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Immunocytochemical localization of β1,4 galactosyltransferase in epithelial cells from bovine tissues using monoclonal antibodies

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Post-embedding immunocytochemistry was employed to investigate the distribution of UDP-galactose:*N*-acetylglucosamine galactosyltransferase (β1,4-GT) in epithelial cells from various bovine organs. Several well characterized monoclonal antibodies previously demonstrated to recognize distinct polypeptide epitopes within the primary structure of β1,4-GT were applied to thin sections from tissues embedded in Lowicryl K4M, followed by the protein A–gold technique. Immunoreactivity was observed in the Golgi apparatus of epithelial cells from intestine, thymus and trachea. No immunoreactivity was observed in other intracellular structures, including rough endoplasmic reticulum, nuclear envelope and goblet cell mucus droplets. Within the Golgi apparatus, the staining was restricted to several cisternae in the *trans* region, with most portions of the *trans*-Golgi network appearing unlabelled. However, in thymic epithelial-reticular cells *trans*-Golgi network portions resembling classical GERL elements were stained by the antibodies. Thus, although immunoreactivity was subcompartmentalized within the Golgi apparatus in all epithelial cell types examined, the extent of staining within the *trans*-Golgi network was variable. Immunoreactivity was not detected at the plasma membrane (ecto-galactosyltransferase), except in the case of a subpopulation of tracheal cells that resemble brush cells. These results suggest that in the epithelial cells examined, the subcompartmental distribution of β1,4-GT within the Golgi apparatus is maintained across different types of epithelial cell organization. Moreover, no evidence for a general epithelial cell ecto-galactosyltransferase could be discerned with these reagents.

Key words: epithelial cells/galactosyltransferase/Golgi apparatus/immunocytochemistry/monoclonal antibodies

Introduction

Glycosyltransferases are membrane-bound proteins of the endoplasmic reticulum and Golgi apparatus that function in the coordinate biosynthesis of oligosaccharide chains present on glycoproteins and glycolipids. In many, but not all cell types, glycosyltransferases elaborating complex-type oligosaccharides are restricted in their distribution to subcompartments of the Golgi apparatus (Roth, 1987). This has led to the subcompartmentalization model of the Golgi apparatus (Dunphy and Rothman, 1985), which proposes that glycosyltransferases are distributed in Golgi apparatus subcompartments according to the order in which they act. Direct immunocytochemical evidence supporting this model has been presented for four glycosyltransferases. *N*-Acetylglucosaminyltransferase I was found to be restricted to middle cisternae of the Golgi apparatus in rabbit kidney and liver cells (Dunphy *et al.*, 1985). On the other hand, the terminally acting glycosyltransferases β1,4-galactosyl- (Roth and Berger, 1982), α1,3-*N*-acetylgalactosaminyl- (Roth *et al.*, 1986) and α2,6-sialyltransferase (Roth *et al.*, 1985) have all been localized to portions of the *trans* region of the Golgi apparatus in some cell types. However, in intestinal epithelial absorptive cells, α2,6-sialyltransferase and α1,3-*N*-acetylgalactosaminyltransferase were found distributed throughout the Golgi apparatus cisternal stack (Roth *et al.*, 1986), calling into question the universality of the subcompartmentalization model.

In addition to their localization in Golgi apparatus membranes, individual glycosyltransferases have also been immunocytochemically detected in post-Golgi apparatus structures, including lysosomes, goblet cell mucus droplets and plasma membranes (Pestalozzi *et al.*, 1982; Davis *et al.*, 1984; Roth *et al.*, 1985; Shaper *et al.*, 1985; Taatjes *et al.*, 1988; Suganuma *et al.*, 1991). Recently, however, some of these results have been called into question in light of the findings that affinity-purified polyclonal antibodies raised against human milk β1,4-galactosyltransferase (β1,4-GT) contained antibodies directed at blood group-related carbohydrate antigens present on the β1,4-GT protein (Childs *et al.*, 1986; Berger *et al.*, 1987a; Feizi *et al.*, 1987). Indeed, although these affinity-purified antibodies decorated the plasma membrane and mucus droplets in immunofluorescence staining of human intestinal sections, preabsorption of the antibodies with blood group substances prior to immunostaining abolished this post-Golgi apparatus reactivity (Childs *et al.*, 1986; Feizi *et al.*, 1987). Since in general glycosyltransferases are glycoproteins, the potential exists for any polyclonal anti-glycosyltransferase antibody to contain a subset of antibodies directed against carbohydrate epitopes.

We have now attempted to circumvent potential interference from carbohydrate-specific antibodies for the immunocytochemical localization of β1,4-GT by employing a panel of well characterized monoclonal antibodies (mab). Monoclonal antibodies raised against a glycosyltransferase isolated from a biological source can be selected by their ability to recognize a polypeptide epitope, as assessed by Western blotting of the recombinant form produced in *Escherichia coli*. Previously, we produced a panel of mouse monoclonal antibodies directed against a soluble form of native bovine β1,4-GT (Ulrich *et al.*, 1986). In the present study, we have used three of these monoclonal antibodies, which recognize three distinct polypeptide epitopes of the β1,4-GT protein, in a post-embedding immunocytochemical investigation aimed at addressing the following two questions. (i) Is the distribution of β1,4-GT similarly subcompartmentalized within the Golgi apparatus of several different epithelial cell types? (ii) Can we detect the presence

of galactosyltransferase protein immunoreactivity in post-Golgi apparatus structures, including the plasma membrane of different epithelial cells (ecto-galactosyltransferase)?

Results

In this study we have examined several different types of epithelial organization: small and large intestine (simple columnar epithelium), trachea (ciliated pseudostratified columnar epithelium) and thymus (epithelial-reticular cells). Examination of intestine and trachea provides the added advantage that several different types of epithelial cells are present, including absorptive enterocytes, goblet cells, brush cells and ciliated cells. Since the monoclonal antibodies used in this study recognize only bovine β1,4-GT, we restricted our analysis to tissues of bovine origin. The three monoclonal antibodies yielded qualitatively similar results; however, the intensity of the immunolabelling was always greatest with mab H5. Thus, unless otherwise specified, the following results were obtained with mab H5.

Intestine

On ultrathin sections from the large intestine (proximal colon), immunoreactivity for β1,4-GT was detectable in the Golgi apparatus of both goblet and absorptive cells (Figure 1a and b). Within the Golgi apparatus cisternal stack, the labelling was restricted to several cisternae on the *trans* side of the stack.

