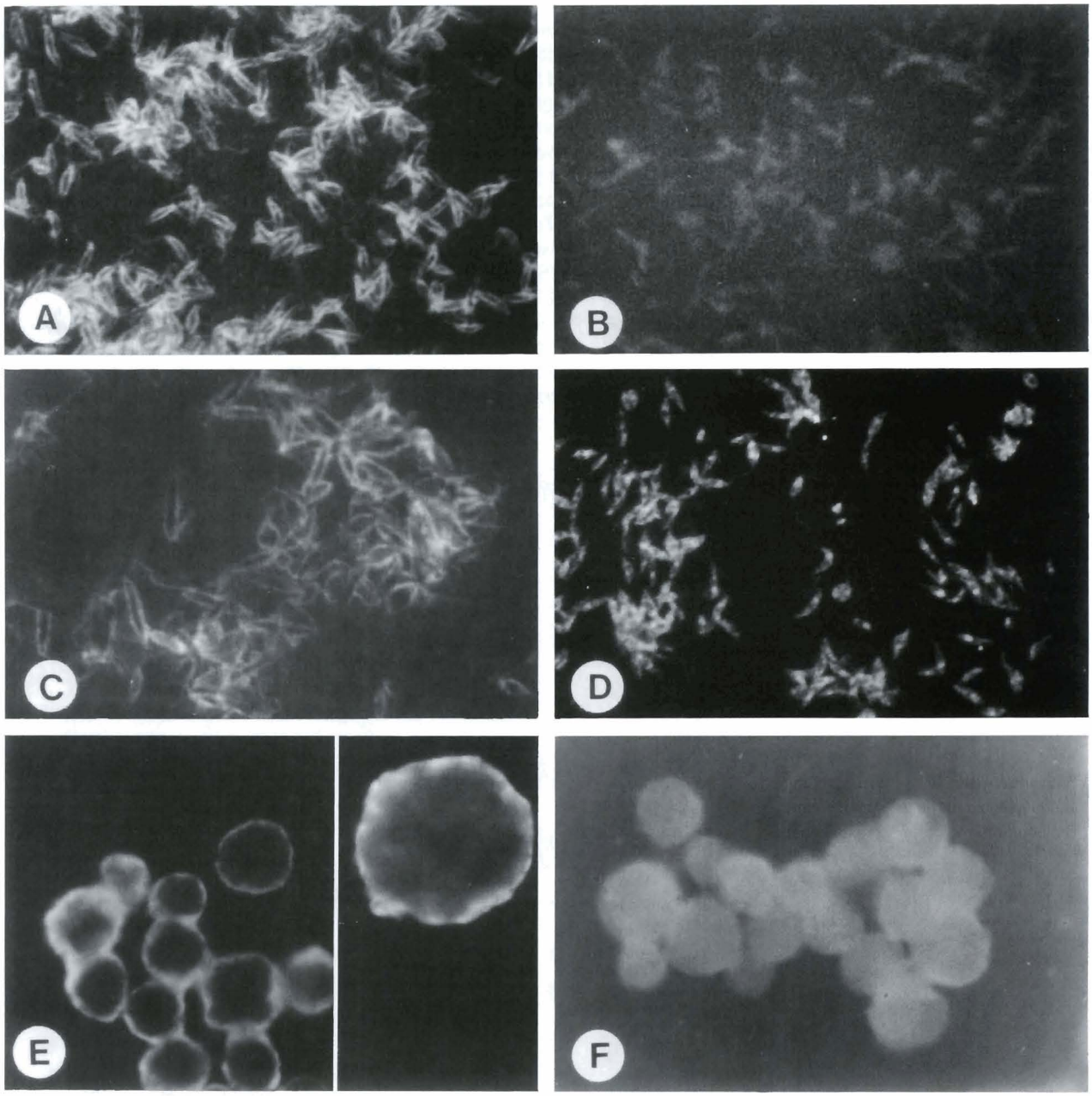

Fig. 9. Visualization of specific binding sites for ß-N-acetylgalactosaminylated and ß-N-acetylglucosaminylated bovine serum albumin after subsequent incubation of promastigotes of *Leishmania donovani* stock LRC L-51 with rabbit anti-albumin and fluorescent goat anti-rabbit antibodies (A, C). Omission of the neoglycoprotein yielded assessment of the background value (B). The guinea pig-specific *Leishmania enriettii* stock 826 specifically bound fluorescent α-mannosylated albumin (D). *Entamoeba histolytica* stock HM 1 specifically bound ß-N-acetylgalactosaminylated albumin, detected by anti-albumin and fluorescent anti-antibody (E), the background level was defined by omission of the incubation step with the neoglycoprotein (F). Scale of magnification: A-D, (x 400); E and F, x 64 (inset in E, x 160)

