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Modulation of Cell-Surface Antigens of a Murine Neuroblastoma

(membrane/indirect immunofluorescence/neural antigens)

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ABSTRACT **Antisera were produced in rabbits to morphologically differentiated cells from the C1300 murine neuroblastoma (i.e., cells in which process formation was induced by maintenance on serum-free medium for 5 days). These antisera reacted more strongly in the complement fixation reaction with such "differentiated" cells than with "undifferentiated" (nonprocess-bearing) neuroblastoma cells. Adsorption of the antisera with undifferentiated cells removed the reactivity to cells without processes, while the reactivity with serum-free cells which possess processes was retained. Indirect immunofluorescence studies confirmed the results obtained by complement fixation and demonstrated that antibodies to the surface antigens of process-bearing cells could be adsorbed by particulate preparations from brain but not liver, spleen, or kidney. This is the first description of neural-associated cell-surface changes that correlate with the morphological differentiation in culture of neuroblastoma cells.**

Seeds *et al.* (1) originally described the phenomenon of induced process formation in culture by C1300 murine neuroblastoma cells. They referred to the processes as "axons", "dendrites", and "neurites", because of their morphological resemblance to these components of normal neural tissue in culture. Several groups have searched for cell-surface molecular changes that would correlate with this morphological "differentiation". Schubert *et al.* (2) observed changes on sodium dodecyl sulfate–acrylamide gels in the electrophoretic patterns of leucine-labeled total membrane protein fractions when neuroblastoma cells extend processes. Although a new surface glycopeptide has been reported on "differentiated" neuroblastoma cells (3), no changes in gross glycosphingolipid composition could be detected (4). Glick *et al.* demonstrated changes in the Sephadex G-50 chromatographic pattern of plasma-membrane tryptic glycopeptides of "differentiated" neuroblastoma cells (5). In contrast, no difference in the serologic reactivity of "differentiated" and "undifferentiated" cells could be detected by various antisera to murine cell-surface antigens (6). Several other studies on possible electrophysiological and enzymatic correlates of morphological differentiation of murine neuroblastoma cells in culture have been reported (7–12).

None of these studies of membrane components has monitored molecules specific to neural tissue. We produced rabbit antisera to morphologically differentiated (i.e., serum-free, process-bearing) cells derived from clone N18 of the C1300 murine neuroblastoma (1). We investigated two questions: (*i*) are there cell-surface antigenic changes that correlate with the morphological differentiation of neuroblastoma cells, and (*ii*) what is the relationship of the antigens involved in such changes to those of normal neural tissue?

Abbreviations: DME, Dulbecco's modified Eagle's medium; N18-SF, N18 cells maintained for 5 days in serum-free medium; N18-sus, N18 cells grown in suspension culture; 10D-SF, 10D cells maintained for 5 days in serum-free medium; RGG, rabbit gamma globulin.

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MATERIALS AND METHODS

Cell Culture. Cells described as N18 in this study were selected from clone N18 (from Dr. David Schubert) for their process-forming ability. The cells obtained were grown in Dulbecco's modified Eagle's medium (DME, ref. 13) without serum for 6–7 days. Most N18 cells that do not extend processes under these conditions die. The remaining population was replated in DME containing 10% fetal-calf serum. After proliferation, the selection in serum-free medium was repeated. Clone 10D was selected from another C1300 stock for its inability to extend processes in serum-free medium. Cell-culture techniques were those of Schubert *et al.* (2) with these exceptions: (*i*) 100-mm tissue-culture plates and bacteriological dishes were used, (*ii*) the atmosphere was 95% air, 5% CO_2, and (*iii*) the fetal-calf serum suppliers were Grand Island Biological Co. and Reheis Chemical Co. Cells were passaged weekly. The cells used are shown in Fig. 1.