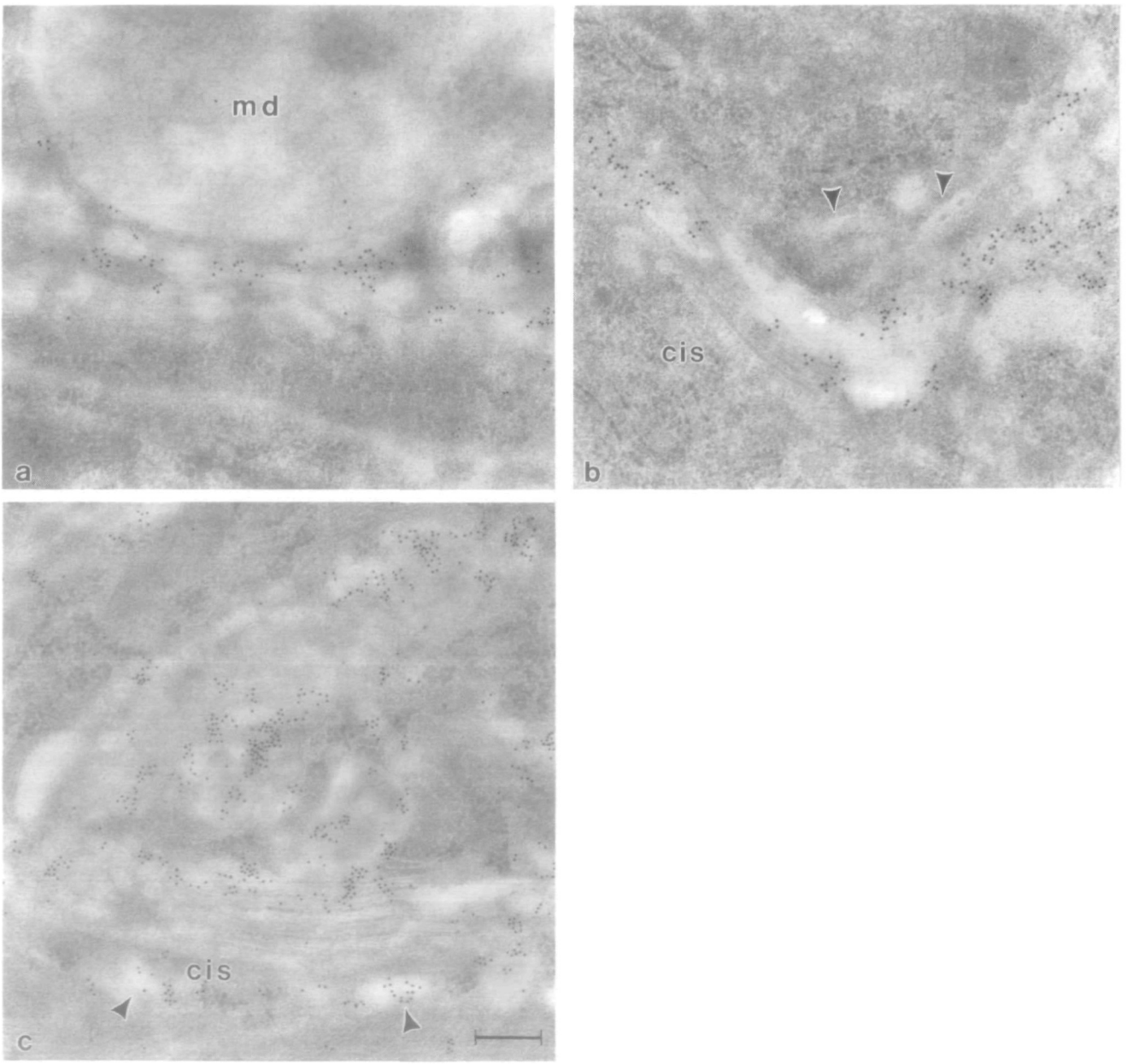

Fig. 1. Localization of β1,4-GT and β-galactose residues on sections from bovine proximal colon. In both goblet cells (**a**) and absorptive cells (**b**), β1,4-GT immunoreactivity detected with mab H5 is restricted to *trans* regions of the Golgi apparatus. Note the absence of staining over goblet cell mucus droplets (md) and unstained regions of the absorptive cell *trans*-Golgi network (arrowheads). The distribution of β-galactose residues, as detected by RCL I followed by asialofetuin–gold, mirrors that of β1,4-GT in the *trans*-Golgi apparatus, as shown in an absorptive cell (**c**) Note the staining of vesicles at the *cis* side of the Golgi apparatus with RCL I (arrowheads). The *cis* side of the Golgi apparatus is labelled 'cis' in this and all subsequent figures. Bars 0 22 μm (a–c).

However, elements of the *trans*-tubular network (*trans*-Golgi network) did not display immunoreactive sites for β1,4-GT (Figure 1b). All other cellular organelles, apical and basolateral plasma membranes, and goblet cell mucus droplets were also not stained by the antibody. β-Galactose residues, visualized with *Ricinus communis* lectin I (RCL I) followed by asialofetuin–gold, were similarly restricted to the *trans* region of the Golgi apparatus (Figure 1c), indicating that this is the site where galactosylation occurs.

On ultrathin sections from the small intestine (duodenum) of the same animals, *trans*-Golgi apparatus cisternae of goblet cells were intensely stained by mab H5 while, surprisingly, adjacent absorptive cell Golgi apparatus was not stained (Figure 2a and b). As in the large intestine, no immunoreactivity was detectable in other organelles, plasma membranes or goblet cell mucus. However, *trans* regions of the absorptive cell Golgi apparatus were intensely stained with RCL I followed by asialofetuin–gold (not shown), indicating the presence of abundant β-galactose residues. RCL I also labelled apical and basolateral plasma membranes, lysosomes and apical cytoplasmic vesicles of the small intestinal absorptive cells.

Thymus

On sections from the thymus, the only cells displaying immunoreactivity for mab H5 were the epithelial-reticular cells; all other cells, including the abundant lymphocytes, were not stained. Within the epithelial-reticular cells, the staining was restricted to *trans* cisternae of the Golgi apparatus (Figure 3a). As observed for the intestinal epithelial cells, portions of the

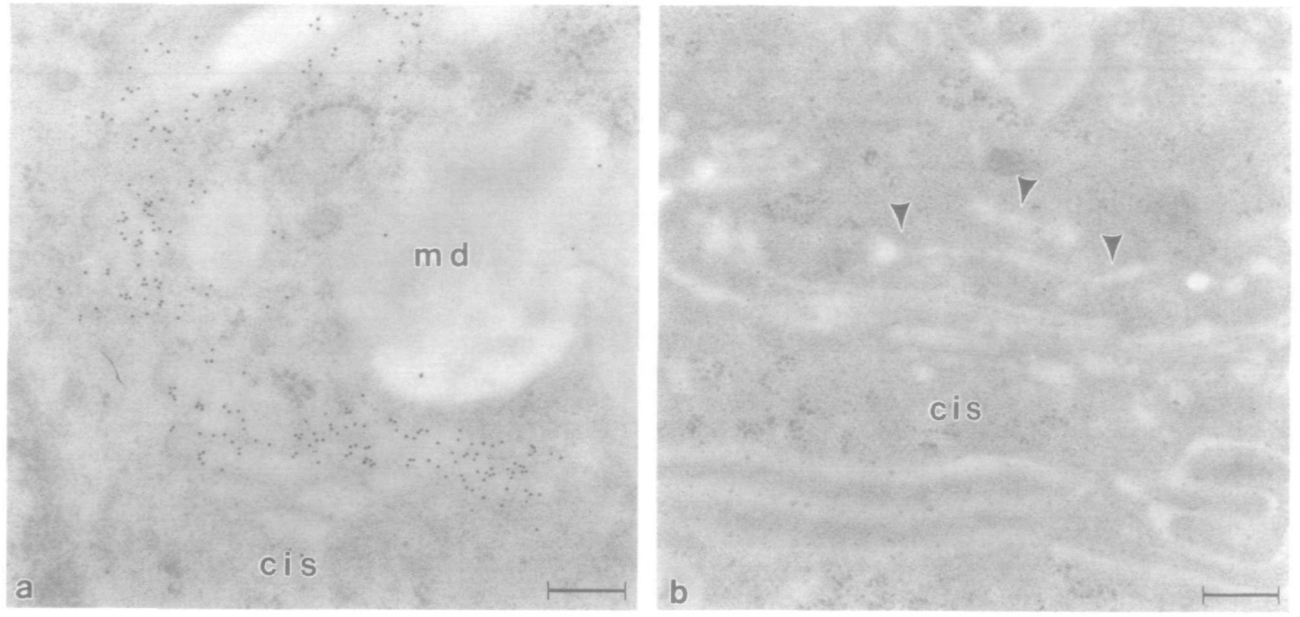

Fig. 2. Immunocytochemical detection of β1,4-GT with mab H5 on sections from bovine duodenum. Gold particle label is restricted to *trans* cisternae of the goblet cell Golgi apparatus (**a**). Note the absence of staining in the goblet cell mucus droplets (md). β1,4-GT was not detectable in absorptive cell Golgi apparatus from the same animals (**b**). Arrowheads indicate portions of the *trans*-Golgi network (GERL elements). Bars 0.21 μm (a) and 0.3 μm (b).