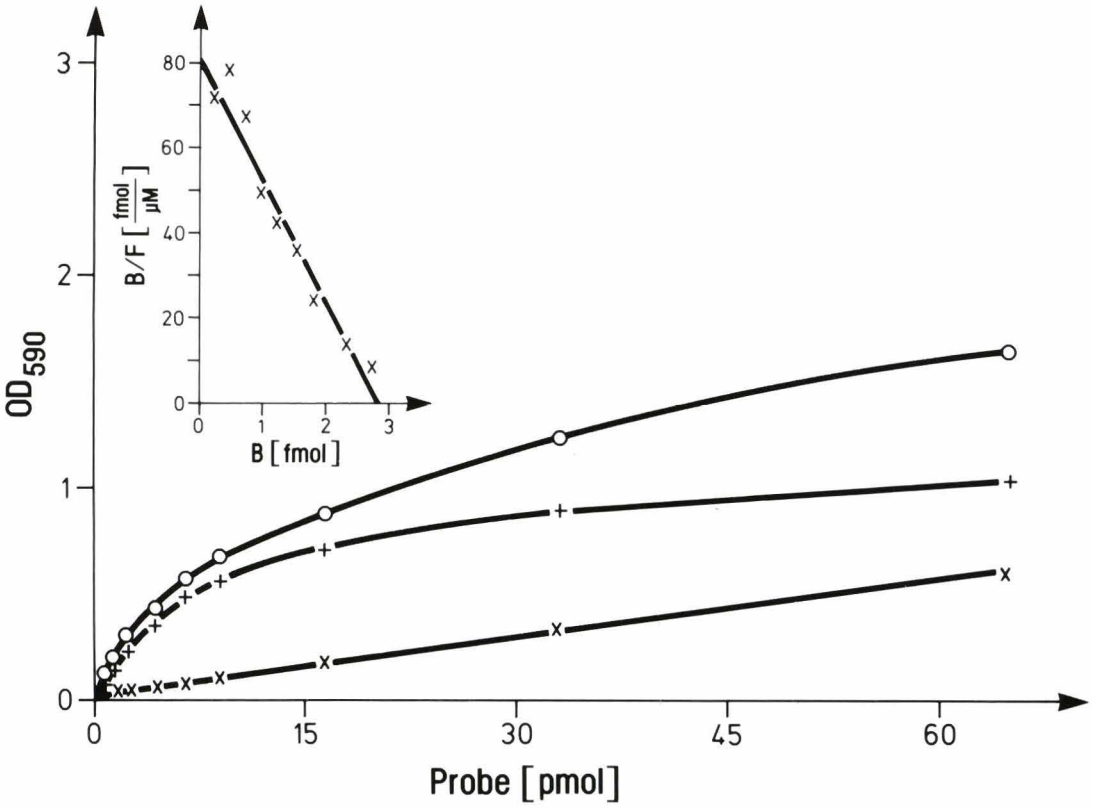

Fig. 10. Determination of total binding (o), non-specific binding (x) and specific binding (+) of fucosylated *E. coli* ß-galactosidase to B-lymphoblastoid Croco II cells and Scatchard plot analysis of the binding data (inset; K_D= 34 nM, B_{max}= 2.5 x 10^4 bound enzymes per cell at saturation).

glycoligands in different areas of interest; for example, research on metastatic spread, homing of stem cells to the bone marrow or melanosome passage to keratinocytes (Gabius et al., 1990c; Tavassoli and Hardy, 1990; Cerdan et al., 1991). Concurrently, presence of lectins can be exploited therapeutically by lectin-mediated drug targeting (Gabius, 1988; Monsigny et al., 1988). Last, but not least these results direct the selection of affinity ligands for the chromatographic purification of the respective proteins and their unambiguous identification as lectins by excluding enzymatic properties or identity to antibodies, thereby opening a new line of research for histology.

When chemical modification by group-specific reagents ascertains lack of detrimental effect by derivative formation, they can be conveniently labelled like any other already popular lectin (Kuchler et al., 1989; Avellana-Adalid et al., 1990; Gabius et al., 1991 c,d). The vertebrate lectins are supposed to eventually substitute exogenous agglutinins in research on tissues, in which they are detectable.

