Differential expression of cell surface sialoglycoconjugates on wild-type and cultured Ehrlich tumor cells as revealed by quantitative lectin–gold ultrastructural cytochemistry

(Sambucus nigra/electron microscopy)

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ABSTRACT Three variants of the classical Ehrlich ascites tumor (EAT) cell have been studied by quantitative, sialic acid-specific, lectin–gold ultrastructural cytochemistry. Electron microscopic examination revealed pronounced differences in the surface morphology of the three cell variants. The wild-type Ehrlich cells (EAT-wt), grown in the peritoneal cavity of mice, exhibited a smooth surface profile. A variant form selected for growth as monolayer on basement membrane (EAT-c) showed a complex surface profile with numerous microvilli. The third variant (EAT-c/m), the cultured cells reisolated into mice and passages 20–25 times as ascites, presented a smooth surface profile similar to the EAT-wt cells. Quantitative single as well as double lectin–gold labeling revealed significant differences in the nature of cell surface sialoglycoproteins. The most significant finding was the presence of cell surface Neu5Acα2-6Gal residues as detected with the Sambucus nigra lectin on EAT-c and EAT-c/m cells, whereas EAT-wt cells contained little or none of such carbohydrate sequences. On the contrary, labeling by Maackia amurensis lectin, which recognizes the Neu5Acα2-3Galβ1-4GlcNAc sequence, was intense on all three Ehrlich cell variants; it was 20–60 times greater than α-2,6-linked sialic acid-containing glycoconjugates. Specific cell surface lectin binding combined with morphologic study appears to have identified a small subpopulation of cells within the ascites tumor that are capable of attaching to and growing on a basement membrane.

In previous communications, we reported on the isolation, by selection of growth on bovine corneal endothelial basement membrane, of a second variant of the Ehrlich ascites tumor cell (EAT cell) (1–3). Unlike the classical “wild-type” Ehrlich cells (EAT-wt), which grow only in suspension, generally in the peritoneum of mice, the variant we isolated adheres to culture plastic and grows to confluence as a monolayer. We term this variant EAT-c (for culture). A third variant EAT cell has been generated by inoculation of the EAT-c cells into the peritoneal cavity of mice and passageing them 20–25 times in mice; these we term EAT-c/m (1) (for cultured in mice).

Additionally, we have reported, by means of agglutination and cell binding studies with lectins, that the EAT-c express a greater quantity of α-2,6-linked sialic acid reactive with Sambucus nigra agglutinin I (SNA), which recognizes the Neu5Acα2-6Gal/GalNAc sequence (1-3). Finally, the adherence of the EAT-c cells to the extracellular matrix molecules laminin and fibronectin has been shown to be mediated by β1 integrins (4).

In this communication we report on differences in the morphology of the three EAT cell variants as observed by electron microscopy and present visual evidence of the differences in the expression of sialylated carbohydrate groups on the surface of the three variant Ehrlich cells by using the technique of quantitative lectin–gold electron microscopy (5).

MATERIALS AND METHODS

Reagents. SNA was isolated from the bark of elderberry trees and purified by affinity chromatography on fenitin-Sepharose (6), the Maackia amurensis leukoagglutinin (MAL) was isolated from seeds of this species and purified by affinity chromatography on murine laminin (1), and amaranthin (Amaranthus caudatus agglutinin; ACA) was isolated from A. caudatus seeds and purified by affinity chromatography on Synsorb-T beads (Chemibomed, Alberta, Canada) (7). The lectins were directly labeled with particles of colloidal gold as described (8–10). Digoxigenin (Dig)-conjugated SNA, MAL, and ACA as well as polyclonal sheep anti-Dig (IgG fraction) antibodies were obtained from Boehringer Mannheim. Gold-labeled anti-Dig was prepared as described (11) using 8-nm gold particles. Silver acetate, Carbowax 20 M, and glutaraldehyde (vacuum distilled) were obtained from Fluka; paraformaldehyde, tetrachloroauric acid, hydroquinone, and trisodium citrate were from Merck. All other reagents were of the highest available purity.

Cells and Low-Temperature Lowicryl K4M Embedding. Ehrlich tumor cells grown in mice as ascites (EAT-wt) were washed twice with Earle’s balanced salt solution (EBSS, pH 7.5) at 37°C. An aliquot of cells was maintained in Ca2+/Mg2+-free EBSS containing 0.02% EDTA for 5 min at 37°C to exactly parallel the treatment of cultured cells. Cells were fixed in 30 volumes of 2% paraformaldehyde/0.1% glutaraldehyde in PBS (10 mM phosphate buffer, pH 7.4/0.15 M NaCl) for 15 min at 20°C followed by an additional fixation for 15 min at 4°C. Afterwards cells were washed with EBSS and PBS, incubated with 50 mM NH4Cl in PBS for a total of 30 min, washed with PBS, and stored in PBS containing 0.02% Na2MoO4 at 4°C until Lowicryl K4M embedding (12) or preembedding cell surface lectin labeling.

