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QUARTERLY

Immunofluorescent detection of CD15-fucosylated glycoconjugates in primary cerebellar cultures and their function in glial-neuronal adhesion in the central nervous system★○

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Expression of CD15 antigen (also referred to as stage specific embryonic antigen, SSEA-1, or Lewis^x) was analyzed in cerebellar cultures prepared from seven day old rats by double immunostaining with anti-CD15 mAb7A and cell-specific antibodies to glial fibrillary acidic protein (GFAP) and Vimentin. The immunocytochemical data suggest that the expression of CD15 antigen is restricted to some GFAP-positive cells with fibroblast-like morphology characteristic of Type-1 astrocytes. In order to explore the involvement of CD15 antigen in glial-neuronal interactions, the ability of mAb7A antibody to interfere with granule cell adhesion to a monolayer of astrocytes was tested in comparison with anti-GFAP. The adhesion of cerebellar granule cells to astrocytes, as determined by the number of bound cells, was decreased by 39% following preincubation with mAb7A. Anti-GFAP did not alter cell adhesion, indicating the specificity of the anti-CD15 antibody effect. These results are consistent with the hypothesis that CD15 antigen participates in glial-neuronal interactions in the developing cerebellum. Furthermore, it may be speculated that the modulation of cell-surface CD15 expression contributes to the altered strength of glial-neuronal interaction, facilitating cell migration and differentiation.

Fucosylated glycoconjugates, sharing the α-(1,3)-fucosyl-*N*-acetyllactosamine epitope and referred to as SSEA-1 (stage specific embryonic antigen), Le^x (Lewis^x) or CD15 antigen,

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Abbreviations: SSEA-1, stage specific embryonic antigen or Lewis^x (Le^x); Fuc-T, fucosyltransferase; GFAP, glial fibrillary acidic protein; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; CFDA, carboxymethyl fluorescein diacetate; MAP, Microtubule Associated Protein; FITC, fluorescein isothiocyanate; Cy3, cyanine 3.

are expressed on the cell surface of both normal and neoplastic tissues (Itzkowitz *et al.*, 1986; Fukuda, 1992) and have been implicated in playing a role in cell adhesion. Changes in CD15 expression during embryonic development (Fenderson *et al.*, 1990) and in association with malignant transformation (Kim *et al.*, 1988) coincide with altered cell adhesion. Studies by the Hakomori group (Eggens *et al.*, 1989a, 1989b) have documented that CD15-mediated cell adhesion involves homophilic interactions of the fucosylated residues (Hakomori, 1992).

Immunohistological data (Marani & Mai, 1992; Mai *et al.*, 1992) suggests that CD15- glycoconjugates may play a crucial role in cell–cell interactions in the CNS. CD15 glycolipids, glycoprotein and proteoglycans are expressed in the brain (Fox *et al.*, 1981; Fenderson *et al.*, 1990; Feizi, 1991; Marani & Mai, 1992). The temporal- and brain region-specific pattern of expression of CD15 antigen in the central nervous system (CNS) (Yamamoto *et al.*, 1985; Feizi, 1991; Marani & Mai, 1992) is consistent with the hypothesis that it may play an important role in the neurodevelopmental process. Specifically, this antigen has been proposed to participate in cerebellar granule cell migration (Altman, 1982).

Crucial to our understanding of the role of CD15 in the CNS is its cellular distribution. Despite numerous studies, the cellular distribution of CD15-glycoconjugates in the CNS has not been fully characterized. While both immunohistochemical and immunocytochemical data indicate the expression of this antigen on astrocytes (Lagenaur *et al.*, 1982; Stark *et al.*, 1992; Gocht & Lohler, 1993; Gocht *et al.*, 1994; Schonlau & Mai, 1995), the presence of this epitope on neuronal cells has not been firmly established. The *in vivo* distribution of CD15 antigen on distinct astroglial contact sites in the rat optic nerve sections (Gocht *et al.*, 1994) supports the notion that CD15 could act in cell-to-cell recognition processes in the CNS.

