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Changes in S-type lectin localization in neuroblastoma cells (N1E115) upon differentiation

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The distribution of a 14.4 kDa S-type lectin was examined in murine neuroblastoma cells, either undifferentiated or after differentiation induced by dibutyryl-cyclic adenosine monophosphate. In undifferentiated cells the immunoreactivity was detected extracellularly, associated with the plasma membrane and in bulges released into the extracellular milieu. Important modifications of the lectin localization were associated with the differentiation process that induced an increased cytosolic expression and a decreased externalization. Possible functions for the lectin expressed intracellularly in the differentiated cells are also considered.

Keywords: endogenous lectin, neuroblastoma cells, differentiation, glycoconjugates, immunocytochemistry.

Introduction

Endogenous carbohydrate-binding proteins (lectins) have been characterized in various vertebrate tissues and cells (for reviews see: [1–4]). They are found both intra and extracellularly and are comprised of several members [3–7], including the 14.4 kDa lectin (S-type lectin), a highly conserved polypeptide.

Fourteen kDa lectin is abundantly expressed in a variety of vertebrate organs [1, 8] including rat brain [9–11] and human brain [12, 13]. Its distribution in the central nervous system (CNS) is developmentally regulated [10, 14–16]. In rat brain, this lectin is predominantly expressed inside the neuron cytosol and in neuritic processes [15, 16]. It is also transiently secreted and found on the surface of neuronal cells [15, 16]. The neuroblastoma cell line N1E115, which can be differentiated by an appropriate treatment with $N^6O^{2'}$-dibutyryl-cyclic adenosine monophosphate (Bt_2cAMP) [17], was chosen to study further the impact of differentiation processes on the localization of S-type lectin in nerve cells.

Materials and methods

Lectins and antibody. Crude extract of adult mouse brain was prepared as previously described [18]. Mouse brain lectin was purified by affinity chromatography on Lactogel (E. Y. Laboratories, San Mateo, CA, USA) as previously described [12]. Molecular weight and isoelectric point (pI) of the purified mouse lectin were determined on PhastSystem (Pharmacia-Biotech, Saint-Quentin-en-Yvelines, France) as previously described [19]. The carbohydrate specificity of the purified lectin was determined as described [12]. Rabbit antibody directed against human brain lectin (HBL 14) was used for immunocytochemical studies. Antibody specificity for immunogen, HBL 14, has been previously reported [15], and its reactivity for mouse brain lectin (MBL14) in crude mouse brain extract and after purification was confirmed by Western blotting.

The mouse N1E115 clone, derived from the C1300 mouse neuroblastoma, was cultivated on plastic substrate in T 81-flask (Corning) with DMEM (Gibco 741600) buffered with sodium bicarbonate (24 mM), and supplemented by 1% glucose (v/v) and 10% (v/v) heat inactivated fetal calf serum (FCS). Cells from confluent cultures were transferred at low density to 24 well plates (Costar) containing sterile glass coverslips without artificial substratum, and incubated for 24 h. To induce differentiation, cells were cultured for 48 h in a fresh medium containing 3% FCS (v/v) and 10^{-3} M Bt_2cAMP [17]. Controls were performed without Bt_2cAMP and referred to as undifferentiated cells.

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Immunocytochemistry. Cells were cultured as described above. After 48 h of differentiation, coverslips were washed with phosphate saline buffer (PBS, 7.5 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, 150 mM NaCl, pH 7.2), and non specific binding-sites were blocked by incubation in 3% BSA (w/v) in PBS, for 15 min at room temperature.