Abbreviations: ACA, Amaranthus caudatus agglutinin; BSA, bovine serum albumin; Dig, digoxigenin; EAT cells, Ehrlich ascites tumor cells; EAT-c, Ehrlich cells grown in tissue culture; EAT-wt, wild-type Ehrlich cells, which grow in the peritoneal cavity of mice; EAT-c/m, EAT-c Ehrlich cells passaged in mice; MAL, Maackia amurensis leukoagglutinin; SNA, Sambucus nigra agglutinin I.

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Two additional variants of the Ehrlich tumor cell were also studied. The first variant is a culture cell line (EAT-c) isolated from ascitic tumor cells. Briefly, ascitic tumor cells were withdrawn from the peritoneal cavity of mice and washed in EBSS, and $6 \times 10^6$ cells were added to 25-cm² culture flasks covered by an extracellular matrix/basement membrane deposited by bovine corneal endothelial cells (13). The cells were maintained in culture for 3 days after which non-adherent cells were decanted. Less than 100 cells remained attached to the extracellular matrix. The cultures were fed twice a week for 3 weeks during which time $\approx 10$ colonies composed of similar-appearing cells were observed. The colonies were allowed to grow for an additional 2 weeks, after which they were detached from the extracellular matrix by incubating them in 5 mM EDTA in Ca²⁺/Mg²⁺-free EBSS (pH 7.4) for 5 min at 37°C. The isolated cells were cultured an additional three passages on extracellular matrix and thereafter on conventional tissue culture plastic. The EAT-c

Fig. 1. Surface morphology and detection of plasma membrane sialoglycoconjugates by double lectin-gold labeling in the three Ehrlich tumor cell variants. Cell surface labeling for MAL is visualized by 12-nm gold particles and that for SNA is visualized by 8-nm gold particles (indicated by arrowheads in b, d, f, and h). (a and b) EAT-wt. (c and d) EAT-c. EAT-c/m Ehrlich cells exhibiting smooth (e and f) or microvilli-rich (g and h) surface profiles are shown. (a, c, e, g, x4500; b, d, f, h, x45,000.)
cells have been carried in the laboratory for 10 years; they retain the morphological and growth characteristics of the early passage cells. The cells are routinely grown in Dulbecco's modified minimal essential medium with 10% (vol/vol) fetal bovine serum and gentamicin sulfate (50 μg/ml).

For this study, confluent cultures of EAT-c were rinsed in 0.02% EDTA in Ca²⁺/Mg²⁺-free EBSS and subsequently detached from the plastic cell culture substratum by incubating the cells in the same EDTA solution for 5 min at 37°C. The detached cells were washed and fixed as described above.

The second variant of the EAT cells was an ascitic tumor cell grown for 25 passages in mice after intraperitoneal injection of the EAT-c cells. This variant ascitic tumor cell derived from the EAT-c is termed EAT-c/m. The EAT-c/m variant was treated in an identical fashion as that described above for the parent EAT.

For Lowicryl K4M embedding, fixed cells were pelleted and enclosed in 2% agar. Small pieces of agar containing the cells were dehydrated in graded ethanol at progressively lower temperature (down to −40°C) and infiltrated with Lowicryl K4M at −40°C followed by indirect UV light-induced polymerization at −40°C as described in detail (13, 14). Ultrathin sections (60 nm) were cut and mounted on Parlodion/carbon-coated nickel grids (150 mesh).

**Lectin-Gold Labeling Techniques.** For preembedding cell surface labeling, fixed cells were placed into PBS containing 1% (wt/vol) bovine serum albumin (BSA) for 5 min and incubated for 45 min at room temperature with gold-labeled lectins diluted, with PBS containing 1% BSA, to an optical density at 525 nm of 0.3 (SNA), 0.5 (MAL), or 0.1 (ACA). In addition, double labeling using SNA complexed to 8-nm gold particles and MAL complexed to 12-nm gold particles was performed. For this, equal volumes of SNA-gold (diluted to an optical density of 0.6 at 525 nm) and MAL-gold (diluted to an optical density of 1.0 at 525 nm) were mixed, and cells were incubated for 45 min at room temperature. Afterwards, cells were washed twice with PBS containing 1% BSA for 5 min each, fixed with 1% glutaraldehyde/2% OsO₄ in PBS for 1 h each, enclosed in 2% agar, and embedded in Epon following standard protocols.

