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Subcellular distribution of terminal α-D- and β-D-galactosyl residues in Ehrlich tumour cells studied by lectin-gold techniques

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We have studied by high resolution *in situ* light and electron microscopic lectin-gold techniques the subcellular distribution of α-D-Gal residues using the *Griffonia simplicifolia* I-B_4 isolectin and compared it with that of β-D-Gal residues as detected with the *Datura stramonium* lectin in Ehrlich tumour cells grown as ascites or monolayer. The microvillar but not the smooth plasma membrane regions were labelled with the *Griffonia simplicifolia* I-B_4 isolectin whereas both plasma membrane regions were equally well labelled with the *Datura stramonium* lectin. Elements of the endocytotic/lysosomal system such as coated membrane invaginations and vesicles, early and late endosomes and secondary lysosomes were positive for both α-D-Gal and β-D-Gal residues. A particular feature of Ehrlich tumour cells is an elaborate tubular membrane system located in the pericentriolar region which is labelled throughout by both lectins and represents part of the endosomal system. In the Golgi apparatus labelling with both lectins was observed to commence in trans cisternae which is indirect evidence for a joint distribution of the sequentially acting β1,4 and α1,3-galactosyltransferases.

Keywords: Ehrlich tumour cells, galactosyl residues, *Griffonia simplicifolia* I-B_4 isolectin, lectin-gold, electron microscopy

Introduction

Ehrlich tumour cells are derived from a spontaneous murine mammary adenocarcinoma and have been adapted to grow in ascites form in mice or as adherent monolayers in culture dishes [1–3]. Previous biochemical analyses have demonstrated that Ehrlich tumour cells contain a number of glycosyltransferases involved in the biosynthesis of the N-acetyllactosamine sequence [4, 5] and also an α1,3-galactosyltransferase that catalyses the synthesis of α-D-Gal(1,3)-β-D-Gal(1,4)-D-GlcNAc-R units [6]. This oligosaccharide sequence occurs uncommonly on the surface of cells [7, 8] but in addition to Ehrlich tumour cells [9] several other murine cell lines have been shown to express oligosaccharides terminated by α-D-Gal residues [10–13]. Interestingly, it has been demonstrated that in murine tumour cell lines a positive correlation exists between their malignant potential and the levels of α-D-Gal residues present on the cell surface [14–16]. Further, the α-D-Gal(1,3)-β-D-Gal(1,4)-D-GlcNAc-R sequence could be detected in human tumour cells and samples [17].

Eckhardt and Goldstein [18–20] isolated and characterized a series of α-D-Gal*p*-containing glycoproteins from Ehrlich ascites tumour cell membranes which ranged in molecular weight from 50 to 200 kDa with a major glycoprotein of 130 kDa. The tentative structure of the glycopeptides was proposed to be tetraantennary branched complex type asparagine-linked oligosaccharides. Each of the keratan-like branches terminated with α-D-Gal units. The distribution and quantitation of α-D-Gal*p* groups on the Ehrlich tumour cell surface was determined by using the α-D-galactopyranosyl-specific isolectin *Griffonia simplicifolia* I-B_4 labelled with fluorescein isothiocyanate, ferritin, or (^{3}H) propionate [19]. A minimum of 18.1×10^6 α-D-Gal*p* groups was estimated per Ehrlich tumour cell surface and found to be randomly distributed over the cell surface. It is not yet known if other internal membranes of Ehrlich tumour cells contain glycoproteins with α-D-Gal*p* residues.

In the present paper we report studies on the subcellular distribution of α-D-Gal*p* groups on the cell surface and intracellular organelles of Ehrlich tumour cells. For this purpose a gold-labelled *Griffonia simplicifolia* I-B_4 was prepared and

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used to label ultrathin sections of Lowicryl K4M embedded tumour cells. In addition, the *Datura stramonium* lectin was applied to reveal the subcellular distribution of β-D-Gal (1,4)-D-GlcNAc sequences [21–23] applying the same technique [24]. Our results demonstrate the presence of glycoconjugates reactive with both lectins in the Golgi apparatus and various post Golgi regions including a specialized highly developed endosomal system.

Materials and methods

Reagents The *Griffonia simplicifolia* I-B_4 isolectin (GS I-B_4) was purified as described by Hayes and Goldstein [25] and Murphy and Goldstein [26]. The lectin was directly labelled with colloidal gold (particle size 15 nm) according to established protocols [27, 28]. In brief, the colloidal gold was adjusted to a pH of 6.0. To stabilize 10 ml of colloidal gold 330 μg of GS I-B_4 dissolved in distilled water was rapidly added and after 1 min 1 ml of 1% aqueous carbowax. Purification and concentration of the crude lectin-gold complex was performed by ultracentrifugation in an angle rotor at 60 000 × **g** for 45 min. The sedimented lectin-gold complex was resuspended in PBS (10 mM phosphate buffer, pH 7.2, 0.15 M NaCl) and stored at 4°C.

