LECTIN BINDING TO NEURAL CREST CELLS

Changes of the Cell Surface during Differentiation In Vitro

MAYA SIEBER-BLUM and ALAN M. COHEN

From the Department of Cell Biology and Anatomy, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205

ABSTRACT

To examine possible changes in cell surface carbohydrates, fluorescent lectins were applied at various times during differentiation of neural crest cells in vitro. The pattern and intensity of binding of several lectins changed as the crest cells developed into melanocytes and adrenergic cells. Considerable amounts of concanavalin A (Con A) and wheat germ agglutinin (WGA) bound to all unpigmented cells throughout the culture period. Melanocytes, however, bound much less of these lectins. Soy bean agglutinin (SBA), unlike Con A and WGA, only bound later in development to unpigmented cells at about the time when catecholamines were detected histochemically. Binding of SBA could be induced in younger cultures by pretreating the cells with neuraminidase. Melanocytes, however, did not bind detectable amounts of SBA even if treated with neuraminidase. The SBA-binding sites were often concentrated on cytoplasmic extensions and on contact points between neighboring cells, even when receptor mobility was restricted by prefixation of the cells or adsorption of lectin at 0°C. All three lectins bound to cell processes resembling nerve fibers in particularly high amounts.

KEY WORDS neural crest cells · lectins · differentiation · cell surface · catecholamines · neurogenesis

The neural crest of the vertebrate embryo lies along the dorsal ridge of the neural tube. Soon after fusion of the neural folds, crest cells start to migrate through the embryo, locate at various distances, and give rise to such divergent tissues and cell types as sympathetic ganglia, adrenal medulla, pigment cells, spinal ganglia, and visceral cartilage (23, 24). Whether the neural crest cells are already determined before they leave the neural tube is not yet known, nor are the mechanisms understood which guide the cells along their paths (23, 24). Direct contacts with neighboring cells, substrates, and soluble factors are thought to play an important role in regulating differentiation and migration. Complex carbohydrates attract particular attention as a probable site for recognition processes involved in the above events (14).

Over the past few years several observations of developmentally regulated cell surface changes have been reported. For instance, the composition of the membrane protein of maturing erythroid cells from chick embryos changes qualitatively and quantitatively (1). In adult rats there is a progressive increase in Con A-binding and a decrease in colloidal iron-binding after successive divisions of erythroblasts. Upon maturation of the orthochromatic erythroblast to reticulocyte, Con A-binding decreases and colloidal iron-binding increases (18). Before gastrulation, hybrid sea

urchin embryos exclusively adhere to cells of the maternal genotype. Beginning at gastrulation, hybrid cells recognize and adhere to embryonic cells of both parental genotypes, a phenomenon apparently acocmpanied by the expression of new adhesive determinants on the cell surface (11). Fucosyltransferase activity located on the cell surface is very high in chick primitive streak cells. In stage-10 embryos, considerable activity is located at the neural tube, cranial crest cells, and skin ectoderm. No significant fucosyltransferase activity, however, was found in stage-17 embryos (16). In addition, temporal and spatial changes in extracellular materials are found in avian and murine embryos and are thought to affect migration and localization of neural crest cells (4, 24).

In this communication we describe studies of the relationship between morphogenesis and changes in cell surface carbohydrates of avian neural crest cells grown in vitro. As a probe for cell surface carbohydrates, fluorescein-tagged plant lectins were used that recognize and specifically bind mono- or oligo-saccharides. We show that morphologically undifferentiated neural crest cells are homogeneous with respect to lectin binding but that binding changes markedly during differentiation into melanocytes and adrenergic cells.

MATERIALS AND METHODS

Cells

Neural crest cells from Japanese quail (*Coturnix coturnix japonica*) were cultured in vitro as described previously (3). In brief, isolated neural tubes of the trunk region were placed in collagen-coated plastic Petri dishes containing culture medium (Eagle's Minimal Essential medium, horse serum, embryo extract, penicillin, streptomycin but no fungizone [3]). After 2–3 h the neural crest cells started to migrate and after 18 h the neural tubes were removed.