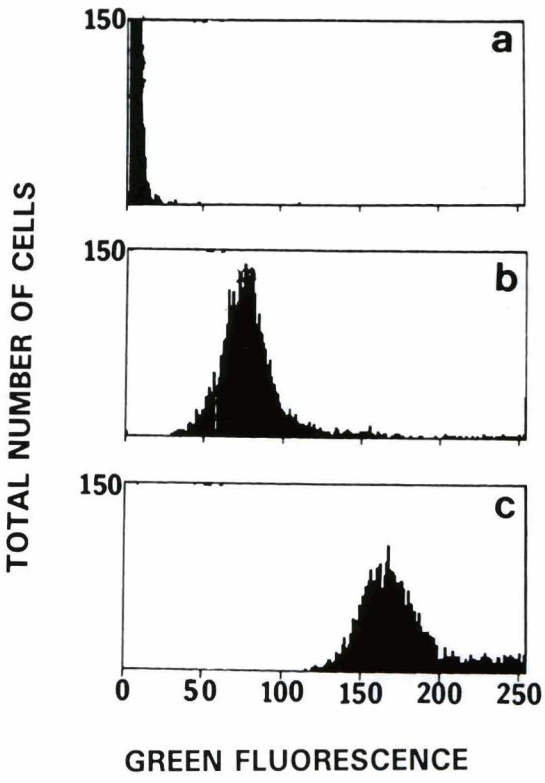

Fig. 11. Flow cytometric analysis of murine breast carcinoma cells after subsequent incubation steps of 10^6 cells with biotinylated neoglycoproteins (maltosylated or ß-N-acetylgalactosaminylated albumin: b, c) and fluorescent avidin. Control cells were only treated with fluorescent avidin, not with the biotinylated probe (a).

Lectin histochemistry with tissue lectins

The purpose of lectin histochemistry on animal tissue is to localize defined glycoconjugate elements and to contribute to the understanding of the functions of these structures. Plant lectins fulfill the first requirement. However, the implication of a functional interplay between the glycostructure, visualized by a lectin from a quite different source, and a putative tissue lectin presupposes the identity of the carbohydrate specificities of the lectins. Comparison of plant and mammalian mannose- and galactose-specific lectins clearly demonstrates that the ligand properties of cellular glycoconjugates can differ markedly (Mori et al., 1988; Ezekowitz et al., 1989; Kuchler et al., 1989; Lee et al., 1992). Thus, tissue lectins are in these cases clearly