However, immunohistological and immunocytochemical data suggest also that the CD15 antigen expression in the CNS not only varies with the age (Yamamoto *et al.*, 1985) and exhibits a brain region-specific distribution pattern (Gocht *et al.*, 1994) but also exhibits a species specific pattern (Marani & Mai, 1992). Furthermore, differences in antigenic marker profile have been observed between astrocytes derived from different brain regions and species (Nagata *et al.*, 1986; Johnstone *et al.*, 1986). Thus, there is an urgent need to characterize cell-specific CD15 antigen expression and to establish its role in glial-neuronal interactions in well-characterized CNS systems.

Present studies were designed to examine cell-specific expression of this antigen in cell cultures derived from the developing rat cerebellum and to explore the possibility of this antigen being involved in glial-neuronal adhesion. The immunocytochemical data suggest that the expression of CD15 antigen is restricted to a subpopulation of astrocytes. The results of studies on binding between the astrocytes and cerebellar granule cells are consistent with the hypothesis of CD15 antigen being involved in glial-neuronal adhesion. Furthermore, it may be speculated that the modulation of cell-surface CD15 expression contributes to the altered strength of glial-neuronal interaction, facilitating cerebellar cell migration and differentiation.

MATERIALS AND METHODS

Animals. Sprague Dawley rats were used in all experiments. They were housed in a controlled environment with food and water available *ad libitum*. Rat pups were euthanized by decapitation at postnatal day 7 (P7) and the cerebellar tissue was used to prepare astrocyte cultures.

Materials. Cell culture reagents and fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY, U.S.A.). Polylysine was ob-

tained from Sigma. The primary anti-CD-15 antibodies: 7A (IgM) was obtained by courtesy of Drs. Gerald Schwarting, Miyuki Yamamoto and Keiko Yoshida (Shriver Center), while mAb 43B11 from Drs. McCaffrey and Dragger; anti-GFAP was purchased from Boehringer-Mannheim; anti-Vimentin (IgG) was purchased from Sigma. Cyanine 3 (Cy3)-conjugated IgM and fluorescein isothiocyanate (FITC)-conjugated IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (U.S.A.).

Primary cerebellar cultures. Cells were isolated from Sprague Dawley P7 rats by a modification of published procedures (Messer *et al.*, 1981; Messer, 1989; Gocht *et al.*, 1994). Briefly, cerebellar tissue was transferred to Dulbecco's modified Eagle's medium (DMEM), freed of meninges, minced, washed, digested with trypsin/DNase at 37°C for 30 min, centrifuged and suspended in DMEM containing 10% fetal calf serum. This suspension was triturated, filtered through a 22.4-μm mesh and centrifuged. The cell pellet was suspended in the DMEM containing 10% fetal calf serum and plated at a density of 1.5×10^6 cells/ml onto polylysine-covered glass cover slips in tissue culture dishes. The culture medium was changed after 24 h. The cultures were maintained in a CO_2 incubator for a total of 7 days.

Immunocytochemistry. Cerebellar cultures grown on polylysine-coated cover slips were used at 7 days following plating. After washing with phosphate-buffered saline (pH 7.4), cells were fixed with 4% paraformaldehyde for 15 min. Thereafter, cells were preincubated with 1% BSA for 15 min, then immunostained for 60 min either with mAb7A (1:50) or mAb 43B11, and Cy3-conjugated goat anti-mouse IgM (1:250) for 60 min. The cells were subsequently labeled with a second antibody (anti-GFAP or anti-Vimentin) for 30 min, followed by FITC-conjugated IgG. Before intracellular staining, cells were treated with 0.1% Triton X-100 for 5 min. Fluorescent labeling was studied using a Zeiss epifluorescence microscope and TRITC HiQ filter (610–675 nm) equipped with an automatic camera.

Adhesion of cerebellar granule cells to astrocytes. The adhesion studies followed a procedure modified from that used previously (Hatten, 1987). Rat cerebellar homogenates, fractionated on Percoll gradients, were plated on polylysine coated wells for 24–72 h to establish monolayer cultures. Cerebellar granule cells, purified by preplating on polylysine coated plates (to remove astrocytes) were plated on the monolayer of astrocytes for 30 min in the presence or absence of mAb7A, or control antibody (GFAP). The unbound cells were removed by controlled vibrations on a rotary platform and counted in a hemocytometer. Alternatively, granule cells were labeled with a fluorescent dye, carboxymethyl fluorescein diacetate (CFDA), prior to plating (Hatten, 1987) and the bound granule cells were counted in several fields using Zeiss epifluorescence microscope.