For postembedding labeling of Lowicryl K4M-embedded cells, thin sections were floated for 5 min on a droplet of PBS containing 1% BSA and 0.05% Tween 20 and then incubated for 45 min at room temperature with one of the various lectin-Dig conjugates diluted with PBS containing 1% BSA and 0.05% Tween 20 to the following concentrations: SNA, 5 μg/ml; MAL, 25 μg/ml; ACA, 10 μg/ml. Subsequently, the thin sections were rinsed twice with PBS for 5 min each and incubated with anti-Dig-gold complexes for 45 min at room temperature. The gold-labeled anti-Dig IgG was diluted to an optical density at 525 nm of 0.05 with PBS containing 1% BSA, 0.05% Triton X-100, and 0.05% Tween 20.

**Cytochemical Controls.** Dig-conjugated or directly gold-labeled SNA and MAL were preincubated with 1 mM 6'-sialyllactose and 50 mM 3'-stallylactose, respectively. Dig-conjugated or directly gold-labeled ACA was preincubated with asialoglycoporphin (10 μg/ml), asialofetuin (50 μg/ml), or 10 mM T-antigen neoglycoprotein. Further, sections were incubated with only gold-labeled anti-Dig antibody.

**Quantification of Lectin-Gold Labeling.** The quantification was performed on sections of cells labeled with one of the lectin-gold complexes before Epon embedding. Micrographs were taken at the primary magnification of ×20,000. The length of smooth and microvillar plasma membrane regions was measured with a cursor connected to a personal computer using IMAGE-PRO PLUS (Media Cybernetics, Silver Spring, MD) or manually with a curvimeter. Labeling inten-

![Fig. 2.](image1) (a) EAT-wt, which, in contrast to the majority of the cells, exhibit groups of microvilli. (b) The cell surface labeling for MAL is shown at higher magnification. (a, ×3600; b, ×49,500.)

![Fig. 3.](image2) Detection of cell surface binding sites for ACA-gold complexes. (a) EAT-wt Ehrlich cells. EAT-c/m exhibiting smooth (b) or microvilli-rich (c) surface profiles are shown. (×36,000.)
Table 1. Quantification of cell surface lectin-gold labeling in Ehrlich tumor cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Lectin</th>
<th>Smooth plasma membrane</th>
<th>Microvillar plasma membrane</th>
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<tr>
<td></td>
<td></td>
<td>WT vs. C or C/M</td>
<td>PT vs. C/M</td>
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<tr>
<td></td>
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<td>Lectin-gold</td>
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<tr>
<td></td>
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<td>labeling*</td>
<td>PT</td>
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<tr>
<td>WT</td>
<td>SNA</td>
<td>0.03 ± 0.09</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C</td>
<td>SNA</td>
<td>0.32 ± 0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C/M</td>
<td>SNA</td>
<td>0.14 ± 0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>WT</td>
<td>MAL</td>
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<td>MAL</td>
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<td>ACA</td>
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<td>NS</td>
</tr>
<tr>
<td>C/M</td>
<td>ACA</td>
<td>3.55 ± 0.14</td>
<td>NS</td>
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</table>

WT, EAT-wt; C, EAT-c; C/M, EAT-c/m; NS, not significantly different. P values were determined by a paired *t* test.

*Lectin-gold labeling expressed as gold particles per micrometer length of plasma membrane regions (mean value ± SE).

Surface morphology was expressed as the number of gold particles per micrometer length of the plasma membrane region. Statistical evaluations were performed using STATVIEW II (Abacus Concepts, Berkeley, CA). The data are based on the evaluation of the entire plasma membrane circumference of 30 equatorially sectioned cells from each group of Ehrlich tumor cells: EAT-wt, EAT-c, and EAT-c/m.

**RESULTS**

Surface Morphology of the Ehrlich Tumor Cells. Ehrlich tumor cells grown in mice as ascites, EAT-wt, exhibited a smooth surface with occasional single microvilli (Fig. 1a and b). In this group, a few cells (<6%) showed a different morphology with many microvilli and a well-developed subplasmalemma microfilament network (Fig. 2). In contrast to the majority of cells grown as ascites, Ehrlich tumor cells grown as monolayer cultures, EAT-c, to confluency had a surface that was characterized by the presence of numerous microvilli and only small regions of smooth plasma membrane (Fig. 1c and d). In addition, they exhibited a well-developed subplasmalemma microfilament network not seen in the great majority of cells grown as ascites. Ehrlich tumor cells derived from a single inoculum of cultured cells (EAT-c/m).