To immunostain only extracellular antigens, each coverslip was incubated with 500 µl of a 1:100 (v/v) of primary antibody (anti-HBL 14) diluted in culture medium supplemented with 3% bovine serum albumin (BSA) instead of FCS, for 2 h at 37 °C. Coverslips were washed thoroughly in PBS, and fixed for 15 min at 20°C with Carson fixative (40% formaldehyde; 2.56% NaOH; 2.26% Na_2HPO_4, $12H_2O$, 11 ml, 17 ml and 83 ml respectively). This fixative was chosen because it permeabilized cell membranes, conserved antibody access to 14 kDa antigen, and gave adequate preservation of cellular morphology. After fixation, the coverslips were rinsed twice with PBS. Endogenous peroxidases were eliminated by treatment with 5 ml per coverslip of a mixture of PBS:methanol (1:1) containing 1% H_2O_2 (v/v) for 15 min at 20°C, in the dark. After careful washing with PBS, coverslips were saturated in T-PBS (PBS, 3% BSA (w/v) and 0.3% (v/v) Tween 20) for 15 min, and incubated with 500 µl per coverslip of 1:200 (v/v) swine anti-rabbit IgG coupled to peroxidase (Dakopatts, Denmark) diluted in T-PBS, for 1 h at 20 °C. After several washings the peroxidase reaction was developed in PBS containing 0.5 mg/ml diamino-benzidine (DAB) (BDH Chemicals, Poole, England) and 0.02% (v/v) hydrogen peroxide [20].

For immunohistochemical staining of intracellular antigens, coverslips were fixed prior to incubation with the primary antibody diluted in T-PBS for 1 h at 20 °C. Other steps were performed as described above. Controls were performed by first replacing anti-HBL 14 antiserum by preimmune serum or PBS and second by substitution of the anti-rabbit immunoglobulin by PBS.

The immunolabelling index representing the percentage of cells labelled with anti-HBL 14 antibody was determined by quantification using a count grid. The number of labelled and unlabelled cells was analysed field by field. For each different preparation a minimum of 500 cells were counted.

Microscopic analysis and microphotography. The immunocytochemical staining was analysed with a Zeiss microscope equipped with a Zeiss Axiophot photographic system. Micrographs were realized using AGFA PAN 25 film.

Results

Purification and characterization of mouse brain lectin

Twelve ml of crude mouse brain extract were applied at 20 °C to a column containing 5 ml of Lactogel. After adsorption and washing, bound lectin was eluted at 4 °C with 50 mM Tris-HCl pH 7.6 containing 100 mM lactose, 4 mM β-mercaptoethanol, 0.25 mM phenyl-methyl-sulfonyl-chloride. The yield of purified soluble lectin was 260 µg, i.e. approximately 0.16% of the total soluble proteins of the crude extract. SDS-PAGE in denaturing conditions showed a single polypeptide with an M_r of 14400 (Fig. 1c), resolved by isoelectric focussing in polyacrylamide Phastgel IEF 4-6.5 into five acidic forms of pH 4.7, 4.8, 4.9, 5.0 and 5.2 respectively. The specificity was determined from the inhibitory effect of saccharides on rabbit erthyrocytes agglutination by MBL14 used at 15 µg ml^{-1}. The most potent inhibitors were thiodigalactoside, Galβ1-4GlcNAc, and lactose. The sugar concentrations for 50% inhibition were 0.05 mM, 0.08 mM and 0.2 mM respectively.

Anti-lectin antibody specificity

As shown on Fig. 1c, immunoblotting using rabbit antiserum to HBL 14 gave a reaction with purified MBL 14. Only one band of 14.4 kDa was detected in crude mouse brain extract. This antibody was then used for the immunolocalization of lectin immunoreactivity in mouse neuroblastoma cells. The binding was monitored by swine anti-rabbit IgG conjugated to peroxidase. Control experiments performed as described in material and methods were completely negative (data not shown).