RESULTS

Detection of CD15 antigen in rat cerebellar primary cultures by immunofluorescence

CD15 antigen expression in the rat cerebellum-derived cultures was analyzed using a double immunofluorescence labeling technique with anti-CD15 primary antibodies (7A or 43B11) and Cy3-conjugated goat anti-mouse IgM followed by cell specific antibodies (anti-GFAP, anti-Vimentin) and fluorescein-conjugated IgG. At 7 days following plating, the cerebellar cultures were predominantly composed of GFAP positive, mature astrocytes (Fig. 1, Panel C and D; Fig. 2, Panels C and F; Fig. 3, Panel C). The immunostaining with anti-CD15 antibody, mAb7A, appeared to be restricted to the surface of some glial cells (Fig. 1, Panels B and D; Fig. 2, Panel B; Fig. 3, Panels B and E). The staining with mAb7A showed fibroblast-like morphology character-

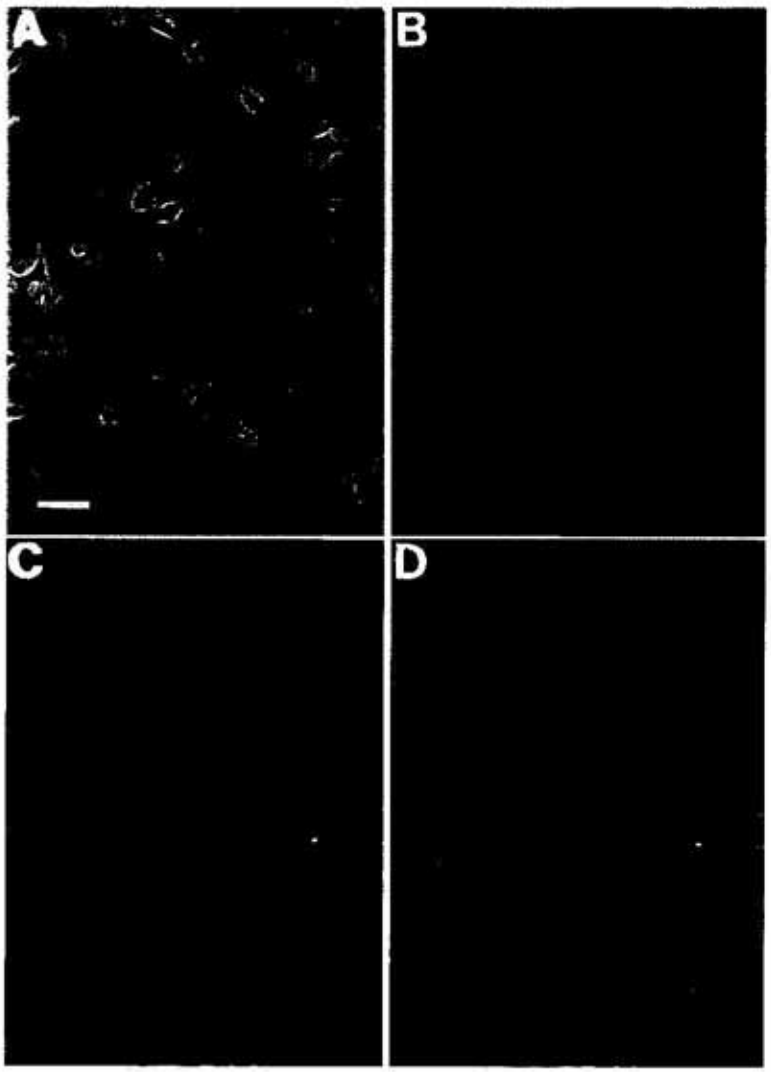

Figure 1. Double immunofluorescence labeling with anti-CD15 mAb (7A) and anti-GFAP in primary rat cerebellar cultures.

