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Microbial Carbohydrate Specific Antibodies Distinguish Between Different Stages of Differentiating Mouse Cerebellum

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High titered anticarbohydrate antibodies were used to identify cell surface carbohydrates during different stages in histogenesis of mouse cerebellum in a micro tissue-culture system which mimics selected features of in vivo cerebellum development. Blockage of fiber formation within the first few days in vitro and inhibition of cell migrations by carbohydrate-specific antibodies served as an assay system for possible contributions of surface carbohydrates to the behavior of developing cerebellar cells. Microbial strains were selected on the basis of carbohydrate structures of their cell wall antigens, and anticarbohydrate antibodies were raised against treated whole bacteria and yeast in rabbits. We found that antibodies to mannan were active at all stages of development tested (embryonic day 13, E13; the day of birth, P0; and postnatal day 7, P7). Antibodies to sialic acids prepared against strains B and C of Neisseria meningitidis distinguish different subterminal structures: anti-B reacted with E13 and P0 cerebellar cells, and anti-C mostly with cells older than P7. Anti-fetuin antibody recognized E13 and P0 but not P7 cell populations. Pneumococcus C strain R36A-specific antibodies were effective only after coating cells to C type carbohydrate before application of the antibody. The results demonstrate that anti-microbial carbohydrate antibodies cross-react with mammalian cell surface carbohydrate structures and therefore can be used as a powerful tool in tissue culture to analyze those structures which might control cell behaviors pertinent to cerebellar development.

Key words: mouse cerebellum, development, surface carbohydrates, antibodies

The recognition sites of external signals acting on cell surfaces or cell-cell interactions are frequently found to be associated with membrane carbohydrates (1–6). The characterization of mammalian cell surface carbohydrates responsible for cell recognition is therefore important in order to understand fundamental features in development, such as cell migration of cells into a tissue structure.

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Antibodies are unique tools to detect specific determinants present in a mixture with many other structurally unrelated molecules (7) provided that a pure immunogen is used for immunization. Therefore, in order to identify cell surface carbohydrates with immunological techniques hapten-specific antibodies are required. Relatively low titered carbohydrate-specific antibodies can be obtained either by immunizing rabbits with carbohydrate determinants coupled to protein carrier molecules or isolating natural antibodies from serum using affinity chromatography (8). In this study a group of microbial strains were selected on the basis of sialic acid and mannose determinants in the carbohydrate structures of their cell wall antigens to raise high titered antibody in rabbit.

Developing mouse cerebellum was chosen to assay the activity of carbohydrate-specific antibody. The developmental stages of cerebellum are well characterized (9), and it is composed of a relatively limited number of neuronal and glial cell types in a highly stereotyped geometric pattern (10, 11). In order to analyze and manipulate cell surfaces during the onset of development, a micro tissue-culture system was designed which mimics certain features of in vivo development, such as synapse and growth cone formation, migration of granule cells, and integration of large neuronal cells, interneurons, and differentiated granule cells into cell aggregates (12, 13). The alteration of these events, for example, blocking of fiber formation and inhibition of migrating cells by carbohydrate-specific antibodies, were used in this study to explore the contribution of surface carbohydrates in cerebellar differentiation.

MATERIALS AND METHODS

Preparation of Microcultures

Preparation of single cell suspensions was performed as described by Barkley et al. (14). Cerebellum from P0 or P7 C57BL/6J mice were washed 3 times in Ca^{2+} and Mg^{2+} Tyrode solution (CMF) after dissection and incubated in 1% trypsin in CMF at room temperature for 8 min or 14 min respectively. Trypsin was removed by washing the tissue 3 times in CMF. Tissue was transferred into Eagle's Glucose (EG) [Basal medium Eagle's (Gibco) supplemented with 0.25% glucose] containing 0.05% DNase and triturated with fire-polished long-tip Pasteur pipettes with decreasing pore sizes. The cell suspension was centrifuged at 600 rpm for 8 min at 4°C. The pellet was resuspended in culture medium [EG, supplemented with glutamine (2 mM), penicillin (25 units/ml), streptomycin (25 units/ml), and horse serum (10%) (Gibco)].