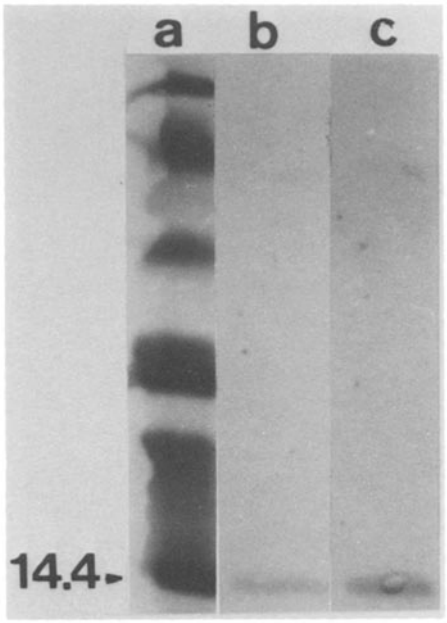

Figure 1. Immunoblot detection of mouse antigens using anti-HBL 14 antibody. Lane a: biotinylated calibration proteins used as molecular mass markers, from top to bottom: phosphorylase B (94000); bovine serum albumin (67000); ovalbumin (43000); carbonic anhydrase (30000) soybean trypsin inhibitor (20100); alpha lactalbumin (14400). Lane b: mouse brain crude extract. Lane c: purified mouse brain lectin.

Immunocytochemical localization of mouse lectin in undifferentiated N1E115 cultured cells

The culture coverslips were examined by phase contrast optic and light microscopy. Cells cultured in the presence of 10% FCS on glass coverslips appeared more or less rounded and formed clusters. Under these conditions, generally no neuritic extentions were seen.

Extracellular detection. When cells were incubated with anti-lectin antibody before fixation, immunoreactive material was revealed as a faint extracellular labelling visible at contact sites between cells in clusters. In 15% of the cultured cells the antigen was detected on the plasma membrane external face. Bulges containing lectin were released at the same sites (Fig. 2a, b).

Intracellular detection. Intracellular and extracellular antigen was detected after cell permeabilization. Immunoenzymatic visualization of lectin revealed a cytosolic localization into patches close to the plasma membrane