An ovomucoid-gold complex using 8 nm gold particles was prepared as described [24]. The *Datura stramonium* lectin, ovomucoid and methyl α-D-galactopyranoside were purchased from Sigma (St Louis, MI), coffee bean α-galactosidase from Boehringer Mannheim (Mannheim, Germany); glutaraldehyde (vacuum distilled), silver acetate and carbowax 20 M from Fluka (Buchs, Switzerland), paraformaldehyde, hydroquinone, trisodium citrate and tetrachloroauric acid from Merck (Darmstadt, Germany) and *N*-acetyllactosamine from Biocarb Chemicals (Lund, Sweden). *N,N'*-diacetylchitobiose was available from previous experiments in the laboratory of Dr I. J. Goldstein.

Cells and low temperature Lowicryl K4M embedding Ehrlich tumour cells grown in mice as ascites or as a monolayer in culture dishes were washed in Hanks balanced salt solution and fixed with 2% formaldehyde (freshly prepared from paraformaldehyde) and 0.1% glutaraldehyde at 37 °C for 30 min, washed with PBS twice for 5 min each time, and treated with 50 mM NH_4Cl in PBS for 30 min to amidinate free aldehyde groups. Ascites cells or mechanically removed monolayer tumour cells were stored in PBS at 4°C until embedding. For embedding, cells were pelleted and enclosed in 2% agar. Small pieces of agar with the enclosed cells were dehydrated in graded ethanol at progressively lowered temperatures (down to –40°C), infiltrated with Lowicryl K4M at –40°C and polymerized at –40°C by indirect UV-light irradiation as described [29, 30]. Semithin (1 μm) sections were mounted on poly-L-lysine activated glass slides and ultrathin sections on parlodion/carbon-coated nickel grids.

Lectin-gold labelling techniques Identical labelling conditions were applied for semithin and ultrathin sections of Lowicryl K4M-embedded Ehrlich tumour cells.

Sections were conditioned with PBS containing 0.05% Tween 20 for 5–10 min at room temperature. The GS I-B_4-gold complex was diluted with PBS containing 0.05% Tween 20 to an absorbance of 0.1 at 525 nm and sections incubated for 45 min at room temperature. Afterwards, sections were rinsed with buffer and distilled water and air dried before counterstaining with uranyl acetate and lead acetate [31]. For light microscopic demonstration of the lectin-gold labelling a silver intensification technique was used [32].

For labelling with DSA, sections were incubated as described previously [24]. In brief, sections were conditioned as described above and incubated with DSA (75–100 μg ml^{-1} in PBS containing 0.05% Tween 20) for 45 min at room temperature. Following two rinses with PBS for 5 min each, incubation with ovomucoid-gold complex (diluted with PBS containing 1% BSA, 0.05% Triton X-100 and 0.05% Tween 20 to an absorbance of 0.2 at 525 nm) was performed for 45 min at room temperature.

Cytochemical controls Specificity controls for the GS I-B_4-gold labelling included treatment of sections with coffee bean α-galactosidase, or preabsorption of the GS I-B_4-gold complex with methyl α-D-galactopyranoside. Control incubations for the DSA labelling were those previously reported [24].

Results

Light microscopy The labelling of semithin sections of the ascites or monolayer form of Ehrlich tumour cells with the GS I-B_4-gold complex and the DSA fetuin-gold technique gave indistinguishable results as shown in Fig. 1. Both lectins produced intense cell surface labelling and, in addition to a sparse, fine punctate cytoplasmic staining, labelled intensely a limited region in perinuclear location.

Electron microscopy An identical labelling pattern by the GS I-B_4-gold complex was observed in the ascites and monolayer form of Ehrlich tumour cells. The outer surface of the plasma membrane exhibited gold particle labelling along the microvillar plasma membrane regions whereas the smooth parts of the plasma membrane were virtually free of gold particle labelling (Figs 2a–c and 4b). Occasionally, plasma membrane invaginations were sparsely labelled (Fig. 2a–c). Endocytotic vesicles close to the plasma membrane as well as peripheral endosomes were positive (Fig. 2a,b). Cisternae located at the *trans* side of the Golgi apparatus stack and the *trans* Golgi network were labelled (Fig. 2c). Typical secondary lysosomes in the neighbourhood of the Golgi apparatus and other parts of the cytoplasm presented gold particle labelling along their limiting membrane and over their content (Fig. 2d). A highly developed membrane system consisting of