Cerebellar cultures were isolated from P7 rats, and plated in DMEM containing 10% fetal calf serum at a density of 1.5×10^5 cells/ml onto polylysine-covered glass cover slips. The cultures were maintained for a total of 7 days. Panel A: phase contrast; Panel B: mAb7A and Cy3-conjugated IgM; Panel C: anti-GFAP and FITC-conjugated IgG; Panel D: double immunofluorescence with 7A and Cy3-conjugated IgM followed by anti-GFAP and FITC conjugated IgG, same field as in Panels A, B and C. Fluorescence was studied using 610–675 nm filter. Scale bar = 8 μm.

istic of Type-1 astrocytes in the optic tract. When immunocytochemical characterization of CD15-expressing cells was carried out with anti-CD15, mAb 43B11, a similarly selective staining pattern was observed (Fig. 2, Panel E). Double immunofluorescence staining with mAb7A and Cy3-conjugated IgM followed by anti-GFAP and FITC-conjugated IgG (Fig. 1, Panel D and Fig. 3, Panel C) showed CD15 expression by GFAP-positive, mature astrocytes. Double immunofluorescence staining with mAb7A and Cy3-conjugated IgM followed by anti-Vimentin and FITC-conjugated IgG (Fig. 3, Panel F) showed that some of the Vimentin-positive, immature astrocytes also express CD15 antigen. The latter observation confirms a previous report on CD15 expression in mouse cerebellar astrocyte cultures (Lagenaur *et al.*, 1982).

The adhesion of cerebellar granule cells to astrocytes

The results of our analysis of cerebellar granule cell adhesion to cerebellar astrocytes are presented in Fig. 4. The astrocytes were preincubated for 6 h in the absence (–7A) or presence (+7A) of anti-CD15, mAb7A, or anti-GFAP (+GFAP) antibody. Following preincubation, granule cells were added to astrocyte-containing or empty wells, and the cells were further incubated for 30 min. The unbound cells, removed by controlled vibrations, were counted with a hemocytometer (three fields