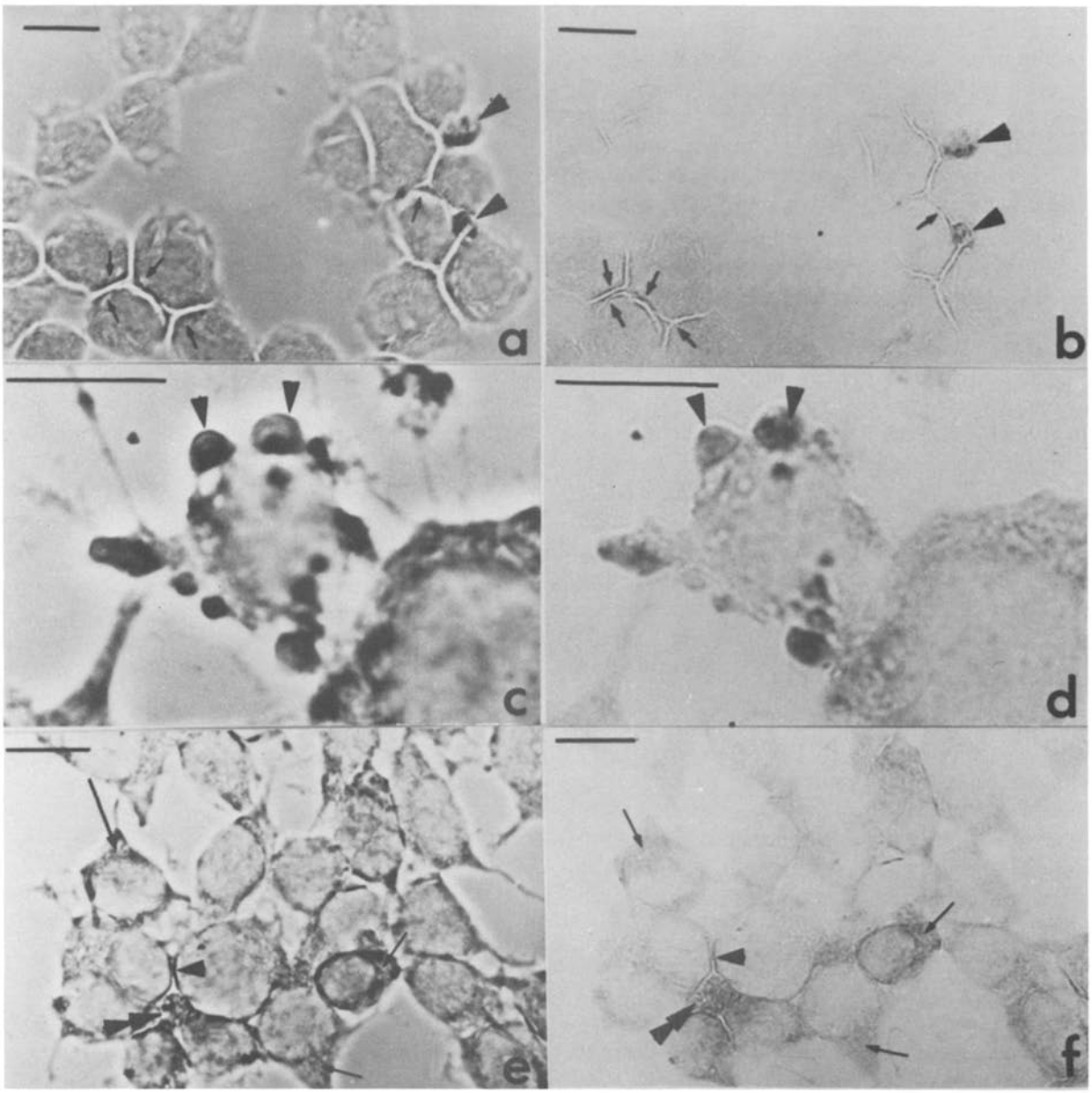

Figure 2. Immunocytochemical localization of mouse lectin in undifferentiated neuroblastoma cells. Figures a, c, e, are phase-contrast micrographs of the fields b, d and f respectively. Extracellular detection is shown in a and b. Note the presence of extracellular antigen in externalized vesicles (a, b, arrow heads) and associated with plasma membrane at contact sites between cells (a, b, arrows). Intracytosolic immunoreactive material is highly concentrated into patches close to the plasma membrane presumably immediately before its externalization in vesicles (c, d arrow heads). Intracellular immunoreactive material is also detected diffusely in the cytoplasm of the cells in clusters (e, f, arrows) and expressed extracellularly by the same cells either on the membrane (e, f, arrow heads) or in deposits between cells (e, f, double arrow heads). Bars = 10 μm.

suggesting that cells could externalize the antigen (Fig. 2c, d). The extracellular localization was very similar to that described above. Generally the same cells expressed the antigen both extra and intracellularly (Fig. 2e, f). We found that only 2% of cells restricted lectin expression to the intracellular compartment.

Immunocytochemical localization of mouse lectin in Bt_2cAMP morphologically differentiated N1E115 cultured cells

In cultures treated with Bt_2cAMP the cells appeared flattened and extended neurite-like process. Some rounded cells remained in little clusters.

Extracellular detection. No extracellular lectin was detectable in morphologically differentiated untreated cultures, and vesicles deposted in the centre of cell clumps were observed (Fig. 3b).