Tissue Culture Condition

Single cell suspensions were plated in microtiter plates (Falcon 3034) at a concentration of $5-7 \times 10^4$ cells per 10 μl medium per well and incubated at 35°C in a moist chamber containing 5% CO_2. When antibodies were applied $5-7 \times 10^4$ cells were added to microwells in 5 μl of medium before 5 μl of various antibody dilutions in culture medium were added.

In order to reverse antibody inhibited culture, antibody containing medium was replaced by culture medium after 1–2 days in vitro. Cultures were incubated for several days. After 2, 3, and 4 days the number of fibers growing out of reaggregates and the number of fibers connecting cell aggregates were determined in order to measure antibody reactivity.

TABLE I. Inhibition of Fiber Formation in Microcultures of Mouse Cerebellar Cells

Antibody	E13	P0		P7	
		A	B	A	B
no antibody	0	140	136	268	249
normal rabbit serum	0	126	135	252	249
rabbit anti-C	±	4	112	0	239
rabbit anti-B	+++[a]	0	118	262	249
rabbit antifetuin	++	0	148	205	223
rabbit anti-S. Thompson	not done	27	106	16	252
rabbit anti-A-1	not done	12	116	8	231
rabbit anti-A-5	++	23	130	0	216

[a]+++ indicates 95% inhibition of fiber formation

TABLE II. Inhibition of Fiber Formation in Microcultures of Mouse Cerebellum Before and After Coating Cells With Pneumococcus C-Carbohydrate

Antibody	Coated		Control	
	P0	P7	P0	P7
mouse anti-R36A	23	14	106	252
Balb/C myeloma S107	36	86	115	246

Each number in Tables I and II represents the number of fibers extending freely from reaggregates or interconnecting reaggregates (12, 13) in 6 microwells after 3 days in culture. E13 cells were cultured following the procedure of Hatten and Sidman (23). PO cells are from newborn and P7 from 7-day-old mice. Row A in Table I represents the number of fibers after cells were treated with various antibodies listed; row B gives the number of fibers grown after antibodies were removed from the medium. Antibody preabsorbed with adult liver and spleen of C57BL/6J mice is uniformly inactive.

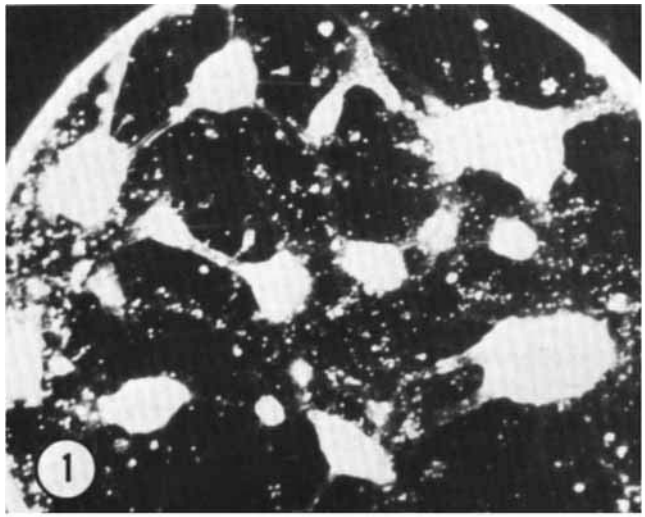
Preparation of Antibody

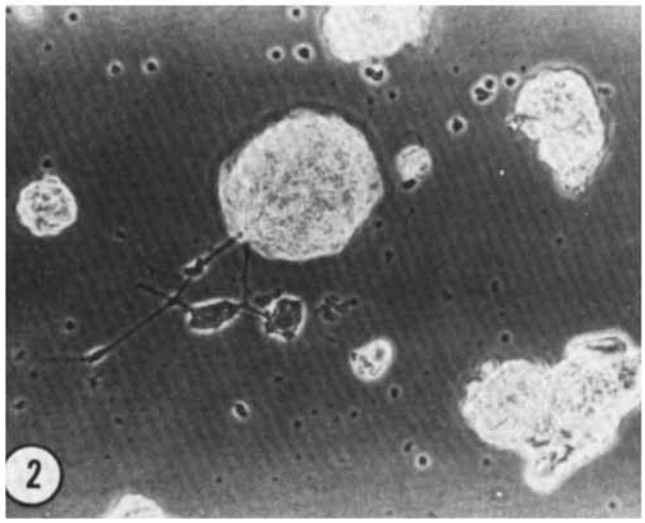
Antibodies containing specificity for polysaccharides rich in sialic acids of Neisseria strains were prepared in rabbits following the schedule described by the Center for Disease Control (15). Mouse antipneumococcus C strain R36A (anti-R36A) were raised in C57BL/6J. Mouse myeloma protein S107 was a gift from Dr. Melvin Cohn. Antibody to fetal calf glycoprotein, fetuin, was prepared as described by Spiro (16). Antibodies were heat inactivated (56°C, 30 min) and absorbed 3 times with adult live and spleen of C57BL/6J (30 min at room temperature) in order to remove unspecific rabbit antimouse reactivity.