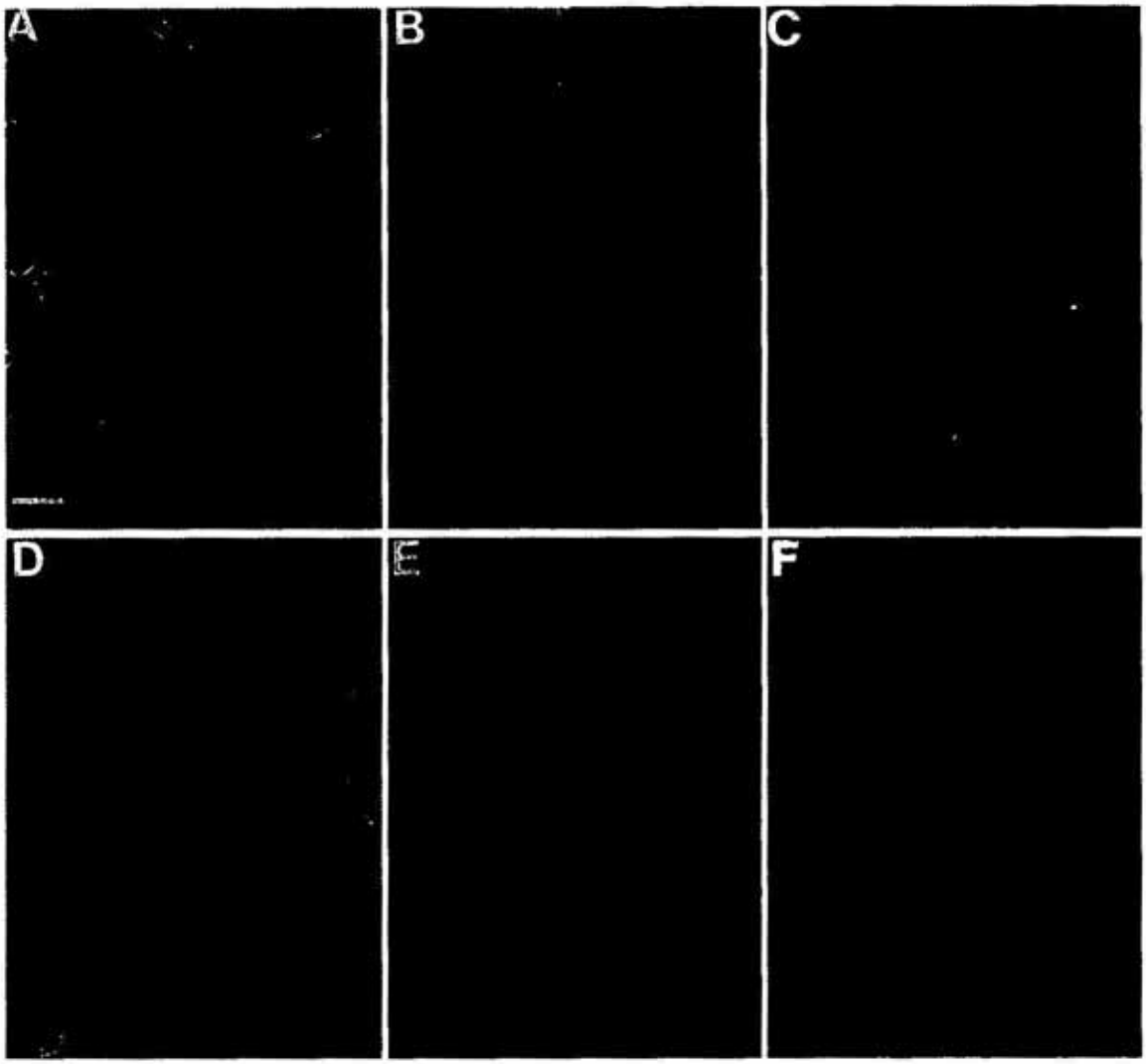

Figure 2. Comparison of immunoreactivity of CD15 antigen with mAb 7A *vs.* 43B11.

Cerebellar cultures from P7 rats maintained under culture conditions described in Fig. 1. Panels A and D: phase contrast; Panel B: mAb7A and Cy3-conjugated IgM; Panel E: mAb 43B11and Cy3-conjugated IgM; Panels C and F: anti-GFAP and FITC-conjugated IgG. Scale bar = 8 μm.

from two wells per data point). The results presented in Fig. 4, Panel A, show that addition of mAb7A results in a 68% increase in the number of unbound cells. Similar results (Fig. 4, Panel B) were obtained when carboxymethyl fluorescein diacetate (CFDA)-labeled granule cells were added to the astrocytes and the results were expressed in terms of bound neurons (fourteen different microscope fields were counted per data point). The data was analyzed using 2-tailed, paired T-test. The number of bound, CFDA labeled granule cells was decreased by 39% ($P < 0.05$) in the presence of anti-CD15 (+7A). Preincubation of astrocytes with anti-GFAP did not significantly alter the number of bound (Fig. 4, Panel B) granule cells ($P < 0.05$). Similar results were obtained when the number of unbound (Fig. 4, Panel A) granule cells were analyzed. These results are consistent with the notion that CD15 antigen mediates glial-neuronal adhesion in CNS derived cultures. The observation that anti-GFAP did not affect cell adhesion indicates the specificity of the anti-CD15 antibody effect.