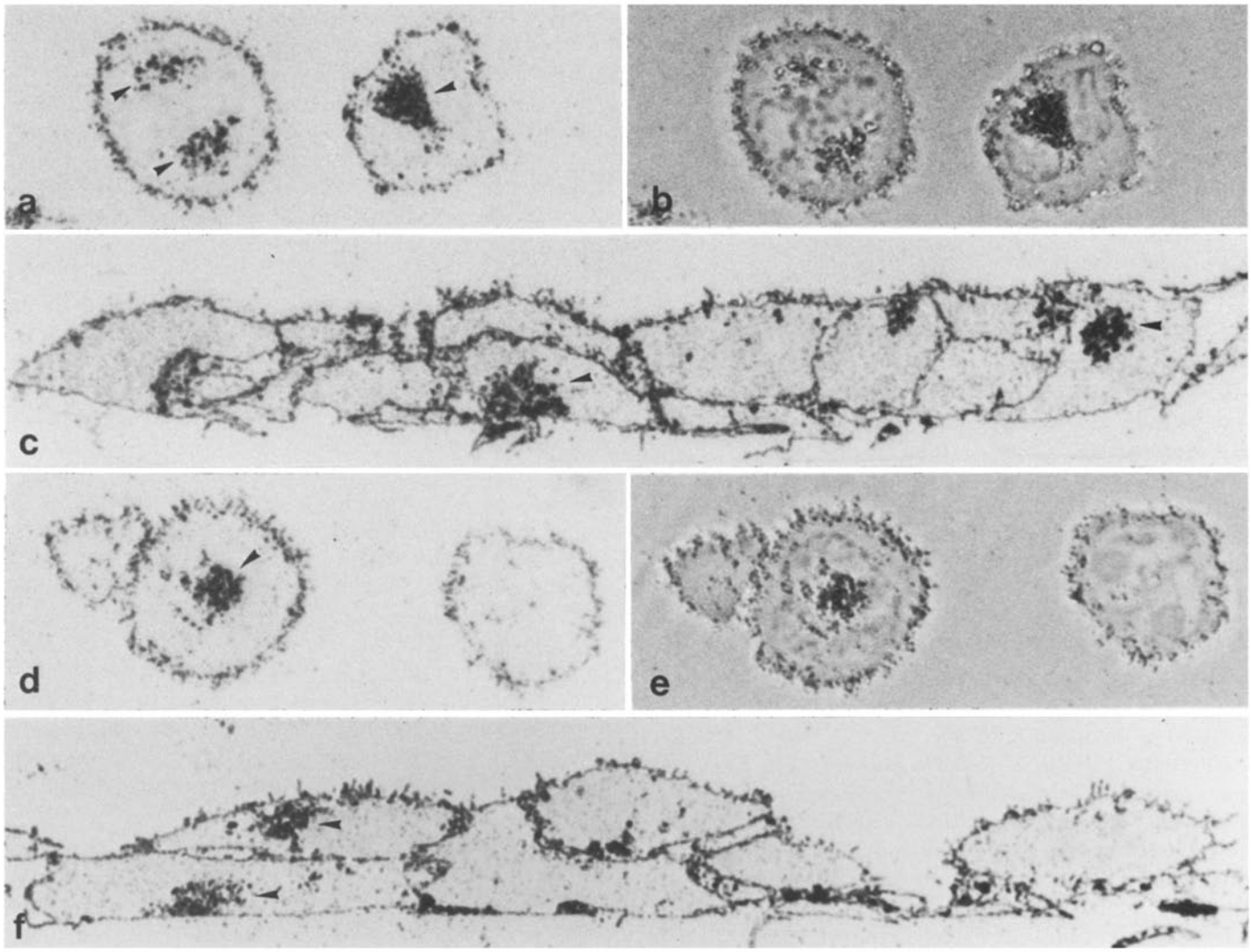


Figure 1. Semithin (1 μm) sections of Lowicryl K4M embedded Ehrlich tumour cells incubated to detect binding sites for GS I-B_4 and DSA. Positive staining occurs in black since the gold particle labelling was silver intensified. Ehrlich tumour cells grown in ascites form exhibit intense cell surface and intracellular staining for GS I-B_4 (a,b) and DSA (d,e). Since the Lowicryl K4M sections were not counterstained, the relation of the intensely positive cytoplasmic region to the nucleus can only be unequivocally appreciated by phase contrast illumination (b,e). Ehrlich tumour cells grown as an adherent monolayer also show intense cell surface staining, sparse punctate cytoplasmic staining and an intensely labelled perinuclear region (arrowheads) for GS I-B_4 (c) and DSA (f). (× 800).

a complex of tubulo-vesicular smooth surfaced membranes was a prominent structure closely related to the Golgi apparatus and centrioles (Figs 3 and 4). This membrane system was thoroughly labelled with the GS I-B_4-gold complex. Labelling was undetectable over the nucleoplasm and nuclear envelope, cisternae of the rough endoplasmic reticulum and mitochondria (Figs 2–4). The above described labelling was undetectable under the various control conditions.