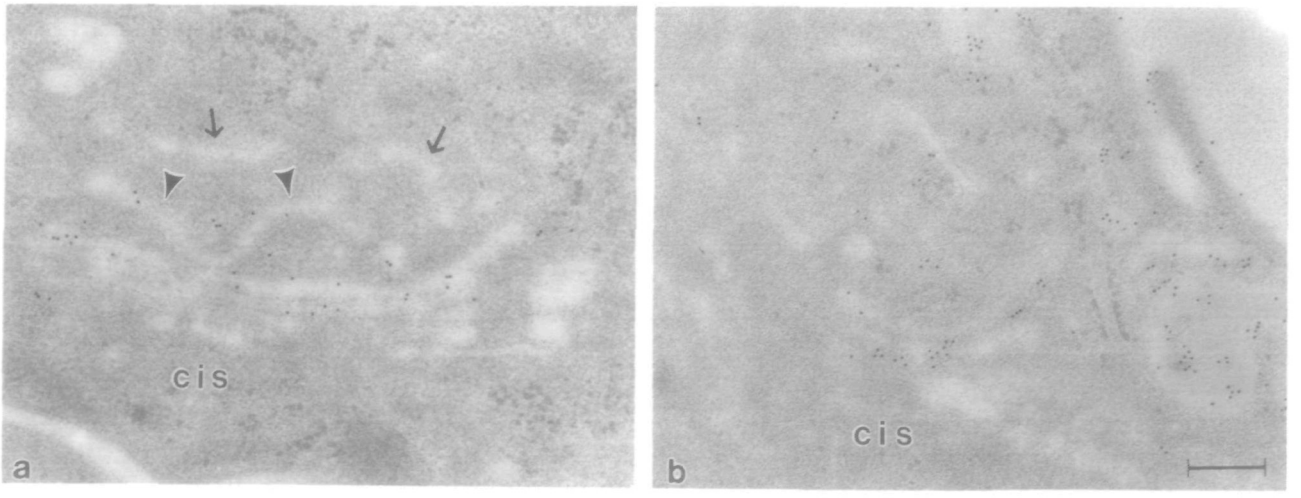

Fig. 3. Detection of β1,4-GT and β-galactose residues on sections from bovine thymus. Immunoreactivity for β1,4-GT using mab H5 is restricted to *trans* regions of the Golgi apparatus in epithelial-reticular cells (**a**). Note immunostaining present in typical GERL element (arrowheads), while other portions of the *trans*-Golgi network are unstained (arrows). β-Galactose residues, as detected with RCL I followed by asialofetuin–gold, were observed over *trans* cisternae of the Golgi apparatus (**b**). Bars 0.21 μm (a, b).

trans-Golgi network were not stained by the antibody (Figure 3a); however, in contrast, typical GERL rigid elements were usually stained (Figure 3a). Incubation of sections with RCL I followed by asialofetuin–gold resulted in staining of *trans*-Golgi apparatus cisternae (Figure 3b). Although thymocytes did not display immunoreactive sites for β1,4-GT, they did display RCL I binding sites in the *trans* region of the Golgi apparatus and along the plasma membrane (not shown).

Trachea

Several tracheal cell types displayed intense immunoreactivity in the Golgi apparatus for anti-β1,4-GT monoclonal antibodies. Owing to the presence of a variety of cell types, as well as good ultrastructural preservation, we decided to focus our attention on this ciliated pseudostratified columnar epithelium. In goblet cells, several cisternae at the *trans* aspect of the Golgi apparatus were intensely stained with mab H5 or H162 (Figure 4a and b). Portions of the *trans*-Golgi network, as well as mucus droplets, were not stained (Figure 4a and b). Similarly, in ciliated cells and brush cells, portions of the *trans* region of the Golgi apparatus displayed intense immunoreactivity for β1,4-GT (Figure 4c and d), although the intensity of immunostaining was usually greater in brush cells. Classical GERL elements were usually not stained with the monoclonal anti-β1,4-GT antibodies (Figures 4a–d). Typically, 2–4 cisternae at the *cis* side of the Golgi apparatus were not stained, followed by 3–4 stained cisternae at the middle to *trans* region of the stack, and finally unstained cisternae or tubules of the *trans*-Golgi network. This pattern of staining was evident in all three of the epithelial cell types examined in the trachea (goblet, ciliated and brush cells). However, owing to variability in the orientation of the Golgi apparatus in different cells in different sections, exact delineation of the subcompartmentation of β1,4-GT immunoreactivity in all cells, as well as comparisons amongst cells, are difficult. Nevertheless, the overall pattern of immunostaining amongst the different cells was similar.