Morphologically differentiated cells (N18-SF) were grown as follows: An N18 stock monolayer culture was treated with 0.25% Viokase in DME until the cells could be removed from the plate by gentle shaking (about 5 min). The cells were diluted with an equal volume of DME plus 10% fetal-calf serum, transferred to a disposable plastic tube, and centrifuged to remove the Viokase. After resuspension and counting, aliquots of 2.5 to 3.0 $\times$ 10^5 cells were plated in 5 ml of DME plus 10% fetal-calf serum. Twenty-four hours later this medium was replaced by 10 ml of DME lacking serum. Fresh serum-free medium was added after 3 days. The cells were maintained for another 2 days in this medium. At this time there were 1.0 to 1.5 $\times$ 10^6 cells per dish. This procedure allowed maximal morphological differentiation of N18 cells. Such cells (N18-SF) will hereafter be referred to as differentiated. N18 cells grown in suspension culture (N18-sus) and 10D cells (maintained with or without serum) will be referred to as undifferentiated cells. This terminology is intended to refer only to cells grown under these conditions, and no comparisons to other preparations of "differentiated" or "undifferentiated" neuroblastoma cells are intended.

Immunization. Before they were harvested for immunization, differentiated cells were washed twice with a 37° solution of 10 mM potassium phosphate buffer pH 7.0 containing 0.15 M

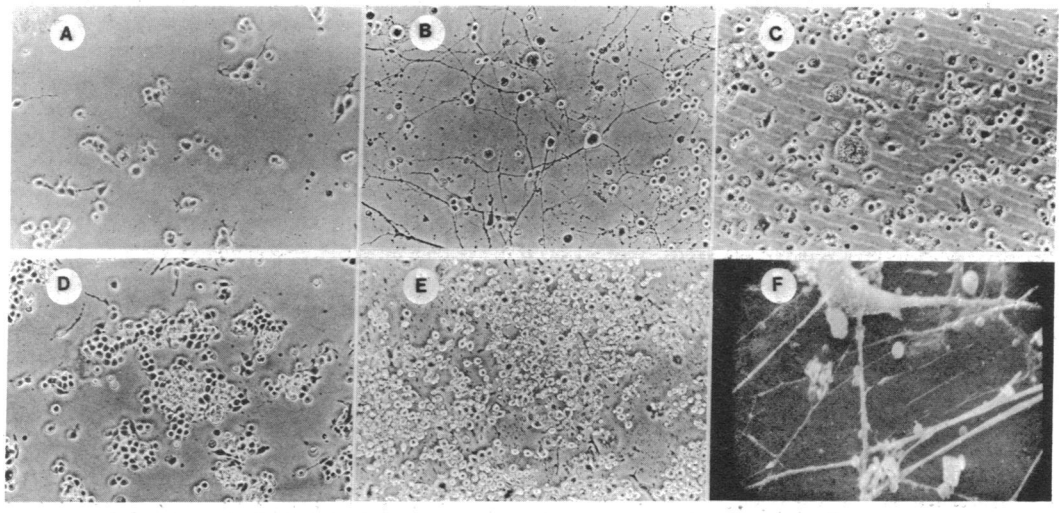

FIG. 1. Phase-contrast photomicrographs of (*A*) N18 culture in exponential growth; (*B*) N18 cells maintained for 5 days on medium without serum (N18-SF); (*C*) N18 cells grown in suspension culture (N18-sus); (*D*) 10D culture in exponential growth; and (*E*) 10D cells maintained for 5 days on medium without serum (10D-SF). (*F*) is a scanning electron micrograph of N18-SF; it illustrates the topological relationships in these cultures. Magnifications *A–E* ×81; *F* ×243.