The labelling pattern with DSA observed in Ehrlich tumour cells was similar to that of GS I-B_4. Examples for the DSA labelling of the plasma membrane (Fig. 5a), coated plasma membrane invaginations (Fig. 5b), endosomes and lysosomes (Fig. 5c, e), *trans* Golgi apparatus cisternae and *trans* Golgi network (Fig. 5d) and the pericentriolar smooth surfaced tubulo-vesicular membrane system (Fig. 5e) are provided. It should be noted that the DSA labelled both microvillar and smooth plasma membrane regions (Fig. 5a). Further, the labelling intensity by DSA was generally higher than with the GS I-B_4-gold complex. Despite differences in the gold particle size, this is probably due to the different labelling techniques used. A two-step technique as compared to a one-step technique usually produces more intense gold particle labelling.

Discussion

The present study was aimed at the precise subcellular localization of terminal non-reducing α-D-Gal residues of glycoproteins in Ehrlich tumour cells to extend existing analytical

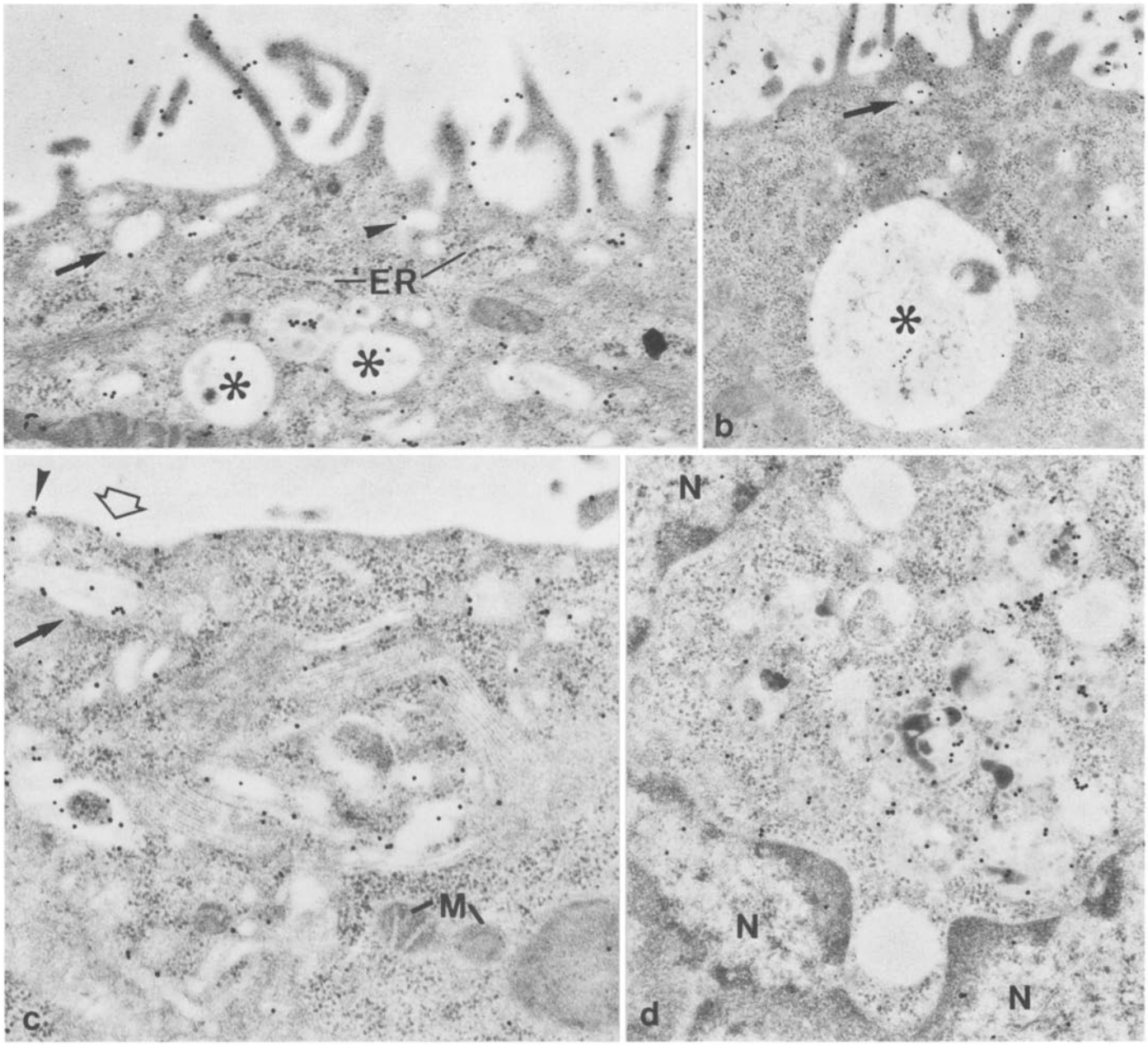
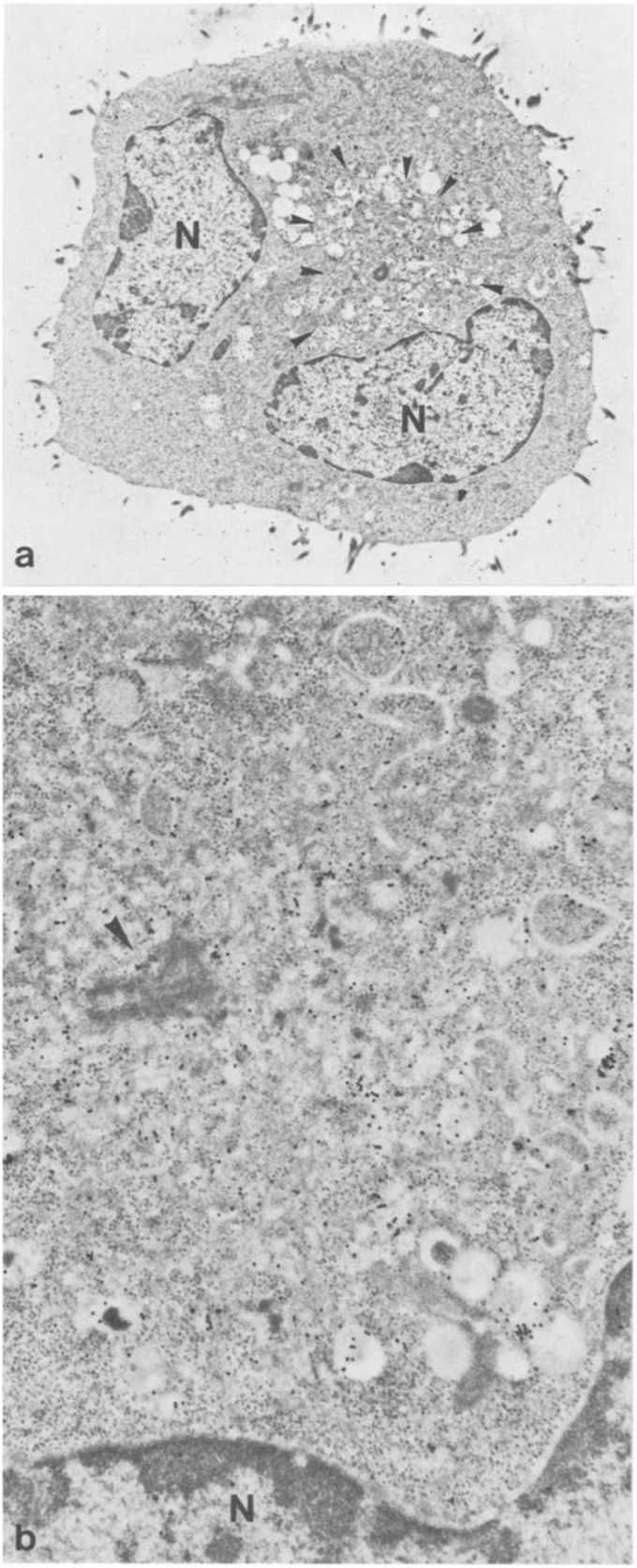