Intracellular detection. The pattern of intracellular labelling by anti-lectin antibody was strikingly different in differentiated cells from that seen in undifferentiated ones. As shown in Fig. 3c, the antibody reacted strongly with almost all cells extending processes. Sixty-five per cent of cultured cells were labelled. Immunoreactive material was diffusely distributed throughout the somata and was not enclosed in

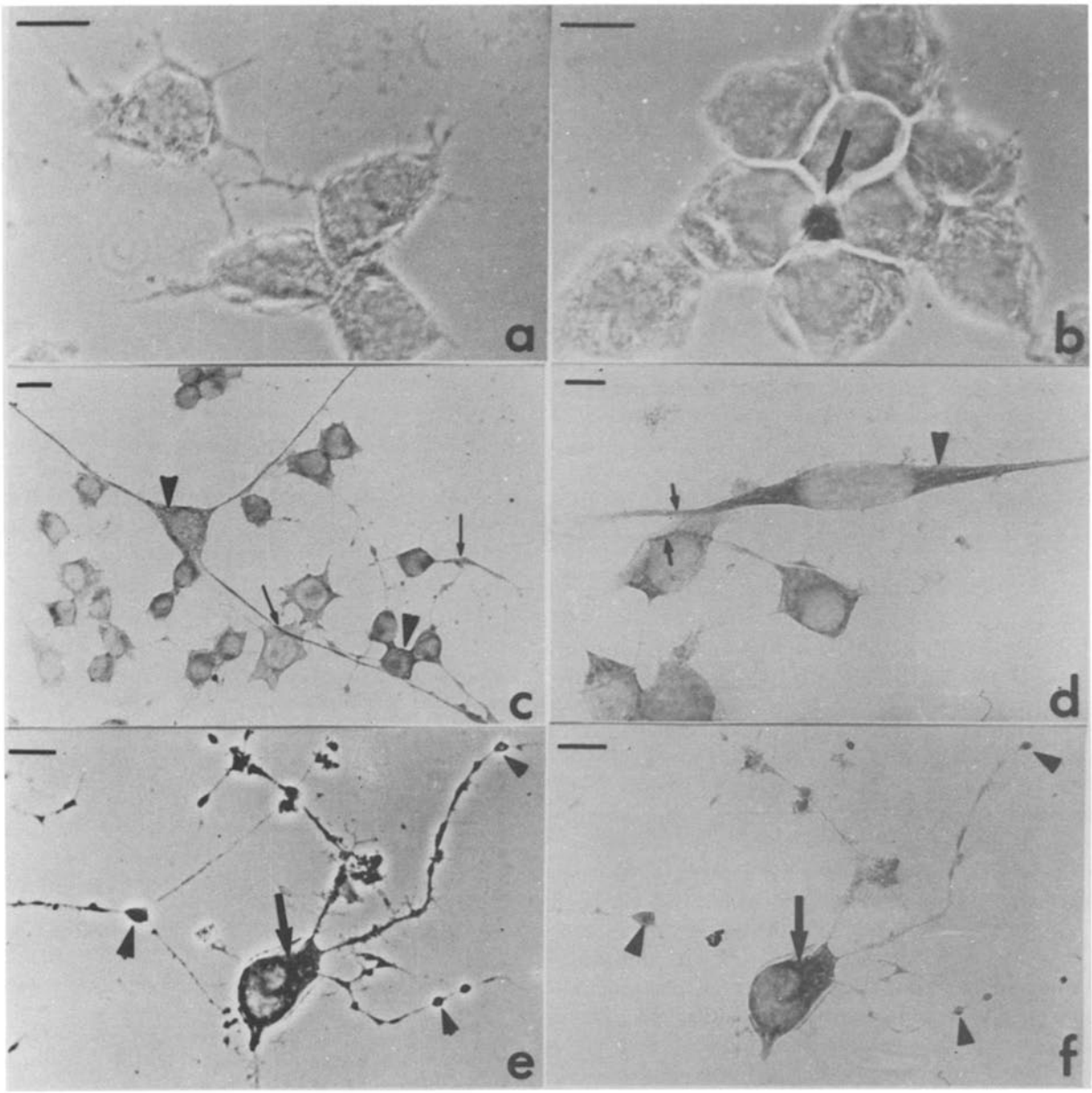

Figure 3. Immunocytochemical localization in differentiated neuroblastoma cells. Antigen is not externalized from N1E115 neuroblastoma cells (a) while secreted vesicles are detected close to the morphologically undifferentiated cells remaining in clusters (b, arrow). Morphologically differentiated cells strongly expressed lectin both in the neuritic extensions (c, d arrows) and in cell somata (c, d, e arrow heads). Figure e shows phase-contrast micrograph of the field f. Lectin is found in somata (e, f arrow) and in distal tips of neurites (e, f arrow heads). Bars = 10 μm.