NaCl and 1 mM $CaCl_2$. Each plate was then incubated in 10 ml of 1 μM $ZnCl_2$ for 10 min at 22°. The cells were then washed with 3.5 ml of a 22° solution of the phosphate buffer lacking $CaCl_2$ and scraped with a rubber policeman into 0.2–0.4 ml of the same buffer. A modification of the immunization protocol of Kim and Reif (14) was used to prepare antisera to intact cells. Four female New Zealand albino rabbits were injected intracardially with differentiated N18 cells on days 0, 7, 14, 21, 40, and 44. Each immunizing injection contained about 10^7 cells per rabbit. Rabbits were bled from the lateral ear vein on days 13, 20, 27, 30, 34, 47, and 50. Sera were heat-inactivated for 20 min at 60° and frozen at −70° until use.

Serologic Procedures. Microcomplement fixation was performed as described (15). Cells were prepared for complement fixation and protein was measured as described (16). Cells and organs used for adsorption of antisera were prepared as above, with the exception that organs were homogenized in a Kontes Duall No. 21 tissue grinder before Dounce homogenization. Adsorbed antisera were prepared by mixing antisera with the appropriate resuspended antigen in 6–8 ml of isotris buffer (16) plus either 3–5 mg/ml of bovine albumin or 10% normal rabbit serum. The antibody–antigen mixture was rotated 6–16 hours at about 10 rpm at 8° and then centrifuged for 25 min at 47,000 × *g*. The supernatant was filtered through a 0.22-μm Millipore filter to remove any remaining particulate material. The dilution of antisera during adsorption was 1/10 or greater. Details of individual adsorptions will be described with each experiment.

Monolayer cells for immunofluorescence were grown on glass coverslips (Corning) sterilized with 95% alcohol in 35-mm tissue-culture dishes. Differentiated cells were grown as described in *Cell Culture*. Cells were fixed with 1% gluteraldehyde in isotris for 15 min at 22°. After a thorough rinsing in isotris they were transferred to a new dish and covered with 1.25 ml of isotris; the appropriately adsorbed rabbit antiserum was added (from rabbit 146 on the 20th day of the protocol). The coverslips were incubated 2 hr at 37°, rinsed thoroughly in isotris, and again transferred to a new dish containing 1.25 ml of isotris. Fluorescein-isothiocyanate-conjugated goat antibody to rabbit gammaglobulin (0.15 ml of a 1/5 dilution, Miles

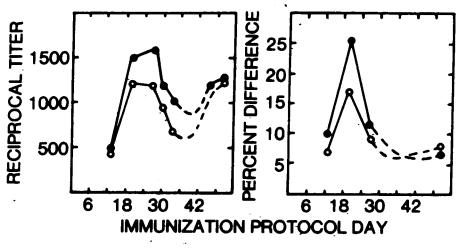

FIG. 2. Immunological responses (*left*) and specific titers (*right*) of rabbits 146 (○) and 147 (●). N18-SF was used as antigen to obtain the titer of the various bleedings. Antiserum titer is defined as the dilution necessary to obtain 80–90% maximal fixation. Each bleeding was then assayed with both N18-SF and N18-sus to determine specific titers. Specific titer is defined as the difference in percent maximal fixation between the antigens N18-SF and N18-sus (N18-SF always fixing more complement).

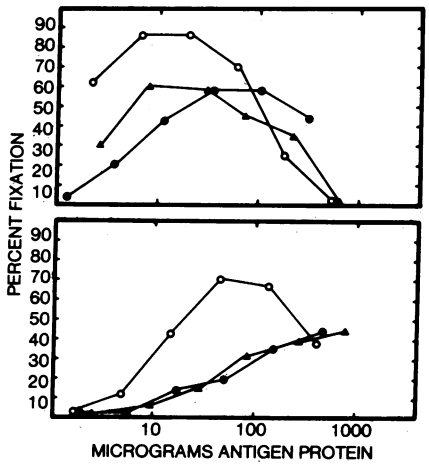

FIG. 3. Complement fixation reaction of particulate fractions from N18-SF (○), N18-sus (●), and 10D (△) with unadsorbed antiserum (*top*) and antiserum adsorbed with 10D cells (*bottom*). Antiserum 147 (200 μl from the 20th day of the protocol) was adsorbed with 2.8 mg of 10D cell particulate material. Antiserum titer was 1:1500 before and 1:300 after adsorption.

