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The developmental regulation of the L2/HNK-1 and L3 carbohydrate epitopes in mouse brain

Evidence for separate control of lipid- and protein-bound epitopes

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The carbohydrate epitopes L2/HNK-1 and L3 have previously been identified on various neural cell adhesion molecules and have been suggested to play a role in the mediation of cell-cell adhesion. In this study, the developmental expression of the two epitopes in soluble, membrane-bound and chloroform/methanol-extracted fractions of the constituent mouse brain regions was examined by enzyme-linked immunosorbent assay (ELISA). The protein-bound epitopes were shown to be uniformly developmentally regulated, with levels peaking at postnatal day 20 (P20). The epitopes in a crude chloroform/methanol fraction, however, demonstrated a different pattern, with L2 peaking earlier at postnatal day zero (P0). These results suggest a possible interaction between the control of the two pools of the epitope.

L2/HNK-1; L3; Carbohydrate epitope; Development; Cell adhesion; (Mouse brain)

1. INTRODUCTION

Nervous tissue contains a large number of lipid- and protein-bound carbohydrate groups which play a major role in both its structuring and functioning. One such carbohydrate group is the sulfated *N*-linked oligosaccharide L2/HNK-1 epitope [1] which is present on both the neural cell adhesion molecule (N-CAM) and other CNS polypeptides including L1, myelin-associated glycoprotein (MAG), P_0, integrin and the adhesion molecule on glia (AMOG) [2–7] as well as being on glycolipids [8]. The epitope itself, a sulfated glucuronic acid derivative [9,10], has been proposed to mediate cell-cell interaction [11–13]. A second carbohydrate epitope, L3, has been identified and is also present on various CAMs including N-CAM, AMOG, integrin and P_0 [2,7,14]. The expression of L2 has been demonstrated to regulate developmentally with respect to both its total and specific polypeptide expression [15–18]. Hence, the developmental expression of protein- and lipid-bound L2 and L3 epitopes was examined in developing mouse brain.

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2. MATERIALS AND METHODS

2.1. *Brain fractionation*

Mice (NMRI strain) of differing developmental ages were killed by decapitation. The brains were dissected and homogenised in ice-cold 20 mM Tris-HCl (pH 7.2) containing 150 mM NaCl, 1 mM EDTA and 1 mM EGTA. All solutions used contained protease inhibitors. The homogenate was centrifuged at 100000 × *g* for 1 h at 4°C resulting in a membrane pellet and a supernatant fraction. A crude lipid extract of the pellet was then prepared. The pellet was resuspended in 1 ml chloroform/methanol (2:1), vortex-mixed, and the protein pelleted in a microfuge. Following extraction, the solvent was evaporated and the lipid content resuspended in 1 ml water. The post-extraction protein-containing pellet was also resuspended in 1 ml water by sonication, and all fractions stored at −70°C until use. The protein content of the soluble and pellet fractions was determined [19] using bovine serum albumin (BSA) as a protein standard.

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2.2. *Carbohydrate epitope determination*

The concentrations of L2 and L3 in the three brain fractions was determined by ELISA. The brain fractions (up to 1 μg protein in 0.1 M $NaHCO_3$) were attached to a solid-phase 96-well polystyrene microtiter plate. The wells were blocked for 1 h with 1% BSA in 0.1 M $NaHCO_3$ and then washed three times in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) containing 0.5% Tween 20 (wash buffer). These were incubated with 100 μl of the respective monoclonal antibodies [1:50 dilution with CMF-PBS containing 0.5% Tween 20 and 1% BSA (dilution buffer)] against L2 and L3 [4,14] for 2 h at room temperature. The plates were then rinsed three times with wash buffer and the wells incubated with 100 μl of a peroxidase-conjugated goat anti-rat antibody (1:50 with diluting buffer) for 2 h and subsequently washed three times as before. The amount of bound immunoglobulin was visualised by the addition of 100 μl acetate buffer, pH 4.2, containing chromogenic substrate, 2,2'-azinodi-3-ethylbenzthiazolinesulfonate [6] (ABTS), and hydrogen peroxide (0.28 μl/ml). The reaction was stopped after 5 min by the addition of 50 μl of 0.6% SDS. The absorbance was then measured at 405 nm. Samples which had been incubated with the goat anti-rat im-