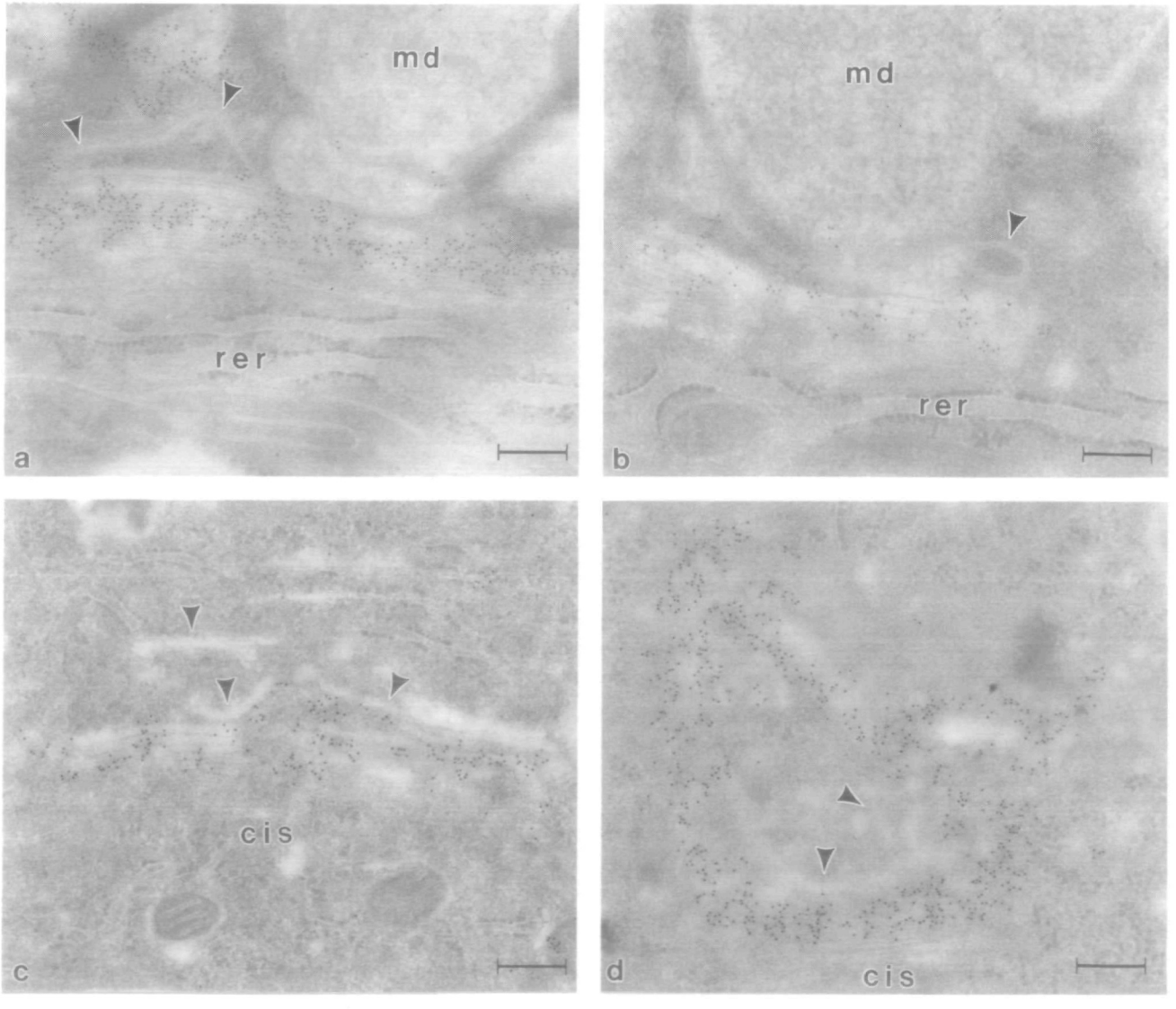

Fig. 4. Immunolocalization of β1,4-GT in bovine tracheal epithelium. In goblet cell Golgi apparatus (**a, b**), gold particle label is distributed in several cisternae in the middle/*trans* regions of the stack. Note the absence of staining in typical GERL elements of the *trans*-Golgi network (arrowheads) and in mucus droplets (md). Similar results were obtained with mab H5 (a) or mab H162 (b). In ciliated cells (c) and brush cells (d), immunostaining is observed in several *trans*-Golgi apparatus cisternae. However, note that typical GERL rigid elements and portions of the *trans*-Golgi network (arrowheads) are not stained. rer, rough endoplasmic reticulum. Bars 0.23 μm (a) and 0.22 μm (b–d).

β-Galactose residues, as visualized with *Datura stramonium* lectin (DSL) followed by asialofetuin–gold, were restricted to regions of the *trans*-Golgi network, lysosomes and plasma membrane. Within the Golgi apparatus, DSL binding sites were generally observed more towards the *trans* side (including GERL elements) than immunoreactivity for β1,4-GT (Figure 5a and b).

Some sections were processed in a double staining procedure to localize β1,4-GT immunoreactivity and β-galactose residues on the same section. As shown in Figure 6, immunoreactivity

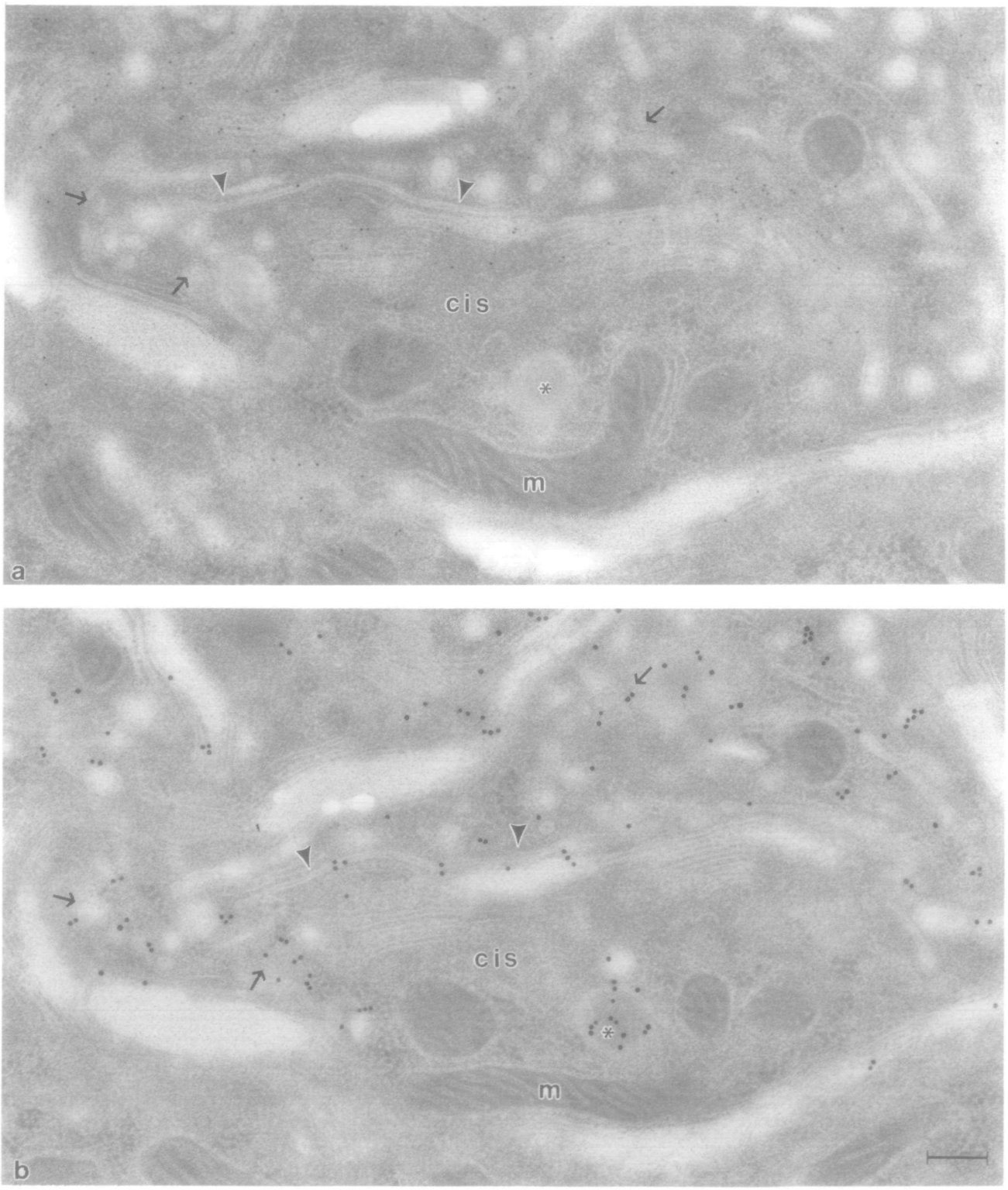

Fig. 5. Serial sections (not consecutive) incubated with mab H5 followed by protein A–gold (a) or DSL followed by asialofetuin–gold (b). Immunoreactivity is restricted to several *trans*-Golgi apparatus cisternae from a tracheal ciliated cell (a). Note that typical GERL elements (arrowheads) are not stained for β1,4-GT. Binding sites for DSL (b) are observed in the distal *trans* region of the Golgi apparatus, including GERL elements (arrowheads). Additionally, lysosomes (asterisk) are also stained with DSL/asialofetuin–gold, but not with mab H5. Vesicles and tubules at the *trans* aspect of the Golgi apparatus are not stained by mab H5 (arrows in 'a'), but do display DSL binding sites (arrows in 'b'). m, mitochondrion. Bar 0.25 μm (a, b).

for β1,4-GT (small gold particles) and Galβ1,4GlcNAc residues [detected with DSL followed by asialofetuin–gold (large gold particles)] overlap within the Golgi apparatus. However, the stainings for enzyme and product were not entirely superimposable; immunoreactivity for β1,4-GT is more widely distributed into middle cisternae of the Golgi apparatus stack. Furthermore, DSL binding sites persist in the *trans*-Golgi network (especially in the GERL element) which are not immunoreactive with the anti-β1,4-GT antibodies (Figure 6). DSL binding sites were also observed in the apical and basolateral plasma membranes, lysosomes and cytoplasmic vesicles.

Of all the numerous cell types examined, immunoreactivity with the three monoclonal antibodies was observed at the plasma membrane in only a subpopulation of tracheal epithelial cells. Based on morphological characteristics, most prominently a tuft of apical microvilli, we believe that these represent tracheal brush cells. Figure 7 shows three serial (not consecutive) sections of the apical plasma membrane of such a cell stained with mab H5. These serial sections demonstrate that the staining at the apical plasma membrane is not the result of a processing artifact, as the same cell is stained from sections processed separately on different grids. Moreover, labelling at the apical plasma membrane of these cells was also observed with mab H162 and mab H12. Indeed, in serial sections the same cell was found to be stained at the apical plasma membrane by both mab H5 and mab H12 (Figure 8). Interestingly, the label was preferentially associated with the microvillar projections of the apical plasma membrane, with little label observed over the smooth membrane regions between individual microvilli (Figures 7 and 8). These cells also contained apical cytoplasmic vesicles that were also stained by the monoclonal antibodies.