Laboratories lot 15) was added and the mixture was incubated for 30 min at 37°. Evans blue (0.15 ml of a 60 mg/ml of solution in isotris) was added and the incubation was continued another 10 min. The coverslips were rinsed thoroughly and mounted on slides in a drop of isotris containing sodium azide. Suspension culture cells were washed in isotris before fixation and then treated similarly, but were washed by gentle centrifugation.

A Leitz Ortholux fluorescence microscope with a Ploem vertical illuminator was used. Filters used were a 2BG38 and a 1GG475 at the illuminator, a 2KP490 filter at the turret, and a 510 filter at the eyepiece. Kodak Tri-X-Pan 35-mm film was used.

RESULTS

Serologic Differences between Differentiated and Undifferentiated Neuroblastoma Cells. All the bleedings taken were assayed by complement fixation against the particulate fraction from N18 cells maintained for 5 days on medium without serum (N18-SF). Fig. 2 (*left*) shows the immune response in rabbits 146 and 147. The antisera were then assayed against the particulate fractions of N18 cells grown in suspension culture (N18-sus). These cells have no processes and should represent the least morphologically differentiated state of the N18 cell in culture. N18-sus fixed less complement than did N18-SF. Fig. 2 (*right*) illustrates the specific titer of the antiserum, i.e., the difference in maximum percent complement fixed between the antigens N18-SF and N18-sus. This difference in fixation indicates a qualitative and/or quantitative antigenic difference between N18-SF and N18-sus, i.e., between cells with and without processes. The greatest difference in complement fixation between these antigens occurs with the bleedings taken on the 20th day of the immunization protocol. Sera from these bleedings of rabbits 146 and 147 were used in all subsequent studies.

We next studied the antigenic difference between N18-SF and N18-sus cells using antisera adsorbed with particulate material from cells without processes. This adsorption should remove antibodies to antigens common to the two morphological states of the cells and leave antibodies only to those antigens (if any) found uniquely on morphologically differentiated cells. In order to insure that the differences in complement fixation observed were not due to the use of a nonplated, dividing, suspension culture cell as a control, we also utilized C1300 clone 10D. Both N18 and 10D cells cease dividing in medium without serum, but 10D cells do not elaborate processes under these conditions (Fig. 1*E*).