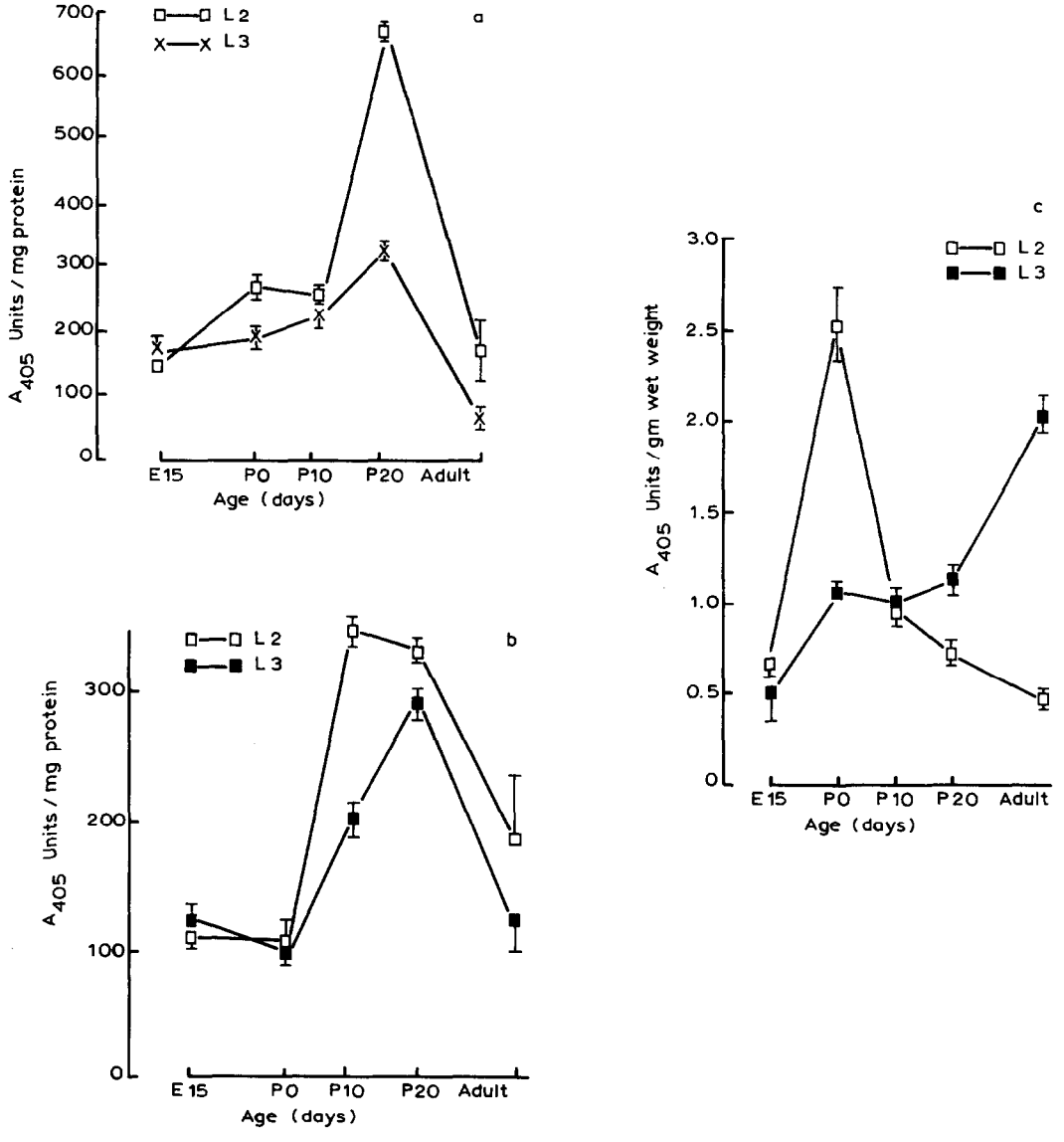

Fig.1. Developmental regulation of the L2 and L3 epitopes in (a) membrane, (b) soluble and (c) chloroform/methanol fractions obtained from developing mouse brain. Points represent mean ± SE ($n = 3$) and are expressed as absorbance units (A_{405}) per mg protein (soluble and membrane-bound fractions) and absorbance units (A_{405}) per g wet wt (chloroform/methanol extract).