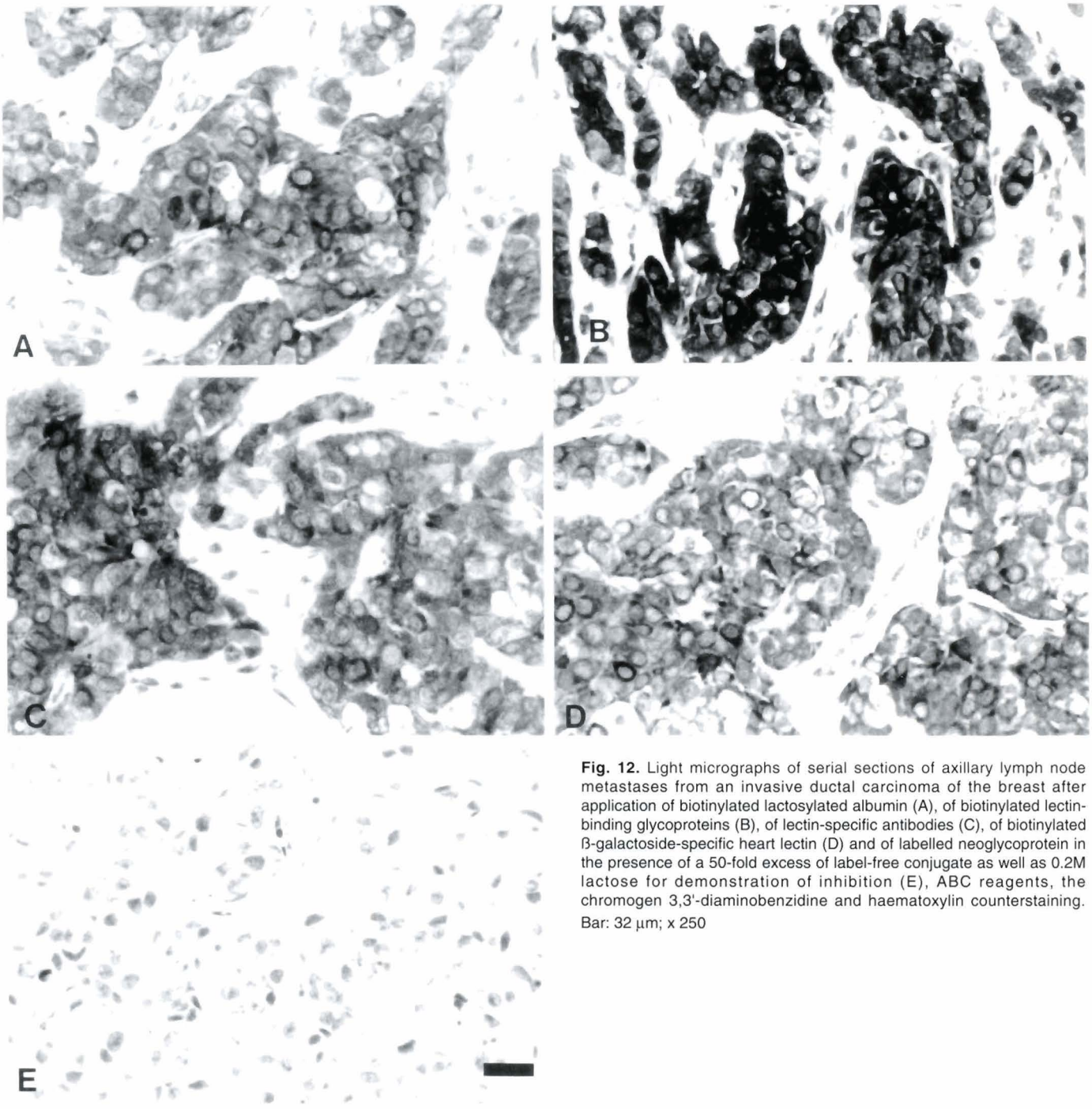

Fig. 12. Light micrographs of serial sections of axillary lymph node metastases from an invasive ductal carcinoma of the breast after application of biotinylated lactosylated albumin (A), of biotinylated lectin-binding glycoproteins (B), of lectin-specific antibodies (C), of biotinylated ß-galactoside-specific heart lectin (D) and of labelled neoglycoprotein in the presence of a 50-fold excess of label-free conjugate as well as 0.2M lactose for demonstration of inhibition (E), ABC reagents, the chromogen 3,3'-diaminobenzidine and haematoxylin counterstaining. Bar: 32 µm; x 250

superior to plant lectins in order to gain functionally valid insights, such as the colocalization of lectin (by antibody, neoglycoprotein, and labelled, biochemically purified tissue ligand) and accessible lectin-binding sites in serial sections can offer (Fig. 12).

Accessible lectin-binding sites in the tissue samples of the species, from which the lectin has been obtained, have already been visualized with different galactoside-specific lectins and a heparin-binding lectin (Beyer and Barondes, 1980; Treichel et al., 1989; Cooper et al., 1991; Gabius et al., 1991b,c,d). Genetic engineering enlarges the panel of markers to pursue this approach. Availability of cloned lectin genes, namely from selectins, has enabled the construction of lectin-IgG chimera, containing the hinge and constant regions of immunoglobulin heavy chains that react with protein A, and its successful histochemical application for ligand detection (Watson et al., 1990; Todderud et al., 1992). No non-mammalian lectin is so far known that matches the specificity of the members of the selectin family, which currently receive a great deal of popularity in the study of leukocyte trafficking (Springer and Lasky, 1991). Tissue lectins as addition to the array of histochemical tools are the adequate response to address this problem.

Perspectives

Advances in separate fields are certain to converge in glycohistochemistry to accelerate the pace of gaining knowledge about the physiological relevance of lectin-carbohydrate recognition. Organic chemistry will supply tailor-made ligands and carriers, biochemistry will make tissue lectins and their ligands available for histochemical use as well as molecular characterization, and molecular biology can frame probes. Deliberate alterations of the carbohydrate-binding specificity of lectins by introduction of mutations into the gene can even be envisaged (Tyrell et al., 1992; Yamamoto et al., 1992). Looking ahead in this direction, molecular modelling of ligands and crystallographic measurements on complexes are powerful techniques to optimize the molecular fit (Calendar et al., 1988; Reeke and Becker, 1988). As the content of this article testifies, these outlined directions of research are not far-fetched wishful thinking, but by all means attainable interdisciplinary project aims.

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