The antibody to yeast mannan was prepared by immunizing rabbits with S. cerevisiae mutant X 2180 – 1A-5 mannan (anti-A-5), X 2180 – 1A-1 (anti-A-1), and Salmonella Thompson (anti-S. Thompson) (17).

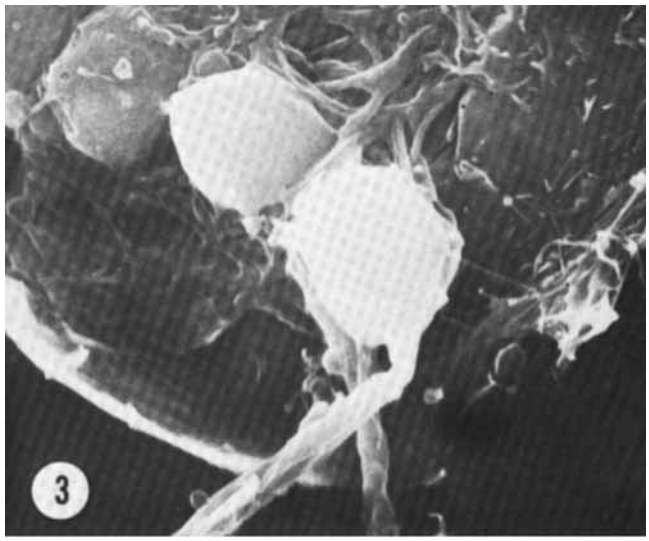
RESULTS

Within each microwell a reproducible series of events leads to a stable pattern of cells interacting with each other. The 3 major components, overlapping in time and probably interdependent are 1) reaggregation of cells (2–16 h after plating), 2) interconnection of reaggregates via sheets of migrating granule cells on the surface of epithelial cells and processes (axonal, dendritic, and glial) oriented in parallel and 3) via straight cables composed of mainly axon-like processes with granule cells migrating along their outer surfaces (12, 13).