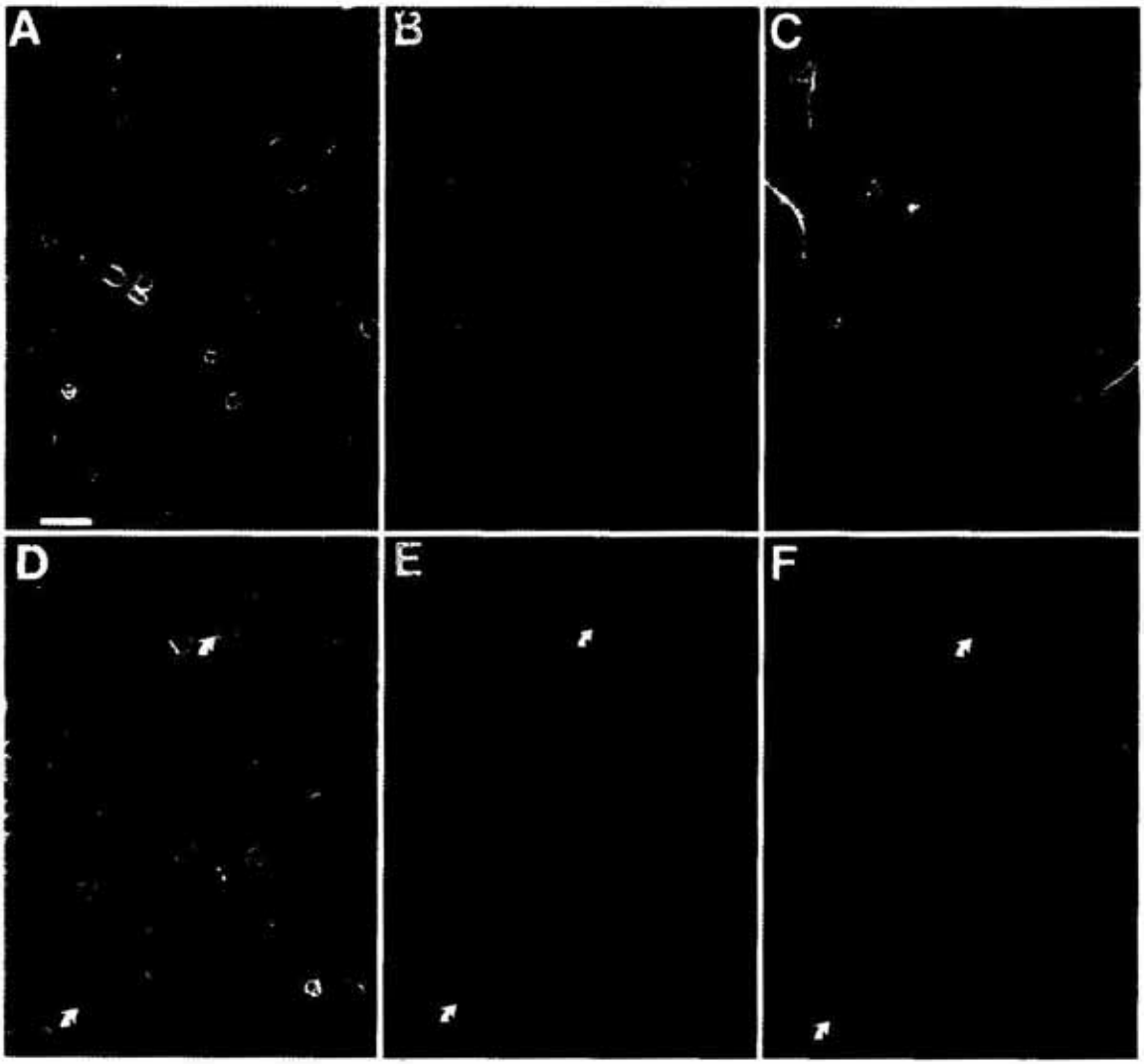

Figure 3. Double immunofluorescence labeling in primary rat cerebellar cultures.

Cerebellar cultures from P7 rats maintained under culture conditions described in Fig. 1. Panels A and D: phase contrast; Panel B and E: mAb7A and Cy3-conjugated IgM; Panel C: double staining with mAb7A and Cy3-conjugated IgM followed by anti-GFAP and FITC-conjugated IgG; Panel F: double staining with mAb7A and Cy3-conjugated IgM followed by anti-Vimentin and FITC-conjugated IgG. Arrows in Panels D–F indicate CD15 and Vimentine positive astrocytes. Scale bar = 8 μm.