Figure 2. Ultrathin sections of Lowicryl K4M embedded Ehrlich ascites tumour cells labelled with GS I-B_4 -gold complexes. Gold particle labelling is present along the microvillar plasma membrane regions (a,b) and undetectable in the smooth plasma membrane regions (a–c) except for some limited portions (open arrow in c) close to labelled membrane invaginations (arrowheads in a and c). Endocytotic (arrows in a–c) and endosomal (asterisks in a and b) as well as lysosomal elements (d) are positive as are *trans* Golgi apparatus cisternae and the *trans* Golgi network (c). N; part of nucleus; M; mitochondria; ER; cisternae of the rough endoplasmic reticulum (a,c,d, × 40 000 (b) × 25 000).

biochemical data [18–20]. For this purpose high resolution *in situ* lectin-gold techniques [27] were employed at the level of light and electron microscopy taking advantage of the specificity of GS I-B_4 for such residues [25, 26]. Our results extend earlier biochemical data by definitely demonstrating the presence of α-D-Gal residues in specific intracellular membranes of Ehrlich tumour cells. Furthermore, the subcellular distribution of such residues was comparable to the terminal nonreducing β-D-Gal 1,4 D-GlcNAc residues as visualized with DSA.

The applied postembedding GS I-B_4-gold technique revealed the presence of cell surface α-D-Gal residues as previously demonstrated with ferritin conjugated GS I-B_4 in a preembedding technique [19]. The general advantages of cytochemical gold labelling techniques over ferritin techniques [27, 28, 33], however, provided evidence that α-D-Gal



residues are not randomly distributed in the plasma membrane of Ehrlich tumour cells grown either in ascites form or as a monolayer culture. The presence of GS I-B_4 binding sites in the microvillar plasma membrane and their virtual absence in the smooth plasma membrane regions contrasts with the appearance of DSA binding sites in both plasma membrane regions. The observed uniform distribution of the DSA labelling excludes the possibility that the GS I-B_4 labelling represents an artefact due to restricted accessibility of lectin binding sites. Our studies on sialoglycoconjugate distribution in Ehrlich tumour cells [34] are of relevance to the present findings. Sialic acid residues either in α 2,6 linkage to Gal/GalNAc as detected with the *Sambucus nigra* lectin or in α 2,3 linkage to Galβ1,3GalNAc-R as detected with the *Amaranthus caudatus* lectin showed a random cell surface distribution. In contrast, sialic acid α 2,3 linked to Galβ1,4(3)GlcNAc-R as demonstrated with the *Maackia amurensis* leukoagglutinin was more abundant in the microvillar as compared to the smooth plasma membrane regions. Collectively, these data demonstrate non-homogeneity in the distribution of certain cell surface glycoconjugates in Ehrlich tumour cells. From the static point of view of electron microscopic lectin labelling this non-homogeneity is most pronounced for α-D-Gal residues which are virtually undetectable in the smooth plasma membrane regions in a single ultrathin section through an Ehrlich tumour cell. However, as part of plasma membrane glycoproteins they must exhibit mobility in the plane of the plasma membrane and consequently should be detectable in endocytotic invaginations of the intermicrovillar plasma membrane. The analysis of our static electron micrographs demonstrated the presence of GS I-B_4 binding sites in endocytotic invaginations of the plasma membrane although this was only rarely observed. The presence of DSA binding sites in coated pits and coated plasma membrane invaginations was a constant finding. Pinched-off subplasmalemmal endocytotic vesicles and others fusing with peripheral endosomes as well as peripheral endosomes exhibited labelling with either lectin at the inner surface of their limiting membrane. Further, late endosomes and typical secondary lysosomes in the vicinity of the Golgi apparatus were labelled with both lectins. Thus, membrane glycoproteins carrying α-D-Gal and β-D-Gal residues can be detected in cellular membranes forming typical structures of the endocytotic compartment [35, 36]. The Ehrlich tumour cell cytoplasm contains an additional elaborate membrane system which consists of numerous, seemingly interconnecting tubules in the pericen-

Figure 3. Same material and labelling as in Fig 2. A low power micrograph showing an Ehrlich ascites tumour cell (a). The pericentriolar membrane system is delineated by arrowheads and corresponds to the intensely labelled cytoplasmic regions seen by light microscopy in Fig. 1. Higher magnification demonstrates details of the intensely GS I-B_4 labelled pericentriolar tubulo-vesicular membrane system (b). The arrowhead in b points to the centriole. N; nucleus; (a × 6500; b × 23 500).