Figs. 1–4. 7×10^4 7-day-old cerebellum cells were cultured for 72 h in 10 μl of Basal Eagle's Medium in Falcon microtiter plates, in the absence of anticarbohydrate antibodies (Fig. 1, 250 ×) or with anti-Neisseria meningitis type C antibody 1:600 diluted (Fig. 2, 250 ×). The formation of fibers connecting reaggregates is blocked in the presence of antibodies. Scanning electronmicrographs demonstrate that the surface of cells and fibers is smooth under normal conditions (Fig. 3, 2,500 ×). Cell surfaces treated with antibody appear ruffled and uncoordinated filopodia and fibers are covering the surfaces of reaggregates (Fig. 4, 5,000 ×).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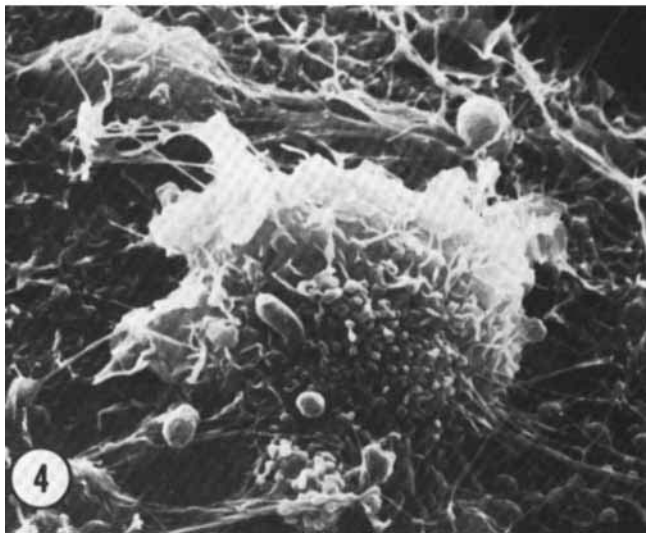
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As demonstrated in this study microbial carbohydrate-specific antibodies cross-react with cerebellar cell surfaces in vitro at different stages of development (Tables I, II), as demonstrated by inhibition of fiber outgrowth (Figs. 1–4). Antibody specific for sialic acid containing determinants in Neisseria meningitidis strains was prepared by immunizing rabbits with sialic acid containing immunodeterminants in their cell wall antigen. Both groups B and C meningococcal polysaccharides were shown to be pure homopolymers of sialic acid. The 2 polysaccharides are, however, noncrossreactive (18) and differ chemically. Acetyl determination indicated that the C-polysaccharide contains both N- and O-acetyl groups, whereas the B-polysaccharide contains only N-acetyl groups. In addition the B-polysaccharide probably consists of α-ketosidic linkages whereas the C-polysaccharide might have β-ketosidic linkages (19).

As shown in Table I, row A rabbit anti-C- and -B-polysaccharide antibodies react with cerebellar cells at different stages in development. Anti-C antibodies inhibit fiber outgrowth in both P0 and P7 cerebellar cultures but not in E13 cultures. In contrast, fiber outgrowth was inhibited in E13 and P0 but not in P7 mice by anti-B and by antifetuin serum.

The mannan chemotype consists of repeated units of either α-1, 6- (mutant X 2185 1-A-5), α-1,3- (mutant X 2185 1-A-1) (20) or of α-1,2-linked mannose (S. Thompson). Antibodies raised against these strains did not cross-react with each other. When applied to cultures of P0 and P7 cerebellar cells these antibodies did not differ in their reactivity and no distinction of developmental stages could be detected (Table I).

Pneumococcus C strain R36A injected into C57BL/6J mice gives rise to antibodies which recognize phosphorylcholine (PC) (21). These antibodies did not react with cells of any stage in development (Table II). This lack of activity was particularly interesting, since cerebellar cell membrane is likely to contain relatively large amounts of PC (22). However, when pneumococcal carbohydrate was artificially inserted into the cell surface by preincubating cells for 1 h with palmitylated derivative of the antigen (21) antipneumococcal mouse antibody (IgG) or PC-specific myeloma protein (IgA) inhibited fiber outgrowth (Table II).

The inhibition due to antibody can be reversed by replacing antibody-containing medium with normal medium not later than day 2 in culture and assayed day 3 and 4 in vitro (Table I, row B).

The reactivity of antibodies was dependent upon antibody concentration added to the cultures (Fig. 5).

The results described above suggest that antimicrobial carbohydrate antibodies react with mammalian cell surface carbohydrates and distinguish prenatal from 7-day-old mouse cerebellar cells.

DISCUSSION

The interaction of cells at different times during histogenesis appears to determine a distinct sequence of events in cell migration and integration of cells into a tissue structure. These interactions are considered to be based on changes in cell surface carbohydrate structures. In order to characterize cell surface changes during development, mouse cerebellum cells were treated in microcultures with various antibodies raised against microbial carbohydrates. As demonstrated in Table I, fiber formation and presumably cell migration were blocked by these antibodies. In particular cerebellum cells of E13 and P0 express surfaces which cross-react with antibodies prepared against polysaccharides or carbohydrates containing predominantly N-acetylated sialic acid moieties (anti-N. meningitidis type B, antifetuin) whereas P7 cells react with antibody specific to both derivatives of neuraminic acids