![Fig. 4. Postembedding localization of intracellular lectin binding sites. (a) EAT-wt exhibit MAL labeling (8-nm gold particles) over trans-Golgi apparatus cisternae (G) and lysosomes (L). (b) In EAT-c, gold particles (15 nm) indicating ACA binding sites are observed over the trans-Golgi, lysosomes (L), and endosomal structures (arrowheads). (a, ×52,000; b, ×36,900.)](image_url)
and maintained for 25 passages as ascites in mice (EAT-c/m) showed a surface morphology that greatly resembled that of the original ascites tumor cells (Fig. 1 e and f). However, in addition to cells showing only a few isolated microvilli, an equal number of cells were seen that exhibited microvilli arranged in small clusters (Fig. 1 g and h).

**Cell Surface Lectin Labeling.** A pronounced difference in the labeling intensity of the cell surface was observed irrespective of the group of Ehrlich tumor cells investigated. Sialic acid residues in α-2,6 linkage as detected with gold-labeled SNA were present in low quantities, whereas α-2,3-linked sialic acid residues as detected with gold-labeled MAL and ACA were abundant (Figs. 1 and 3 and Table 1). However, the few cells rich in microvilli observed in EAT-wt were densely labeled with SNA, which was similar to the one obtained with MAL. However, the number of these cells was too low to permit quantification of the lectin labeling but could represent the extremely low population of cells (EAT-c) that were selected by their ability to adhere to basement membrane components.

A significant increase in SNA labeling was observed in EAT-c as compared to EAT-wt (Fig. 1 b and d and Table 1). The intensity of the SNA cell surface labeling remained significantly higher in EAT-c/m (Fig. 1 f and h). A comparison of the labeling intensity by SNA between the smooth and microvillar plasma membrane regions in each group of Ehrlich tumor cells showed no difference in labeling density between these two membrane profiles (Table 1).

The smooth plasma membrane regions of EAT-wt and of EAT-c/m showed significantly higher labeling with MAL than did EAT-c (Table 1). When the microvillar plasma membrane regions were considered, no significant difference in labeling intensities was observed among the three groups of Ehrlich tumor cells. The labeling intensity of the plasma membrane by ACA (Table 1) was intermediate between the intensities found for SNA and MAL. No significant difference existed between EAT-wt and EAT-c/m.

**Lectin Labeling of Intracellular Structures.** Labeling of intracellular structures by SNA was sparse. In EAT-c, gold particles were detected over the trans-cisternae and the trans-Golgi network, vacuoles, and lysosome-like elements in addition to the plasma membrane. A similar labeling pattern but with higher labeling intensity was observed with MAL and ACA in all three groups of Ehrlich tumor cells, with the gold particle labeling confined to the trans-Golgi apparatus, vacuoles, and lysosome-like elements (Fig. 4). None of the three lectins labeled the endoplasmic reticulum, nucleus, mitochondria, or cis/medial-Golgi apparatus of the three groups of Ehrlich tumor cells.

**DISCUSSION**

This study provides graphic and quantitative confirmation and extension of the biochemical studies we have conducted on three variants of EAT cells (1-3). It also extends our studies to include information on the morphology of these EAT cells and of the possible biochemical events involved in the glycosylation of these cells as visualized by the binding of lectin–gold complexes to various subcellular organelles. It is not surprising that the SNA and MAL labeling was confined to the trans-Golgi apparatus inasmuch as, like other cells that synthesize sialoglycoconjugates, this is the principal site of sialyltransferase localization (15).

The difference in the electron microscopic appearance of the EAT-wt and EAT-c cells was also diagnostic of the two populations of cells; the EAT-wt cells exhibited a rather smooth surface with occasional single microvilli, whereas the EAT-c cells grown as monolayer cultures displayed a surface profile characterized by the presence of numerous microvilli and only small regions of smooth plasma membranes.

One of the most significant findings of this study was that EAT cells selected for growth on basement membrane components (EAT-c cells) were labeled with SNA (which recognizes Neu5Acα2-6Gal/GalNAc residues) to a much greater extent than EAT-wt cells. EAT-c/m cells were also labeled by SNA, indicating that these cells, even after 20–25 passages in mice, retain their ability to express α-2,6-linked sialic acid on their surface. A particularly interesting finding was the presence of a few cells among the ascitic tumor cells that displayed the properties of the EAT-c cells; we believe this may represent a subpopulation of cells in the ascitic tumor that are capable of attaching to a basement membrane and growing in culture.

Using the MAL–colloidal gold complex, it was established that there was a greater expression of α-2,3-linked sialic acid glycoconjugates than the α-2,6-linked isomer. Similarly, differential expression of sialoglycoconjugates has been observed in CHO cells after transfection with an α-2,6-sialyltransferase cDNA and shown to be due to differences in the expression of the respective specific sialyltransferases (16). It remains to be established whether the observed differences in Ehrlich tumor cells are regulated at the transcriptional level or if other factors play a role in the control of sialylation. Our data, however, provide evidence that differences in the conditions of growth may be involved in the expression of cell surface sialoglycoconjugates.

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