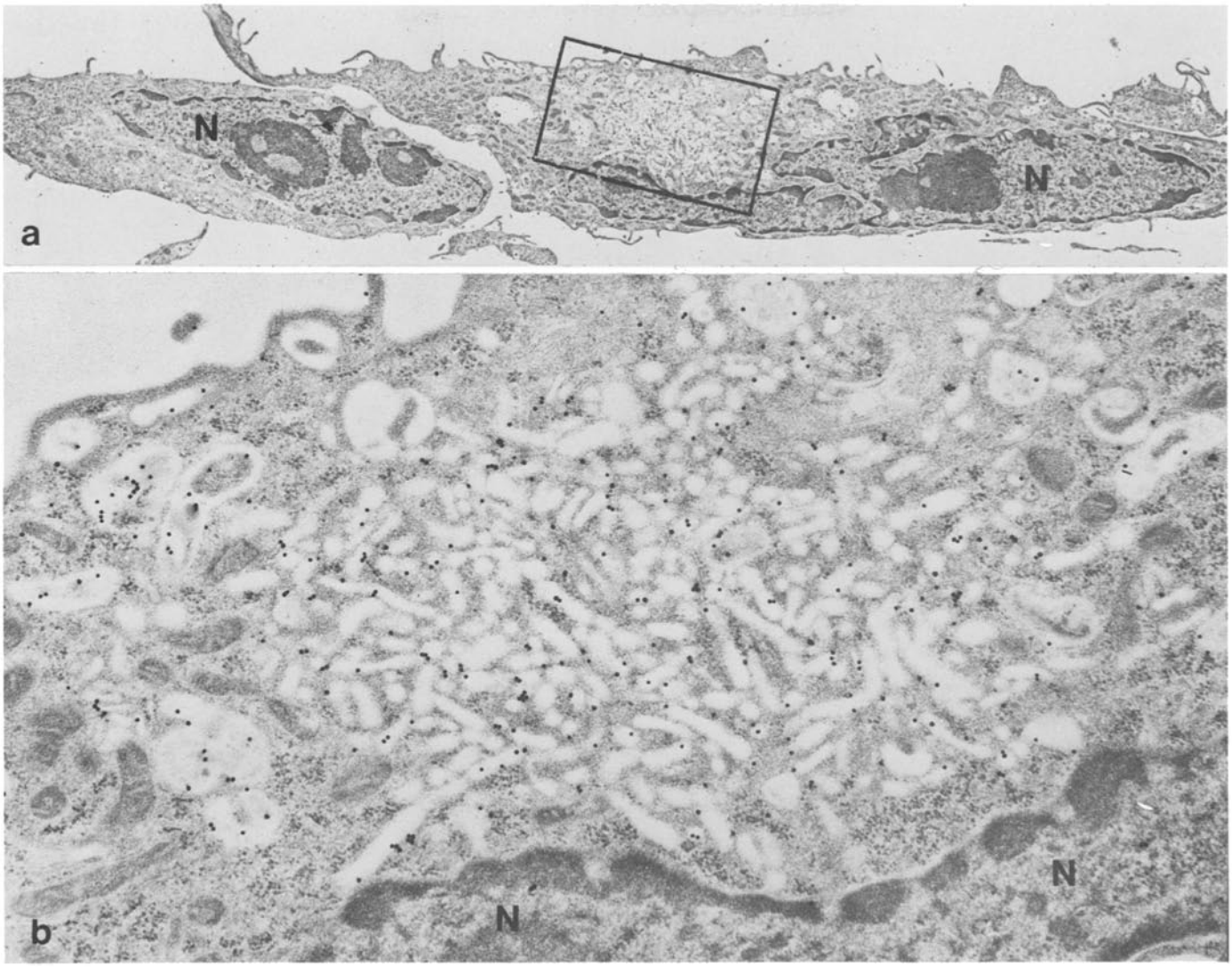


Figure 4. Ultrathin sections of Ehrlich tumour cells grown as adherent monolayer incubated with GS I-B_4-gold complexes. Two Ehrlich tumour cells are shown at low magnification (a) and the pericentriolar membrane system delineated by the rectangle is seen at higher magnification in (b). N; nucleus (a × 7500; b × 33 000).

triolar region. This system which was throughout labelled by GS I-B_4 and DSA corresponds to the strongly labelled perinuclear region observed by light microscopy and should not be confused with Golgi apparatus labelling. Such a concentration of tubules in the pericentriolar cytoplasm has been observed in various cultured cell types experimentally studied with regard to the recycling route of receptors and their respective ligands [37–41]. In these studies, continuity between tubules and endocytotic vacuoles was observed which was rarely seen in resting Ehrlich tumour cells. Our findings demonstrate that this pericentriolar tubular system is endogenous to Ehrlich cells grown either in ascites form or as a monolayer and is not induced by experimental manipulations. Further, our preliminary immunoelectron microscopic studies with antibodies against endosomal/lysosomal membrane glycoproteins [42] revealed the presence of immunoreactivity for such proteins. Therefore, we assume bone fide that the pericentriolar tubular system in Ehrlich tumour cells corresponds to the one found in other cell types and identified as representing a specialized distal segment of the recycling pathway [41].

Positive GS I-B_4 lectin staining indicating the presence of α-D-Gal residues was furthermore observed in the Golgi apparatus including the *trans* Golgi network but not in the endoplasmic reticulum including the nuclear envelope, the nucleoplasm or mitochondria. The labelling of the Golgi apparatus by GS I-B_4 and DSA is not surprising in view of the established location of late glycosylation reactions in this organelle [43]. Specifically, immunolabelling for UDP-gal:GlcNAcβ1,4 gal transferase has localized this glycosyltransferase to *trans* cisternae of the Golgi apparatus in HeLa