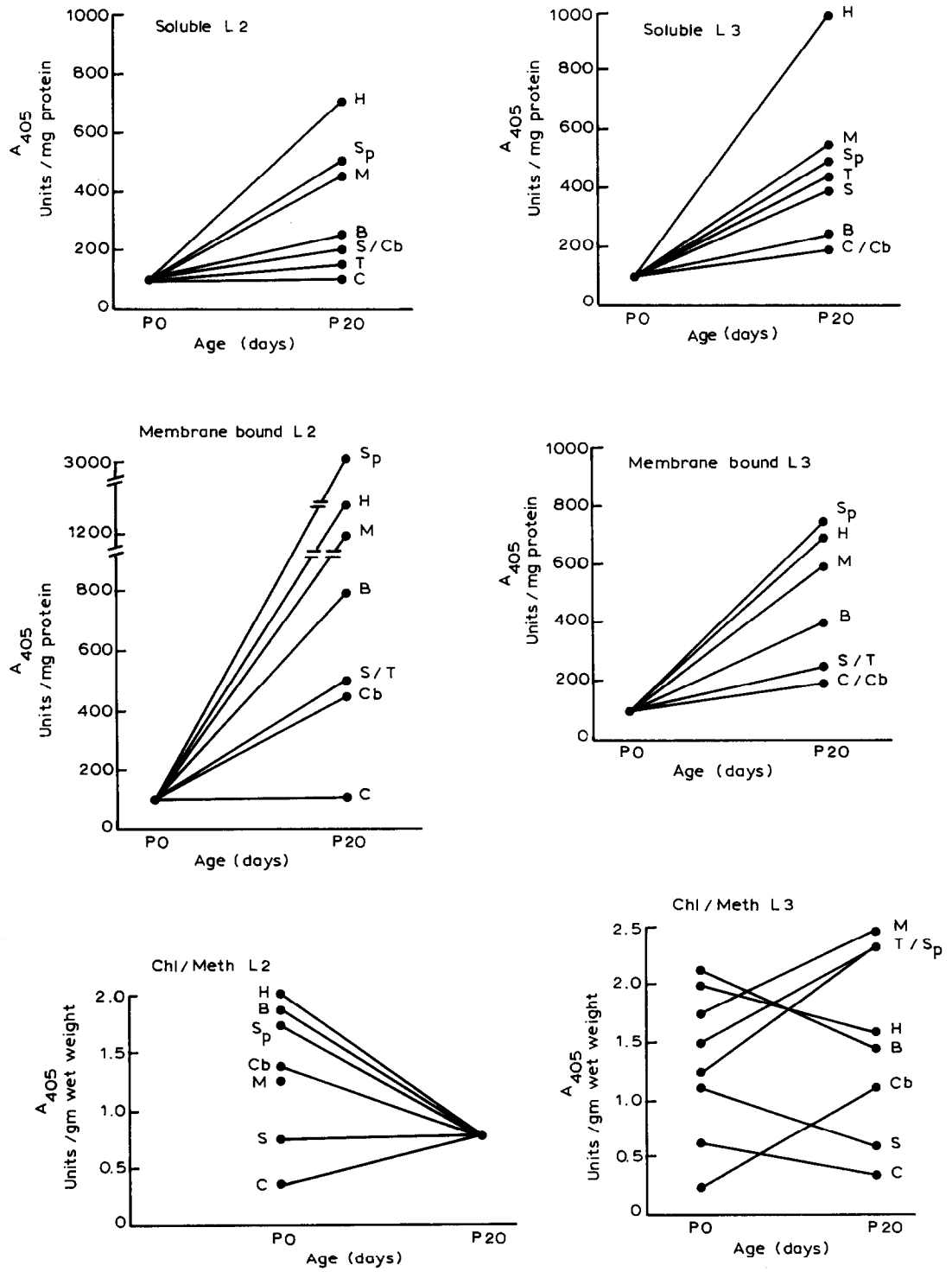

Fig.2. Regional developmental expression of L2 and L3 in constituent brain regions. Points represent mean (n = 3) and are expressed as in fig.1. Brain regions indicated: C, cortex; Cb, cerebellum; T, thalamus; M, midbrain; Sp, spinal cord; S, striatum; H, hypothalamus; B, brain stem.

munoglobulin antibody only served as a measure of non-specific antibody binding. The assay was characterised with respect to the linearity of both antigen detection and lipid/protein binding to the wells. All detections were carried out within the linear range of both variables. The antigen content was expressed as absorbance units (A_{405}) per mg protein for the soluble and pellet fractions and A_{405} per g brain wet wt for the chloroform/methanol fraction. Although expressed in reference to total brain wet wt, there was a direct correlation between brain protein and wet wt in the samples used allowing for a comparison of the epitope levels in the three fractions.

3. RESULTS AND DISCUSSION

In both membrane-bound and soluble protein fractions, there was a developmental increase in expression of both L2 and L3 epitopes coincident with age up to a peak of expression at postnatal day 20. This was followed by a subsequent decrease to adult expression levels (fig.1a,b). These results are in good agreement with those published previously for protein-bound L2 [18]. Further investigation into the developmental regulation of protein-bound sugars in the different brain regions indicated that this followed the same general pattern and was not region-specific (fig.2). A peak enrichment of the two epitopes was seen in the hypothalamic, brainstem, spinal cord and midbrain regions at postnatal day 20.