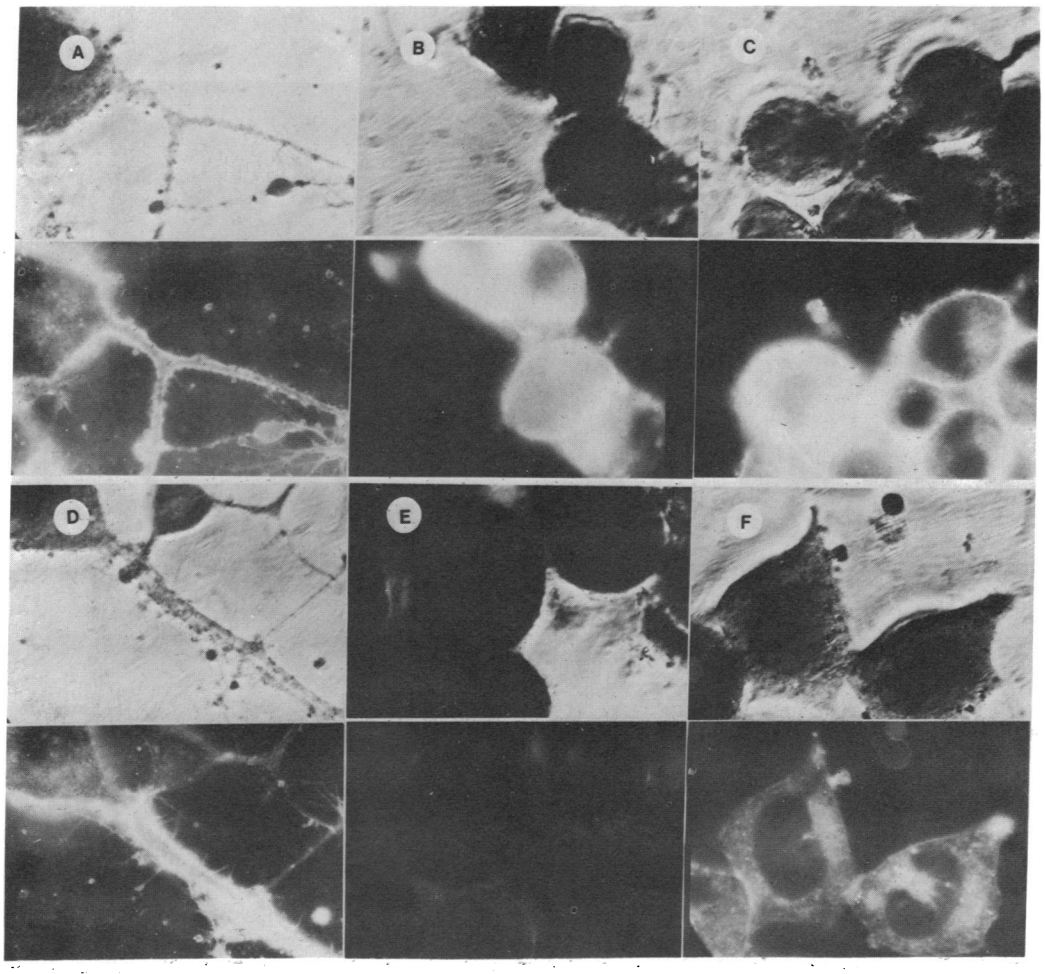

FIG. 4. Bright field (*above*) and fluorescence (*below*) photomicrographs of N18-SF (*A* and *D*), N18-sus (*B* and *E*), and 10D-SF (*C* and *F*) cells treated with unadsorbed antiserum (*A*, *B*, and *C*) and antiserum adsorbed with 10D-cell particulate material (*D*, *E*, and *F*). Antiserum 146 (330 μl) was adsorbed with 4.6 mg of 10D cell particulate material. A volume containing 7 μl of adsorbed antiserum was used for each sample. Each sample treated with unadsorbed serum received a volume containing 5 μl. Exposure time was 120 seconds for the fluorescence photographs. Magnification ×525.

Before adsorption the antiserum reacts strongly with N18-SF, N18-sus, and 10D cells (Fig. 3, *top*). The peaks of fixation are 86%, 60%, and 59%, respectively. After adsorption with 10D cell particulate material (Fig. 3 *bottom*), the antiserum still reacts with N18-SF cells. However, there is little reaction with either N18-sus or 10D. These cells do fix some complement at high protein concentrations, but it is difficult to determine if this fixation is due to the presence of a small amount of the same antigens found on N18-SF cells or to nonspecific interference by the high protein concentrations required. Similar nonsaturating complement fixation curves occur with both adsorbed and unadsorbed antiserum when particulate preparations of mouse L cells, mouse sarcoma 37 cells, rat C_6 astrocytoma cells, or HeLa cells are used as antigen. At the antiserum dilutions used, antigen preparations from all of these cells containing 1 mg/ml of protein fix 25–30% of the added complement. These complement fixation data illustrate the presence of antigens that are either unique to or present in greatly increased levels on N18-SF when compared to N18-sus, 10D, or any of the other cell lines.