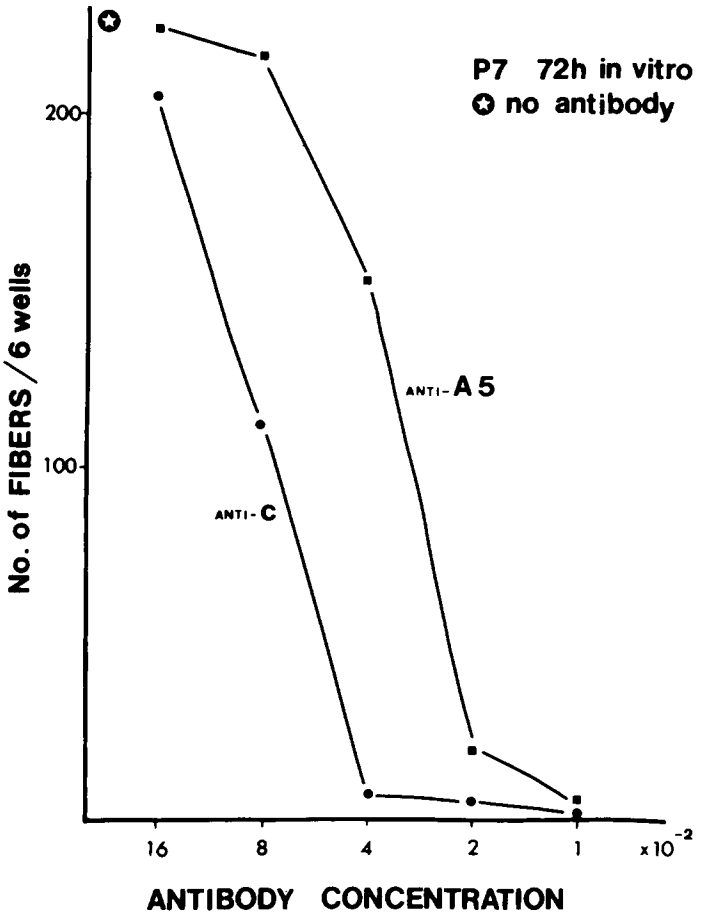

Fig. 5. Inhibition of fiber formation in cerebellar cultures is dependent upon the concentration of antibodies. Anti-C and anti-A-5 antibodies were added to P7 cells at the indicated concentrations 1 h after plating. Fibers were counted after 72 h in vitro. ⋆) Cultures without antibody.

(N-ac and O-ac) (anti-C). Yeast mannan antibodies directed against α-1,3- and α-1,6-linked mannose units and bacterial α-1,2-mannose unit did not discriminate between different stages of development in vitro.

These results raise several questions. First, the extent of similarity between the structures of cross-reacting sialic acid antigens of cerebellar cell surface and bacterial antigens still remains to be determined. The involvement of, for example, mammalian membrane sialic acid structures in many aspects of cellular recognition may make it difficult to ascertain the target structure(s) involved in this particular assay. Recent experiments by Hatten and Sidman (23) using different lectins as probes of surface carbohydrates demonstrated similar results, suggesting that the recognition of sugar specificities by antibodies is very similar to lectin specificities.

Second, what is the mechanism which leads to inhibition of fiber formation? The inhibition is reversible when antibodies are removed not later than 2 days after addition. In-

cubation of cultures with antibodies longer than 2 days leads to degeneration of predominantly neuronal cell population. It is doubtful that the inhibition caused by binding of antibodies to the surface carbohydrates or to artificially inserted molecules (Table II) is due to specific recognition of structures responsible for fiber formation (for example, growth cones); rather it is due to a pleiotropic effect caused by a nonspecific agglutination of carbohydrate-bearing surface proteins. The intensely folded cell surface in the presence of antibodies compared to the smooth surface of control cells (Figs. 3, 4) suggests that the fluidity of surface molecules is reduced or blocked by antigen-antibody reaction.

The rearrangement of surface antigens may be required to form the appropriate growth cone surface. In order to block fiber outgrowth, antibodies had to be added before fiber formation was initiated. When antibodies were added while the cell pattern was forming (1–2 days in culture) pattern formation was arrested. Under light microscopy, inhibition caused by antibodies at first appears to be restricted to the formation of fibers and connecting patterns, including the migration of granule cells but not the formation of reaggregates. Whether these results account for specific interaction of anticarbohydrate antibodies with, for example, migrating cells, still remains to be determined.

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