The immunocytochemical results from all of the tissues examined are summarized in Table I.

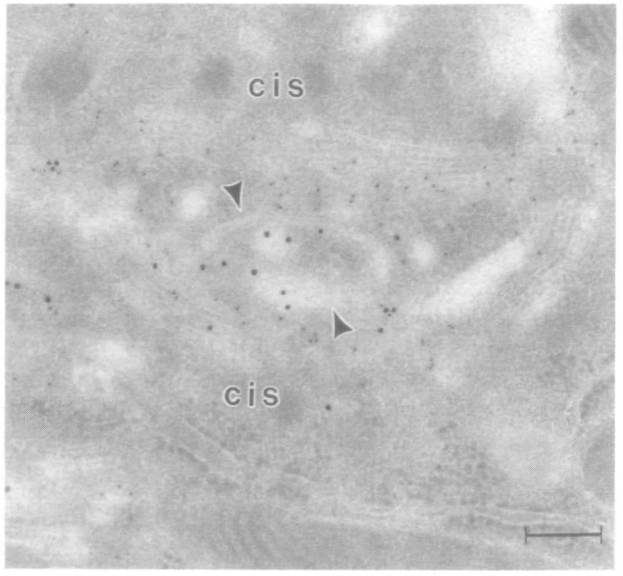

Fig. 6. Double staining for β1,4-GT and β-galactose residues on the same thin section from bovine trachea. Immunoreactivity for mab H5 (small gold particles) and DSL binding sites (large gold particles) are found over the *trans* region of a ciliated cell Golgi apparatus. This Golgi apparatus is horseshoe shaped with *cis* regions found at both the bottom and top of the micrograph, with *trans* region in between. Note that the staining with DSL ensues in more distal *trans*-Golgi apparatus cisternae as compared to β1,4-GT immunoreactivity and that GERL elements (arrowheads) are stained with DSL, but not with mab H5. Bar 0.21 μm.

Discussion

The use of immunocytochemical studies to localize a specific antigen rely for their interpretation on the specificity of the antibodies. In the present investigation we have used a panel of well characterized monoclonal antibodies previously demonstrated to recognize by Western blot analysis three different polypeptide epitopes within the non-glycosylated recombinant bovine β1,4-GT polypeptide expressed in *E.coli*. When β1,4-GT was present in cells in a quantity sufficient for detection by post-embedding immunoelectron microscopy, these monoclonal antibodies were found to be reliable and specific reagents for the localization of both Golgi apparatus and cell surface β1,4-GT.

Subcompartmentation of β1,4-GT in epithelial cell Golgi apparatus

One of the primary aims of this study was to compare the distribution of β1,4-GT in the Golgi apparatus of various epithelial cell types. To date, the Golgi apparatus distribution of β1,4-GT has been documented for only a limited number of cell types: HeLa cells (Roth and Berger, 1982; Strous *et al.*, 1991), $HepG_2$ cells (Geuze *et al.*, 1985), myotubes (Tassin *et al.*, 1985), rat gastric mucous neck cells and rat testis interstitial cells (Suganuma *et al.*, 1991). In the latter three studies, the low resolution of the immunoperoxidase technique makes it difficult to interpret the precise localization of the enzyme as it relates to the structural organization of the Golgi apparatus.

In the present study, we found that the distribution of β1,4-GT was virtually the same in the Golgi apparatus from all of the different epithelial cells examined. Several unlabelled cisternae at the *cis* side were followed by several labelled cisternae in the middle/*trans* region, followed by mostly unlabelled structures as part of the *trans*-Golgi network. Typical rigid elements at the *trans* aspect of the cisternal stack indicative of GERL elements (Novikoff and Novikoff, 1977) were generally unlabelled, as previously observed in HeLa cells by Roth and Berger (1982). However, the rigid GERL elements were labelled by the monoclonal anti-β1,4-GT antibodies in thymic epithelial-reticular cells. Such localizations probably reflect the enormous diversity in Golgi apparatus structure noted in various cell types and under different experimental conditions. Without the aid of extensive serial sectioning analysis and exact control of experimental procedures, it is impossible to precisely compare the distribution of antibody binding sites in different Golgi apparatus. Moreover, the number and extent of Golgi apparatus subcompartments is highly subjective and often arbitrarily defined. Indeed, a still unresolved question concerns the extent or lack of overlap of sequentially acting glycosyltransferases within the *trans*-Golgi apparatus. Double labelling procedures for the localization of galactosyl- and sialyltransferase at the light microscopic level have provided conflicting results (Berger and Hesford, 1985; Berger *et al.*, 1987b; Taatjes *et al.*, 1987). Clearly, double labelling at the electron microscopic level of two sequentially acting glycosyltransferases will be required to settle this conflict and may aid in establishing more precise definitions of Golgi apparatus subcompartments.