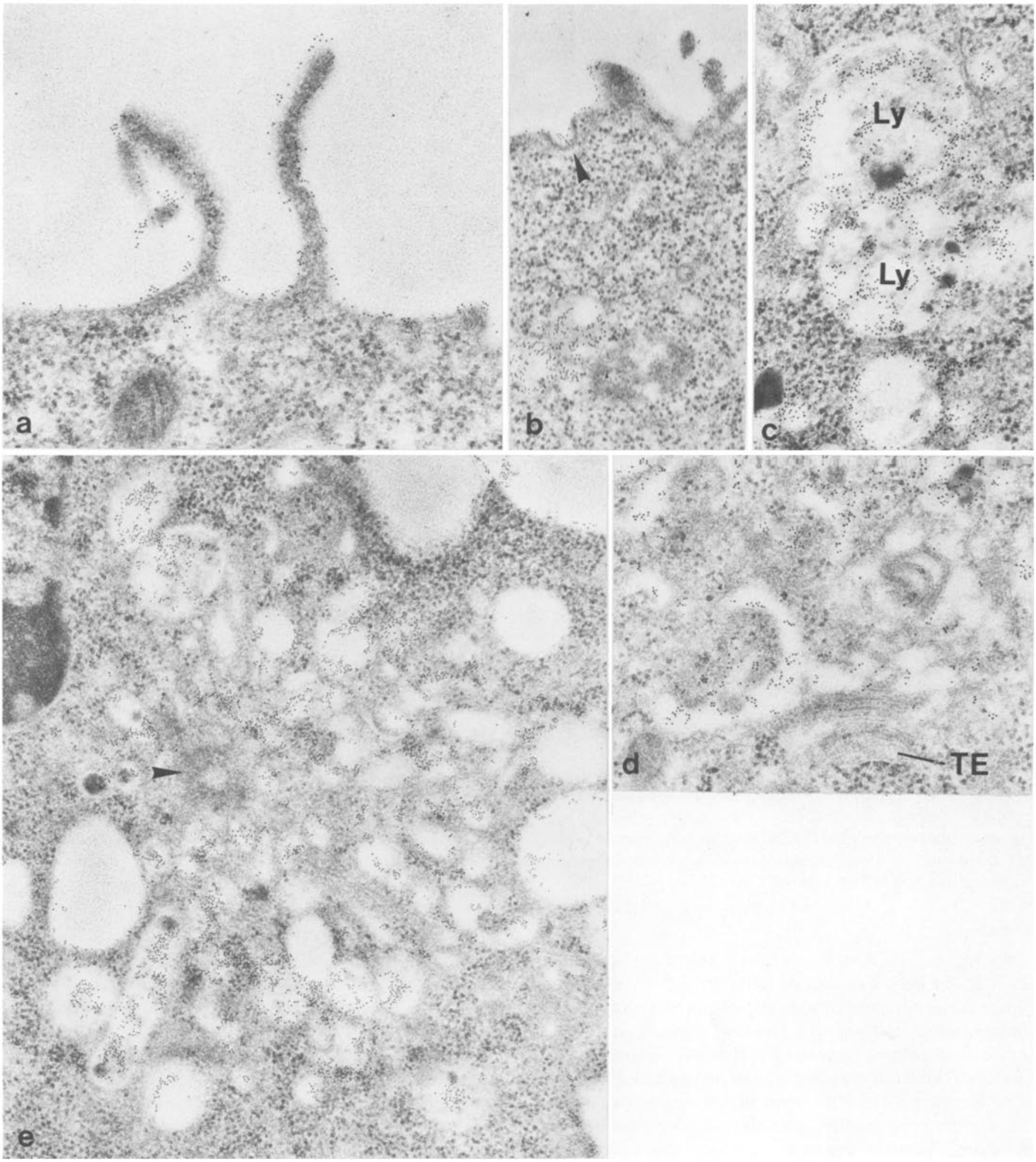


Figure 5. Ultrathin sections of Lowicryl K4M embedded Ehrlich tumour cells incubated with the DSA/ovomucoid-gold technique. Gold particle labelling is present along the entire plasma membrane including coated invaginations (a,b), endosomal (b) and lysosomal elements (c). *Trans* Golgi network and *trans* Golgi cisternae are positive (d) whereas a transitional element (TE in d) of the rough endoplasmic reticulum, *cis* and middle Golgi apparatus is unlabelled. The pericentriolar tubulo-vesicular membrane system exhibits intense gold particle labelling (e). The tangentially sectioned centriole in (e) is marked by an arrowhead. N; part of nucleus (a,c–e × 55 500; b × 43 000).

cells [44] and other cell types [45]. Although a UDP-gal:β gal 1,4 GlcNAc α 1,3 galactosyltransferase has been purified to homogeneity from Ehrlich tumour cells [6], its immunolocalization has not yet been achieved. All currently available evidence indicates that the immunolocalization of a glycosyltransferase in the Golgi apparatus and the distribution of the specific sugar residue transferred by this enzyme to the oligosaccharide side chain mirror each other [46]. Thus, the occurrence of α-D-Gal residues in *trans* Golgi cisternae as visualized with the GS I-B_4-gold complex provides indirect evidence for the localization of the α 1,3 galactosyltransferase in this Golgi apparatus region. It should be emphasized that the β 1,4 and the α 1,3 galactosyltransferases of Ehrlich tumour cells are sequentially acting glycosyltransferases [6] and that the α 1,3 galactosyltransferase shows similarities to the α 2,6 sialyltransferase isolated from different sources [6] in that both have an almost absolute acceptor substrate specificity. The similar distributions of α 1,3 gal and β 1,4 gal residues in the *trans* Golgi apparatus demonstrated in the present study therefore provide *in situ* evidence for a joint localization of the respective galactosyltransferases. Light microscopic immunocytochemical localization of β 1,4 gal transferase and α 2,6 sialyltransferase supports the view of a colocalization in the *trans* Golgi apparatus [47]. However, this point will remain unconfirmed until the localization of these sequentially acting late glycosyltransferases has been achieved by double labelling immunogold electron microscopy.

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