A different developmental pattern was seen, however, for the epitopes in the chloroform/methanol fraction (fig.1c). While this crude fraction contains mainly lipid-bound sugars, one cannot rule out the possibility of some small proteoglycans also being present. The developmental pattern of L2 in this fraction is, however, in good agreement with previous studies on lipid-bound L2 in rat brain [18], indicating that the epitope detected in this fraction is probably lipid-bound. Both in total brain and in the constituent regions, L2 expression peaked at postnatal day zero, followed by a decrease to adult levels. This is in contrast with protein-bound L2 which peaked later at postnatal day 20, thereafter decreasing to adult. No clear overall pattern was seen with L3 levels. In total brain, there was only a slight increase in expression of L3 from postnatal days 0 to 20. At a regional level, however, there was a considerable variation in developmental expression with some regions registering an increase, while others registered a decrease in expression. It is likely, therefore, that these regional variations counteract each other to result in the slight increase seen for total brain. Both epitopes in this fraction displayed a regional enrichment similar to that seen in the membrane-bound and soluble protein fractions (fig.2).

Recent studies have been performed on the control of the $\alpha(2-8)$ polysialic acid chain on N-CAM. These have suggested that this chain is transferred to the polypeptide in total, rather than as individual sialic acid units [20,21] as has previously been described for certain bacterial systems [22,23]. This process is suggested to occur via a membrane lipid intermediate, on which the chain is synthesised and elongated prior to transfer. With L2, we have seen a sequential developmental expression of the sugar in lipid, followed by protein fractions. Furthermore, there is also a good regional agreement between epitope levels in the lipid and protein fractions. It is therefore tempting to speculate that the L2 epitope may be synthesised in total on a lipid intermediate, before being transferred to the final glycoprotein acceptor where it plays its functional role.

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