DISCUSSION

CD15 (SSEA-1, Le^x) antigen, characterized by carbohydrate epitope 3-fucosyl-*N*-acetyllactosamine, has been implicated in cell-cell adhesion during embryo compaction, leukocyte adhesion, tumor cell aggregation (Hakomori, 1992) and during egg fertilization (D'Cruz *et al.*, 1997). In the CNS, brain region-specific modulation of CD15 expression accompanies neurodevelopmental events, but the function of this antigen has not been established. Defining the role of CD15 in the CNS would provide an answer to one of the key questions in developmental neurobiology concerning the mechanism involved in cell migration.

At this point, it is not clear whether the neurons actively migrate to their destination or are passively pushed by further cell division. It has been suggested that the migration process involves both adhesion and repulsion events that guide cells in reaching their destination. Although a number of adhesion mole-

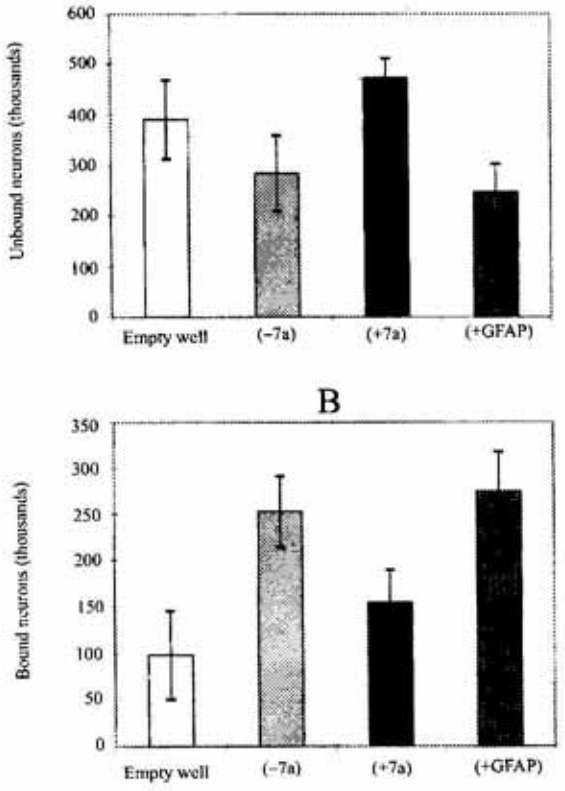

Figure 4. The adhesion of cerebellar granule cells to astrocytes.

Cerebellar astrocytes were plated for 72 h to establish monolayer culture. Cerebellar granule cells were plated on the monolayer of astrocytes for 30 min in absence (-7A) or presence (+7A) of anti-CD15 mAb 7A or anti-GFAP antibody (+GFAP) or empty wells. Panel A: unbound neurons: granule cells were removed by controlled vibrations and counted in hemocytometer; Panel B: bound neurons: granule cells were labeled with CFDA prior to plating and the bound granule cells were counted using Zeiss epifluorescence microscope. Each value is the mean ±S.D. of 14 fields ($P < 0.05$).

cules such as Neuronal Cell Adhesion Molecules (NCAMs), the cadherins, the integrins, and extracellular matrix proteins have been identified in the CNS, none of them fully explain the regulation of cell migration. CD15 is a logical candidate for this important role in cell migration in the CNS because of its temporal and brain region-specific distribution (Yamamoto *et al.*, 1985; Mai *et al.*, 1992; Marani & Mai, 1992). The coincidence of the transient elevation of CD15 glycolipid expression in the rat cerebellum between days P-0 to P-21 (Chou *et al.*, 1996), the period in which external granule cells are migrating (Altman, 1982), and the presence of the antigen in the molecular layer at this time has led to the supposition that CD15 glycolipids may play a role in external granule cell migration. However, it has also been shown that low levels of CD15 expression correlate with the synaptic reorganization processes (Schonlau & Mai, 1995). Therefore, it is possible that a decrease in CD15 expression signals the readiness of the cells to migrate (Allendorfer *et al.*, 1995) and to differentiate.