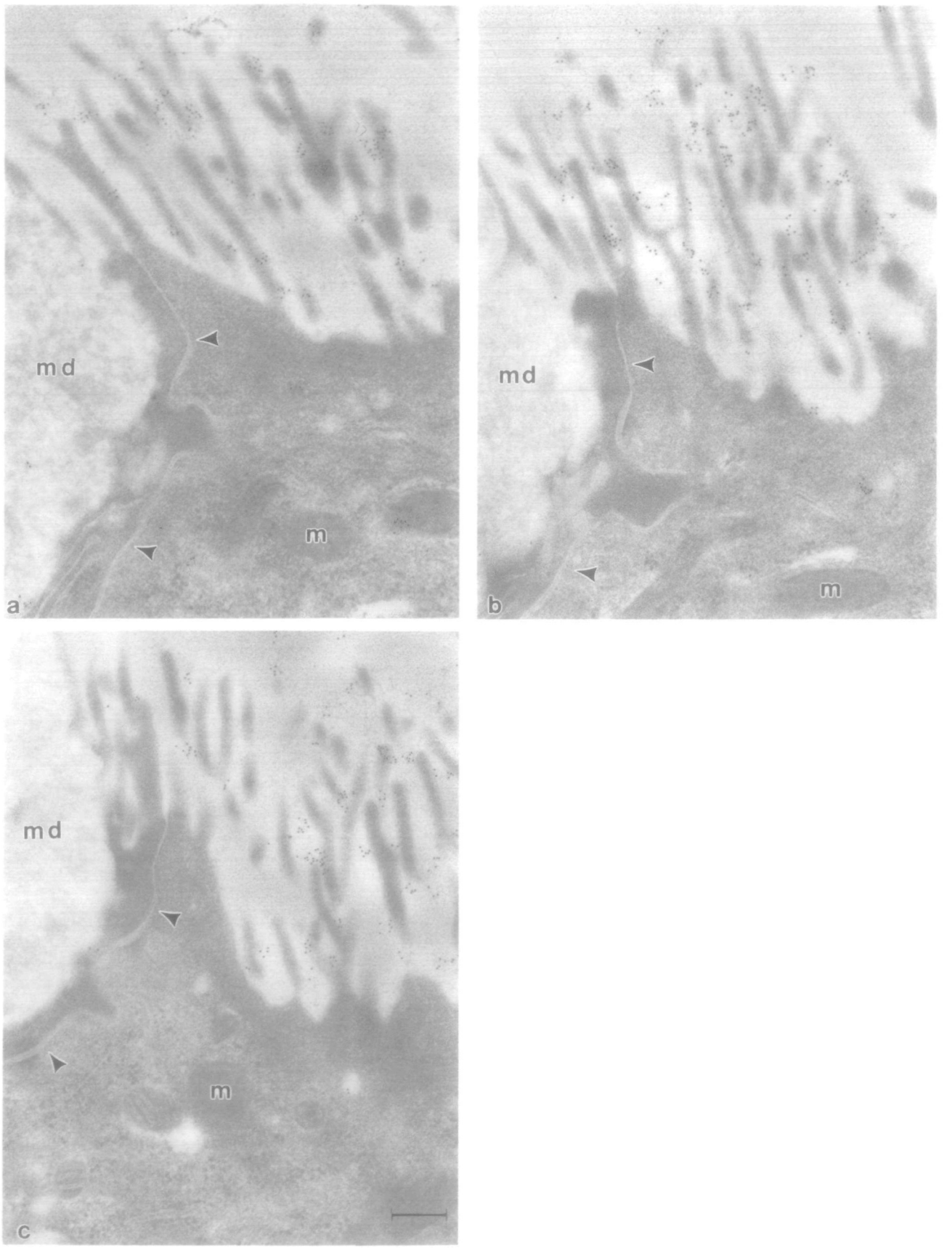

Fig. 7. Three serial sections (not consecutive) showing the apical cytoplasm and plasma membrane of the same tracheal brush cell. Binding sites for mab H5 are observed in the apical plasma membrane in all three sections. Note the absence of staining in the neighbouring goblet cell mucus droplets (md) and along the lateral plasma membrane (arrowheads) separating the brush and goblet cell. m, mitochondrion. Bars 0.22 μm (a–c)

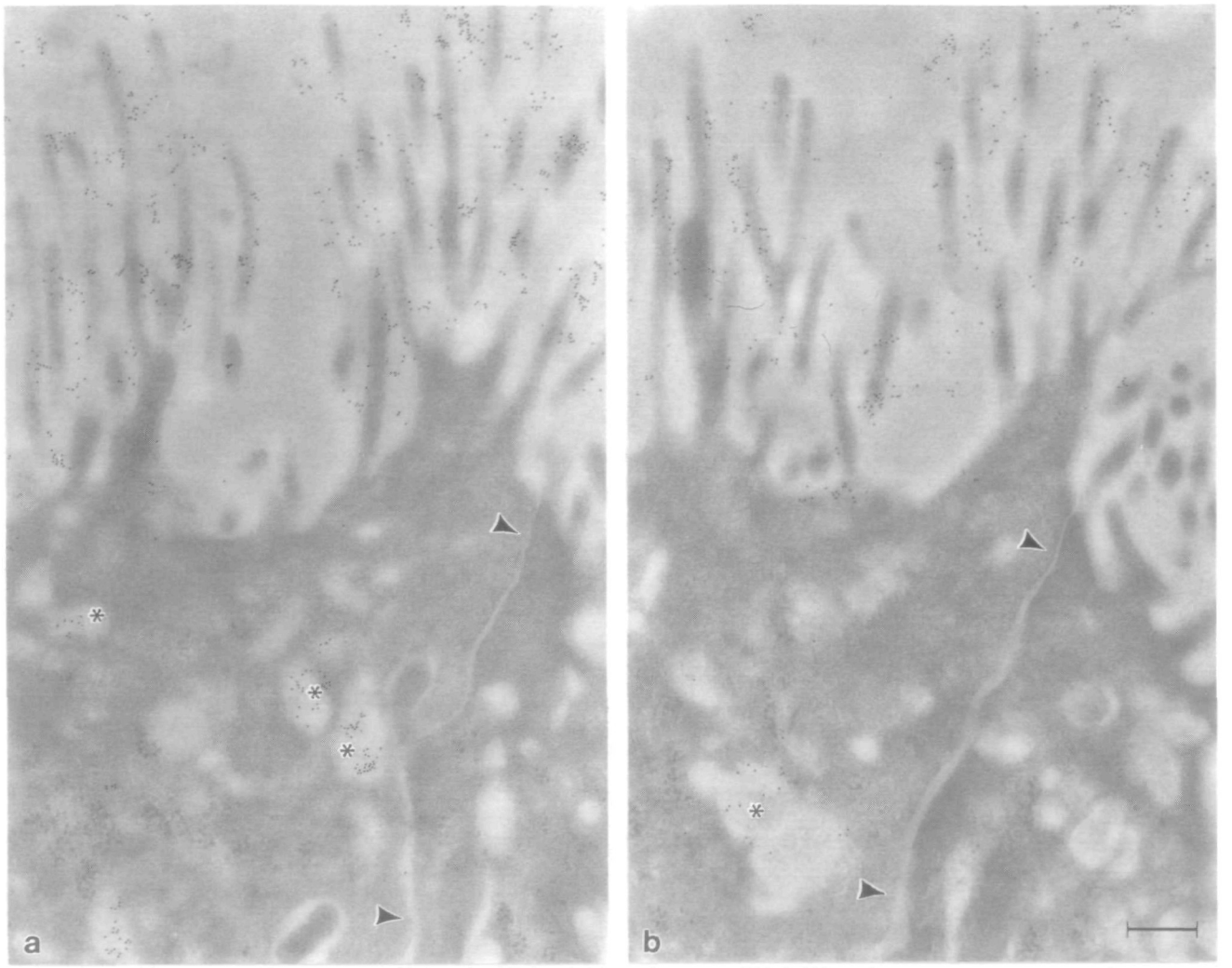

Fig. 8. Two serial sections (not consecutive) showing the apical cytoplasm and plasma membrane of a tracheal brush cell. Binding sites for both mab H5 (a) and mab H12 (b) are observed in the apical plasma membrane of the same brush cell. Apical cytoplasmic vesicles (asterisks) are also stained. Note the absence of staining along the lateral plasma membrane (arrowheads) separating the brush cell from its neighbour. Bar 0.29 μm (a, b).

Table I. Subcellular distribution of β1,4-GT in bovine epithelial cells

Tissue	Cell type	*cis*-Golgi	middle Golgi	*trans*-Golgi	TGN	APM
Proximal colon	Absorptive	−	−	+	−	−
	Goblet	−	−	+	−	−
Duodenum	Absorptive	−	−	−	−	−
	Goblet	−	−	+	−	−
Thymus	Epithelial-reticular	−	−	+	±	−
Trachea	Ciliated	−	±	+	−	−
	Goblet	−	±	+	−	−
	Brush[a]	−	±	+	−	+[b]

This table is based on observed immunostaining obtained with the monoclonal anti-bovine β1,4-GT antibodies. Division of the Golgi apparatus into *cis*, middle, *trans* and *trans*-Golgi network subcompartments is highly subjective; see the text for details.
TGN, *trans*-Golgi network; APM, apical plasma membrane.
(−), no detectable immunolabel; (+), immunolabel present; (±), label present in portions of this Golgi apparatus subcompartment.
[a]Tentatively identified as 'brush' cell based on morphological characteristics
[b]Label present at APM on only a portion of cells.