intracellular vesicles (Fig. 3d). The stained profiles extended up to the neurites elaborated by the differentiated cells but they were less conspicuous in the region of the growth cone (Fig. 3e, f). Distal tips and varicose enlargments of the processes contained antigen. Cells that remained morphologically undifferentiated showed the same expression as that described above for Bt_2cAMP-non-treated culture, except that extracellular vesicles disappeared.

Discussion

Neuroblastoma cells respond to Bt_2cAMP differentiation by stopping their growth which is accompanied by a change in the cytoskeleton protein distribution (such as an assembly of microtubules) leading to an alteration of its adhesion properties with neurite outgrowth [21, 22], and a modified expression of various membrane associated molecules [23, 24].

In this study we showed that Bt_2cAMP-induced differentiation of N1E115 cell line leads to a prominent intracellular expression of a S-type lectin, as determined by immunocytochemistry. The profile of lectin expression in murine neuroblastoma cells was studied using an anti-human brain lectin antibody. To ascertain cross-reactivity with mouse determinants, and to check its specificity, the antibody was assayed by immunoblotting against soluble proteins of crude mouse brain extract. It was found to react with only one band corresponding to 14.4 kDa MBL purified by affinity chromatography. Furthermore this lectin was the only S-type lectin detected in neuroblastoma cells (S20) either undifferentiated or induced to differentiate [25].

The results presented here show that N1E115 S-type lectin could be externalized from undifferentiated cells. How they are externalized by the cells is still unknown. An alternative pathway for secretion has been proposed for the 14 kDa lectin in mouse myoblasts, in which the lectin becomes concentrated in evaginations of plasma membrane, which pinch off to form extracellular vesicles [27, 28]. These studies show interesting similarities to and differences from our present findings. In myoblasts and neuroblastoma cells, respectively, the process of externalization seems similar in that both lectins became concentrated in patches within the cytoplasm before being seen in vesicles budding off the cell surface. In contrast to these similarities the regulation of the phenomenon was rather different. It appeared primarily in undifferentiated neuroblastoma cells while it was prominent in differentiated myotubes [27, 28]. Thus, induction of neuroblastoma differentiation dramatically decreased the secretion of the lectin which was restricted to the intracellular compartment. Such a localization was previously found in differentiated neurons of the CNS [15, 16]. In our experimental conditions, the differentiated N1E115 cells behave as mature neurons with respect to lectin expression.

The demonstration that the localization of a 14 kDa lectin in neuroblastoma cells varies with morphological differentiation suggests that lectin functions differ according to whether it is predominantly reserved in the cytosol or externalized. S-type lectins have been, until now, repeatedly proposed to function by interacting with extracellular matrix, i.e. laminin [27–32], restricting the cytoplasmic molecules to no more than storage forms. Considering the restricted localization of the N1E115 lectin in differentiated cells, it seems to fulfil important functions in the intracellular milieu as well. We have previously hypothesized that 14 kDa lectin participates in intracellular traffic of glycosylated molecules [15, 16]. It is possible that cytosolic lectin may also play a role in the construction of the cytoskeleton by binding to actin molecules [33]. Such a putative function presumably does not involve the carbohydrate-binding domain of the lectin.

Finally, the results presented here, in line with previous reports, suggest that lectin expression as well as secretion may be a part of the characteristic differentiation programme of each cell type.

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