We then attempted to confirm these results by an indirect immunofluorescence technique with intact cells. Fig. 4 illustrates the indirect immunofluorescence analogous to the complement fixation data of Fig. 3. Unadsorbed serum reacts well with N18-SF(*A*), N18-sus(*B*), and 10D-SF(*C*) cells, resulting in sharply outlined cell bodies with some diffuse autofluorescence (visible in all monolayer cultures in Fig. 4; *A*, *C*, *D*, *F*) within these sharp outlines. This autofluorescence is red, and hence easily distinguishable from the yellow-green of the fluorescein-isothiocyanate-conjugated antibody. After adsorption with 10D cells, the antiserum reacts well only with N18-SF (Fig. 4 *D*, *E*, *F*). Although the cell bodies of the N18-SF cells were also strongly immunofluorescent, the fluorescence is not illustrated in the figure (*A* and *D*) because the plane of focus was on the processes. These experiments confirm the presence of unique or highly enriched antigens on

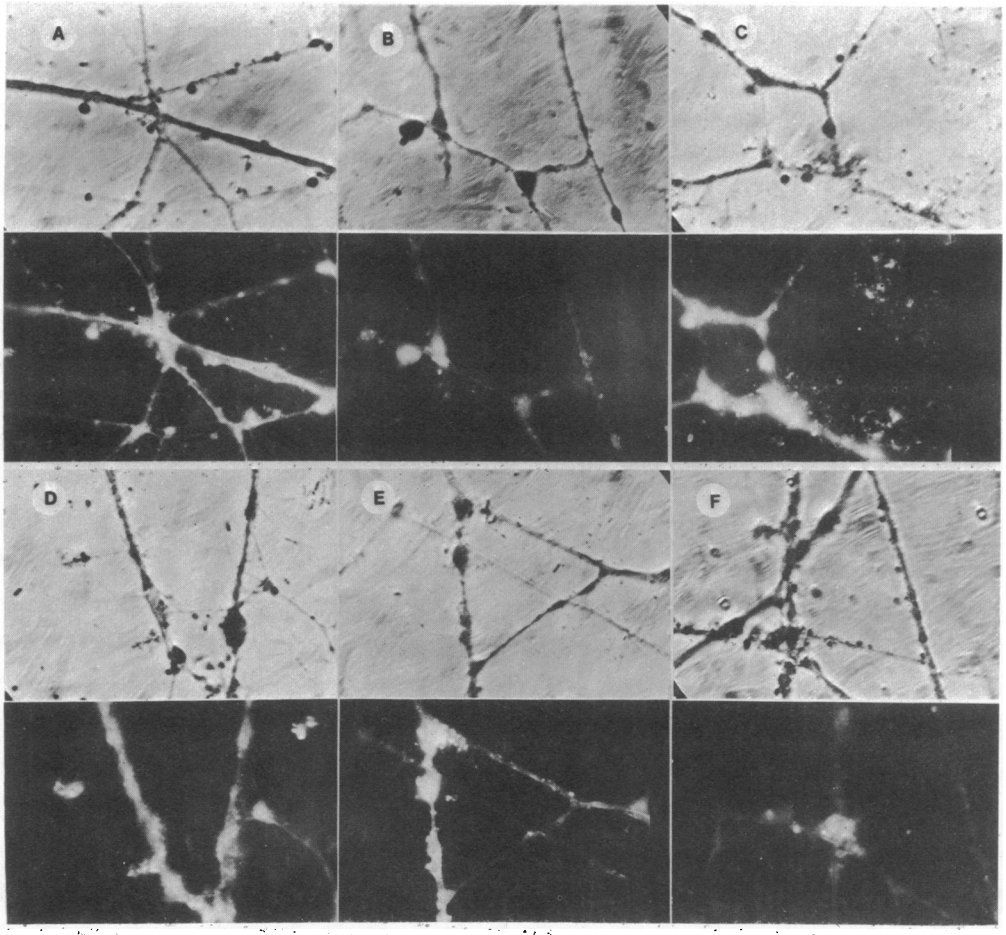

FIG. 5. Binding to N18-SF cells of antisera adsorbed with (*A*) 10D cells only, (*B*) 10D cells then brain, (*C*) 10D cells then spleen, (*D*) 10D cells then kidney, (*E*) 10D cells then liver. (*F*) Control to which only the fluorescein-isothiocyanate-conjugated goat antibody against rabbit gamma globulin (anti-RGG) was added. It illustrates the natural autofluorescence of the tissue after Evans blue treatment. Bright field (*above*); fluorescence (*below*). Other controls which showed only autofluorescence included: (*i*) HeLa cells treated with the rabbit antibody followed by conjugated goat anti-RGG, (*ii*) N18-SF cells treated with nonimmune rabbit serum and then conjugated goat anti-RGG, and (*iii*) N18-SF cells treated with the rabbit antibody, than an excess of unconjugated goat anti-RGG, and then fluorescein-isothiocyanate-conjugated goat anti-RGG. No change in reactivity of any cell type was noted upon gluteraldehyde fixation. Antiserum 146 (400 μl) was adsorbed with 5.0 mg of 10D cell particulate material. After centrifugation, aliquots (the equivalent of 40 μl) were readsorbed with 3.2 mg of particulate material from various organs. This amount of particulate material is about twice the stoichiometric amount necessary to adsorb all the antibodies that bind to brain and 3–5 times the stoichiometric amounts necessary to adsorb antibodies that bind to the other organs. These figures are based on direct binding studies of ^{125}I-labeled immunoglobulin. A volume containing 7.2 μl of adsorbed antiserum was used for each coverslip culture. Exposure time was 240 sec for the fluorescence photographs. Magnification ×525.

N18-SF and illustrate the cell-surface nature of at least some of these antigens.