Nevertheless, our present results indicate that the subcompartmental distribution of β1,4-GT in the Golgi apparatus is restricted to the *trans* region across many different cell types, and within similar cells in different organs. This contrasts with the differential distribution of α2,6-sialyltransferase observed in the Golgi apparatus of rat intestinal absorptive and goblet cells (Roth *et al.*, 1986). In goblet cells, α2,6-sialyltransferase was found to be compartmentalized to *trans* cisternae of the Golgi apparatus, while in adjacent absorptive cells this immunoreactivity for α2,6-sialyltransferase was observed throughout the Golgi apparatus cisternal stack (with the exception of the fenestrated first *cis* cisterna). However, in the present study we did find a regional distribution within the intestinal tract of binding sites for the monoclonal anti-β1,4-GT antibodies. In the large intestine, the Golgi apparatus was stained in both absorptive and goblet cells. In contrast, in the small intestine (duodenum) from the same animals absorptive cell Golgi apparatus was not stained. The Golgi apparatus and apical plasma membrane of absorptive cells were stained with RCL I, indicating the presence of β-galactose residues (Goldstein and Poretz, 1986). However, since RCL I is not a specific probe for β1,4-linked galactose residues (Goldstein and Poretz, 1986), this lectin binding could represent activity from a host of other

galactosyltransferases. β1,4-GT is constitutively expressed in most if not all somatic cells. Why then were we unable to immunocytochemically detect β1,4-GT in duodenal absorptive cells? Although not clear at this point, the answer may lie in the limitations of immunocytochemical techniques. Perhaps this enzyme is maintained in much lower quantities within the Golgi apparatus of duodenal absorptive cells as compared to absorptive cells from the large intestine, or the turnover time of this enzyme is much faster in these cells.

β1,4-GT does not appear to be expressed at the cell surface of most bovine epithelial cells

In addition to its primary location in the Golgi apparatus, β1,4-GT immunoreactivity has also been reported to be present at the cell surface of many tissues and cell types [Berger *et al.*, 1981; Pestalozzi *et al.*, 1982; Davis *et al.*, 1984; Roth *et al.*, 1985; Shaper *et al.*, 1985; see also the recent review by Shur (1991)]. Most epithelial cells displayed cell surface β1,4-GT immunoreactivity when assessed at either the light or electron microscopic levels (Pestalozzi *et al.*, 1982; Davis *et al.*, 1984; Roth *et al.*, 1985; Shaper *et al.*, 1985), while human fibroblasts and HeLa cells in culture showed only Golgi apparatus-associated immunoreactivity (Berger *et al.*, 1981; Roth and Berger, 1982). Indeed, the immunohistochemical localization of β1,4-GT at the cell surface was taken as corroborative evidence for earlier suggestions of ecto-galactosyltransferase based on enzyme activity measurements and autoradiographic incorporation of radioactive substrates (Porter and Bernacki, 1975; Weiser *et al.*, 1978; Cummings *et al.*, 1979). Interestingly, the immunohistochemical studies are linked by the use of affinity-purified polyclonal antibodies raised against a purified soluble form of the enzyme derived from human milk (Berger *et al.*, 1981) or bovine colostrum (Shaper *et al.*, 1985). Although the polyclonal anti-β1,4-GT antibodies raised against the human milk enzyme were affinity purified (Berger *et al.*, 1981), they were subsequently found, via sensitive radiobinding assays, to contain antibody species recognizing blood group-related carbohydrate structures present on the human β1,4-GT polypeptide (Childs *et al.*, 1986; Feizi *et al.*, 1987). Thus, pre-absorption of the affinity-purified anti-β1,4-GT antibody with blood group substances resulted in the abolition of immunofluorescence on sections from human intestine and stomach (Childs *et al.*, 1986; Feizi *et al.*, 1987). We have found similar results with a monospecific affinity-purified polyclonal antibody raised against affinity-purified bovine milk β1,4-GT. Although the antibody decorated the plasma membrane and mucus droplets on identical sections from bovine intestine as used in this study with the monoclonal antibodies, pre-absorption of the antibody with asialomucin virtually abolished all cell surface and mucus droplet staining (D.J.Taatjes, unpublished). These findings might not be totally unexpected since human β1,4-GT is a glycoprotein containing highly immunogenic carbohydrate structures (Childs *et al.*, 1986; Amano *et al.*, 1991). From these studies, it would appear that the majority of the staining observed at the cell surface was due to recognition of carbohydrate epitopes and not β1,4-GT polypeptide.

There are several ways available to potentially avoid antibodies raised against β1,4-GT which may in actuality recognize carbohydrate epitopes present on the polypeptide. The availability of cDNA clones for β1,4-GT (Shaper *et al.*, 1986) would allow the production of non-glycosylated recombinant proteins in bacteria, which could be then used as affinity matrices for the purification of polyclonal antisera. By definition, the resulting affinity-purified antibodies would recognize only protein epitopes. We have used this strategy in a previous study for the selection of polypeptide-specific antibodies against rat α2,6-sialyltransferase (Taatjes *et al.*, 1988). Similarly, the recombinant β1,4-GT could be used as an immunogen for eliciting an antibody response in injected animals. Indeed, Berger and colleagues (Watzele *et al.*, 1991) recently used such techniques for the production of a polyclonal rabbit antibody directed against a non-glycosylated fusion protein of human β1,4-GT produced in *E.coli*. The polyclonal antiserum decorated a peri-nuclear pattern, indicative of the Golgi apparatus, upon immunofluorescence labelling of HeLa and CaCo-2 cells in culture. Alternatively, one could chemically deglycosylate purified β1,4-GT and use this form of the enzyme as an immunogen for injection into rabbits. This method was applied by Berger and colleagues (1987a); however, they still could not demonstrate exclusive protein specificity with the resulting antisera. Moreover, the achievement of total chemical deglycosylation is difficult to establish unequivocally. Finally, monoclonal antibodies could be raised against affinity-purified β1,4-GT and selected for their ability to recognize non-glycosylated recombinant protein produced in bacteria. This is the approach we have used in the present paper. Thus, we have visualized immunocytochemically three different epitopes located within the β1,4-GT polypeptide (Ulrich *et al.*, 1986; Russo, 1990). Berger and colleagues (1986) have also produced a series of monoclonal antibodies against human milk β1,4-GT. While some were shown to recognize a Golgi apparatus-like pattern in HeLa cells by immunofluorescence staining, others were found to stain cytoskeletal elements, nuclei or contact sites of the cell surface (Berger *et al.*, 1986).

Recently, Suganuma and co-workers (1991) reported the immunoelectron microscopic localization of β1,4-GT in a spectrum of murine tissues. They used a rat IgM monoclonal antibody produced against the enzyme purified from mouse F9 cells and observed staining using immunoperoxidase methodology in *trans*-Golgi apparatus cisternae, surprisingly in the nuclear envelope, and in the plasma membrane of F9 EC cells, PYS-2 parietal endoderm cells, STO fibroblasts, gastric mucosal epithelial cells, intestinal goblet cells, spermatocytes, Sertoli cells, spermatids and epididymal epithelial cells (Suganuma *et al.*, 1991). In addition, a general plasma membrane staining was observed on most epithelial cells. In contrast, of all the epithelial cells we examined in the present study, staining was observed at the plasma membrane from only a minority of bovine tracheal epithelial cells. The staining tended to be restricted to a subregion of the plasma membrane comprising the microvillar projections. Importantly, this immunoreactivity was observed with all three of the anti-β1,4-GT monoclonal antibodies, arguing strongly against a carbohydrate determinant being responsible for this plasma membrane staining. Although we cannot rule out the formal possibility that the three different monoclonal antibodies recognize an unrelated cell surface protein, the most direct explanation for these observations is that in these cells the immunoreactivity at the plasma membrane may represent an ecto-galactosyltransferase present in quantities sufficient for detection with our methods. Indeed, immunostaining of serial sections with two of the monoclonal antibodies demonstrated binding to the apical plasma membrane of the same epithelial cell, arguing in favour of the recognition

of β1,4-GT. These results, together with those described above, do not conclusively prove the existence of an ecto-galactosyltransferase; rather a combination of approaches, including molecular biology, biochemistry and immunocytochemistry, may probably be required to solve this controversial issue (Berger, 1991; Suganuma, 1991).