In order to understand the role of CD15 antigen in cell-cell interactions it is important to determine which cell types express the antigen in the CNS. The expression of this antigen on astrocytes has been reported by a number of investigators (Lagenaur *et al.*, 1982; Stark *et al.*, 1992; Gocht & Lohler, 1993; Gocht *et al.*, 1994; Schonlau & Mai, 1995). Immunohistological studies of rat cerebellar cortex (Marani & Mai, 1992) demonstrate that CD15 antigen is expressed on both glial and neuronal cells. The expression of CD15 antigen was observed on neural cells of chick embryo (Andressen *et al.*, 1996; Streit *et al.*, 1996), but the neuronal localization of the antigen was not observed in primary cell cultures isolated from fetal human brains (Satoh & Kim, 1994).

The pattern of CD15 antigen expression is not only brain region- (Gocht *et al.*, 1994) and age-specific (Yamamoto *et al.*, 1985; Yamamoto & Schwarting, 1992), but appears to vary even in the same brain region among closely related species such as rats and mice (Marani & Mai,1992). It is not clear whether these differences reflect expression of the antigen by astrocytes, which also demonstrate brain region-specific and species-characteristic patterns of CD15 distribution (Lagenaur *et al.*, 1982; Gocht *et al.*, 1992). In the rat CNS, CD15-immunoreactive astrocytes are concentrated in some layers of the telencephalic cortex and within the molecular layer

of the cerebellum (Gocht *et al.*, 1994). While earlier studies (Lagenaur *et al.*, 1982) showed CD15 expression by a subpopulation of GFAP-negative and Vimentin-positive mouse cerebellum-derived astrocytes, recent studies (Gocht *et al.*, 1994) using the double labeling technique showed that, in cultures derived from rat optic track, both Type 1 and Type 2 astrocytes express the antigen. Our studies of astrocytes derived from the rat cerebellum indicate that CD15 antigen expression is restricted to some GFAP-positive (Type-1) astrocytes. Some of the Vimentin-positive immature astrocytes derived from rat cerebellum also express CD15 antigen, in agreement with earlier findings on immature astrocytes derived from mouse brain tissue (Lagenaur *et al.*, 1982).

The *in vivo* distribution of CD15 antigen on distinct astroglial contact sites (Gocht *et al.*, 1994) supports the notion that CD15 could act in the astrocyte-astrocyte recognition processes in the CNS (Niedieck & Lohler, 1987; Gocht *et al.*, 1994). The data on binding between cerebellar astrocytes and granule cells presented here further extend this concept and are consistent with the supposition of CD15 antigen mediating glial-neuronal adhesion in the CNS. The interpretation of these results is complicated by the fact that the expression of CD15 antigen is restricted to a subpopulation of astrocytes; initial enrichment in CD15-expressing cells would greatly improve interpretation of the results.

At this point it is not clear whether the cerebellum-derived granule cells express CD15 antigen. If both cell types were to express the antigen, the two cell populations could utilize the homotypic interaction (Hakomori, 1992). It is possible that CD15 on astrocytes interacts with specific, yet unidentified lectin(s) expressed on granule cells, similarly to the hematopoietic system. Although platelet activation-dependent granule-external membrane protein (PAD-GEM) lectin has been found to interact with CD15 antigen (Larsen *et al.*, 1990; Lund-Johansen *et al.*, 1992), its presence in the CNS has not been reported. It is possible that P-selectin may turn out to be involved in a heterotypic interaction with CD15 antigen in the CNS (Larsen *et al.*, 1990; Allendorfer *et al.*, 1995).

In conclusion, our preliminary findings suggest that CD15 antigen is involved in glial-neuronal interactions in the CNS. In the developing rat cerebellum, CD15 antigen, expressed on a subset of astrocytes, is involved in astrocyte-granule cell adhesion. It may be speculated that, as the granule cells migrate along the glial fibers, they are guided by CD15-mediated cell adhesion. Our recent observations of elevated CD15 expression in the hypothyroid rat cerebellum characterized by delayed cell migration would support such a supposition (Sajdel-Sulkowska *et al.*, 1997a). Immunocytochemical studies both *in vitro* and *in vivo*, involving well-characterized species- and brain region-specific cell subpopulations, combined with molecular studies involving recently cloned rat Fuc-T gene (Sajdel-Sulkowska *et al.*, 1997b), will further aid in defining the role of CD15 antigen in cell-cell interactions in the CNS.

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