Adsorption of Antiserum Specific to Differentiated Cells with Mouse Organs. Once the presence of unique or enriched antigens on N18-SF cells was indicated by both complement fixation and indirect immunofluorescence, we attempted to determine their relationship to antigens of normal neural tissue. We first prepared antiserum specific to N18-SF cells by adsorption with 10D particulate material and then adsorbed this antiserum with particulate preparations from various mouse organs. These doubly adsorbed antisera were then tested by immunofluorescence for the presence of antibodies reactive with N18-SF cells (Fig. 5). In each field two or more processes are shown. Adsorption with particulate preparations from brain reduces the level of fluorescence to nearly that of the autofluorescence control. Adsorption with stoichiometric excesses of spleen, kidney, and liver particulate fractions reduces the fluorescence only slightly. Although it is difficult to quantify these results, it is clear that neural tissue contains high levels of the antigens also found uniquely or in high levels on N18-SF, while the other organs tested do not contain such levels.

DISCUSSION

Two conclusions may be drawn from these results. First, morphological differentiation of murine neuroblastoma cells induced by maintenance in serum-free medium results in antigenic alterations in the cell membranes. Cell-surface antigens are present that are unique to and/or highly enriched on morphologically differentiated cells. Second, brain particulate preparations contain antigens that can react with and remove antibodies directed to the antigens found on morphologically differentiated cells, while similar amounts of other organ preparations are unable to adsorb such antibodies. A class of antigens thus exists that is found uniquely or in high levels only on N18-SF cells and brain of all the cells and organs tested.

The fact that, concomitant with morphological and (in some aspects) electrophysiological differentiation (10–12), neuroblastoma cells display new neural antigens strongly implies that these antigens are involved in the functions of the differentiated cells. The immunofluorescence studies do not localize the antigenic changes to only the process or any other specific area of the cell. However, the antisera are clearly multispecific. Some functions, such as increased sensitivity to transmitter and action potential initiation, would be expected to be localized to the cell body. Others, such as action potential conduction, would be localized to the process. Still other antigenic changes might be found only on the growing tip of the process.

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