Materials and methods

Reagents

Staphylococcal protein A was obtained from Pharmacia Fine Chemicals (Piscataway, NJ), RCL I was from Vector Laboratories (Burlingame, CA), and affinity-purified rabbit anti-mouse IgG was from Organon Teknika-Cappel (West Chester, PA). Tetrachlorauric acid, trisodium citrate and paraformaldehyde were from Merck (Darmstadt, FRG), tannic acid (Aleppo tannin, no 1740) from Mallinckrodt (St Louis, MO) and glutaraldehyde (vacuum distilled) came from Fluka (Buchs, Switzerland). Ovalbumin, bovine serum albumin (RIA grade, fraction V), Triton X-100, Tween 20, fetuin, asialofetuin, asialomucin, peroxidase, ovomucoid (trypsin inhibitor) and DSL were purchased from Sigma (St Louis, MO). All other reagents were of the highest available purity.

Antibodies

Three mouse monoclonal antibodies (designated H5, H12 and H162) were developed against a soluble form of bovine UDP-galactose:*N*-acetylglucosamine galactosyltransferase purified to apparent homogeneity by a combination of affinity and immunoadsorption chromatography, as previously described (Ulrich *et al*, 1986). By a combination of competitive binding assays and Western blot analysis with β1,4-GT, these monoclonal antibodies were found to recognize distinct epitopes of the polypeptide (Ulrich *et al.*, 1986). Moreover, recent studies have demonstrated that each of these antibodies can recognize, by Western blot analysis, three different peptide epitopes on the recombinant bovine β1,4-GT polypeptide expressed in *E.coli*, indicating that the three different epitopes are contained within the primary structure (Russo, 1990; Russo *et al.*, 1992).

For comparison, a monospecific affinity-purified rabbit polyclonal antibody raised against affinity-purified bovine milk β1,4-GT (Shaper *et al*, 1985) was also used for immunocytochemical localization experiments.

Tissue processing

Bovine tissues were freshly obtained from a local slaughterhouse in Basel, Switzerland. Small pieces from the trachea, thymus, small and large intestine were excised and quickly immersed in 3% formaldehyde (freshly prepared from paraformaldehyde), 0.1% glutaraldehyde in 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4 [phosphate-buffered saline (PBS)] for 2 h at room temperature. During the fixation period, the tissue pieces were minced into smaller pieces of ~1 mm^3 After two quick rinses with PBS, free aldehyde groups were quenched by immersing the tissue pieces in 50 mM ammonium chloride in PBS for 1 h. Afterwards, the pieces were rinsed with PBS and stored in PBS overnight at 4°C. The next day, the tissue pieces were dehydrated in a series of graded ethanols at progressively lower temperature and embedded in Lowicryl K4M (Carlemalm *et al.*, 1982) at −35°C as previously described (Roth *et al*, 1981).

Preparation of colloidal gold-labelled reagents

Colloidal gold particles were prepared with an average diameter of 10 nm according to the tannic acid/citrate reduction method (Slot and Geuze, 1985) and with an average diameter of 15 nm according to the citrate reduction method (Frens, 1973). Protein A was complexed with 10 nm colloidal gold particles according to standard protocols (Roth *et al.*, 1978). Asialofetuin was complexed with 10 or 15 nm colloidal gold particles as previously described (Taatjes *et al.*, 1990).

Immunolocalization of β1,4-galactosyltransferase

Immunoreactive sites for β1,4-GT were detected with the protein A–gold technique (Roth *et al*, 1978). Ultrathin sections were cut with glass knives and collected on 150 mesh nickel grids coated with parlodion and carbon. Grids were floated section side down on drops of 0.5% ovalbumin in PBS for 20 min, followed by transfer to droplets of one of the anti-galactosyltransferase antibodies and incubated overnight (14–21 h) at 4°C. The antibodies were used at the following concentrations [diluted with PBS containing 0.1% bovine serum albumin (BSA)]: mab H5 (5 μg/ml), mab H12 (0.3 μg/ml) and mab H162 (50 μg/ml). Since mab H12 tended to produce somewhat high background, we also used this antibody diluted with PBS containing 1% BSA, 0.075% Triton X-100 and 0.075% Tween 20 (PBS+++) to a concentration of 12.5 μg/ml. This resulted in a virtual absence of background gold particles. Following two rinses with PBS (5 min each), the grids were either placed directly onto droplets of protein A–gold or incubated for 1 h at room temperature on droplets of affinity-purified rabbit anti-mouse IgG (10 μg/ml). Incubation of grids on the secondary antibody rabbit anti-mouse proved to not be obligatory, since its omittance nevertheless resulted in specific immunostaining with the monoclonal antibodies The grids were incubated for 1 h on droplets of protein A–gold (10 nm colloidal gold particles), diluted with PBS+++ to yield an optical density (OD) of 0.06 at 525 nm. Following rinses with PBS and distilled water, the grids were air dried. Finally, the sections were contrasted with 3% aqueous uranyl acetate for 5 min and lead acetate for 45 s.

The monospecific affinity-purified rabbit polyclonal antibody against β1,4-GT was used at a concentration of 50 μg/ml, followed by protein A–gold as described above for the monoclonal antibodies.

Localization of β-galactose residues

β-Galactose residues were detected on ultrathin sections using lectins in a two-step cytochemical affinity technique as previously described (Taatjes *et al.*, 1990). Sections were floated on drops of PBS for 5 min, followed by transfer to droplets of RCL I [nominal specificity for β-galactose (Goldstein and Poretz, 1986)] or DSL [nominal specificity for Galβ1,4GlcNAc (Goldstein and Poretz, 1986)] for 45 min at room temperature. RCL I was used at a concentration of 75 μg/ml and DSL at a concentration of 25 μg/ml. Following two rinses with PBS (5 min each), the grids were incubated for 30 min on droplets of asialofetuin–gold complex. The asialofetuin–gold complexes (10 or 15 nm) were diluted with PBS+++ to yield an optical density of 0.3 at 525 nm. Finally, the grids were rinsed with PBS and distilled water, and contrasted as described above.

Double labelling procedure

Immunoreactivity for galactosyltransferase and lectin recognition of β-galactose residues were performed on the same sections in a double staining procedure Grids were floated on drops of PBS for 5 min, followed by incubation on droplets of DSL (25 μg/ml) for 30 min at room temperature. After two rinses with PBS, the grids were transferred to droplets of asialofetuin–gold complex (15 nm particles; diluted with PBS+++ to yield an $OD_{525\ nm}$ of 0.3) for 30 min at room temperature. This was followed by rinses with PBS and distilled water, and air drying. The dried grids were then floated for 20 min on drops of PBS containing 0.5% ovalbumin, followed by incubation overnight at 4°C on droplets of mab H5 (5 μg/ml). After two rinses with PBS, the grids were placed on droplets of protein A–gold complex (10 nm particles; diluted with PBS+++ to yield an $OD_{525\ nm}$ of 0.06) for 1 h at room temperature. Finally, the grids were rinsed, air dried and contrasted as described above.

Specificity control incubations

For antibody incubations: (i) grids were incubated on PBS containing 0.1% BSA overnight at 4°C, followed by incubation on droplets of protein A–gold complex for 1 h; (ii) grids were incubated on PBS containing 0.1% BSA overnight at 4°C, followed by sequential incubation on droplets of affinity-purified rabbit anti-mouse IgG for 1 h and protein A–gold complex for 1 h; (iii) antibodies were preabsorbed with various glycoproteins prior to use. Briefly, asialomucin, asialofetuin, fetuin, ovomucoid or peroxidase were reconstituted in PBS containing 0.1% BSA to a concentration of 200 μg/ml. Equal volumes of each of these glycoprotein solutions were mixed with double-strength antibody concentrations 30 min prior to use for incubations

For lectin incubations: (i) grids were floated on drops of PBS for 30 min, followed by incubation on droplets of asialofetuin–gold complex for 30 min.

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Abbreviations

BSA, bovine serum albumin; DSL, *Datura stramonium* lectin; β1,4-GT, β1,4-galactosyltransferase; mab, monoclonal antibody; PBS, phosphate-buffered saline; RCL I, *Ricinus communis* lectin I

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