Adsorption of Lectins

The following fluorescein-labeled lectins (containing no free fluorescein isothiocyanate [FITC]) were purchased from Miles Laboratories (Elkhart, Ind.) and used without further purification: Con A (specific for α-D-mannose and glucose residues [15]), WGA (specific for *N*-acetyl-D-glucosamine residues [12]), and SBA (specific for *N*-acetyl-D-galactosamine and galactose residues [8]). The ratio OD_{280}/OD_{495} was 0.7 for WGA, 1.64 for Con A, and 1.1 for SBA. The cultures were washed twice *in situ* with phosphate-buffered saline (PBS; pH 7.4). The lectins were added to the cells at a concentration of 50 μg/ml in PBS and the cultures were incubated on a heating plate at 37°C or on ice for 20 min. Then the cultures were washed twice with PBS (chilled PBS, when adsorption was carried out at 0°C) and fixed with 2% glutaraldehyde in PBS (Ladd Research Industries, Inc., Burlington, Vt.) for 20 min at 4°C and washed again in PBS. They were then mounted with a few drops of 50% glycerol in PBS and a cover slip and were examined for fluorescence. When cultures were prefixed as controls, they were washed twice with PBS and subsequently incubated in a 1% solution of paraformaldehyde in PBS (Fisher Scientific Co., Fair Lawn, N. J.) for 20 min at 0°C before adsorption of the lectins. In some experiments fluorescein-labeled goat anti-guinea pig γ-globulin (Antibodies Inc., Davis, Calif., kindly provided by Dr. R. Grzanna of this department) was used instead of lectins at a concentration of 50 μg/ml in PBS and incubated with cells for 20 min at 37°C.

The specificity of observed lectin adsorption was tested in two ways: (*a*) Lectins were preincubated with their hapten inhibitors in order to prevent binding to cells. (*b*) Bound lectins were dissociated from their receptor sites on the cells using their appropriate haptenic sugars. The sugars used were *N*-acetyl-D-glucosamine (Sigma Chemical Co., St. Louis, Mo.) for WGA, *N*-acetyl-D-galactosamine (Sigma Chemical Co.) for SBA, and α-methyl-D-mannoside (Sigma Chemical Co.) for Con A. For a preincubation, 50 μg of lectin was incubated in 1 ml of a 0.1-M solution of the respective carbohydrate for 20 min at room temperature. The mixture was then added to the culture, incubated for 20 min at 37°C, washed, and fixed as described above. To examine dissociation by haptenic sugars, the lectins were adsorbed to cells at 37° or at 0°C as described above. 1 ml of 0.1-M solution of the hapten was added to the culture, which then was incubated for an additional 20 min at either 37° or 0°C, then rinsed and fixed.

Histochemistry

To assay surface carbohydrates and the presence of catecholamines in the same cells, a modification of the glyoxilic acid fixation method of Lorén et al. (10) was used. After the adsorption of the lectins, the cells were treated with 2% paraformaldehyde and 2.5% sodium glyoxylate in 0.1 M phosphate buffer (pH 7.5) for 5 min at 0°C. The cultures were dried in a flow of warm air, covered immediately with mineral oil and a cover slip, and examined for lectin-fluorescein fluorescence. After this, the cover slip was removed and the mineral oil was washed off the dish with absolute alcohol. The cells were air-dried again and then exposed to formaldehyde vapors at 80°C for 1 h to induce catecholamine fluorescence.

Fluorescence Microscopy

Fluorescence was examined in a Zeiss Universal microscope (Carl Zeiss, Oberkochen, Wuerttenberg, West Germany) with reflected illumination. For fluorescein fluorescence an excitation filter FITC 490, barrier filter

530, dichroic mirror 500 and a 100 W Osram halogen lamp (Osram, GmbH, Berlin, West Germany) were used. For catecholamine fluorescence an excitation filter S 400 (Leitz, Wetzlar, West Germany), barrier filter 470, dichroic mirror 450 and an HBO 200 mercury lamp were used. Fluorescence was recorded on Tri-X film (Eastman Kodak Co., Rochester, N. Y.) at an exposure time of 30 s for fluorescein fluorescence and 4 s for catecholamine fluorescence; phase contrast was photographed on Panatomic-X film (Eastman Kodak Co.).

Enzyme Treatment of Cells

Washed cultures were incubated before lectin adsorption with 25 U/ml of neuraminidase from *Vibrio cholerae* (500 U/ml; Schwarz/Mann, Orangeburg, N. Y.) in PBS for 15 min at 37°C. Cells were pretreated with hyaluronidase from beef testes (300 USP U/mg, ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) at a concentration of 100 μg/ml in PBS for 30 min at 37°C. Trypsinization of cells was accomplished at a concentration of 50 μg of crystalline trypsin per ml of PBS (three times crystallized trypsin from bovine pancreas, Worthington Biochemical Corp., Freehold, N. J.) at room temperature. The neuraminidase preparation used for these studies was free of measurable amounts of proteolytic contaminants, whereas 100 μg of hyaluronidase hydrolyzed Azocoll (Calbiochem, San Diego, Calif.) at the same rate as 8 ng of trypsin.

Test for Viability of Cells

The viability of cells was tested both by uptake of trypan blue and by staining with acridine orange. In the latter method living cells are identified with green nuclei and dead cells with red nuclei in the fluorescence microscope (9).

RESULTS

In young cultures, all morphologically undifferentiated cells bound considerable amounts of Con A and WGA as early as 18 h after explantation of the neural tube, the earliest stage tested (Fig. 1*a* and *b*, Fig. 2*a* and *b* and Table I). As differentiation proceeded, all unpigmented cells bound these two lectins to at least the same amount, whereas melanocytes, which appeared at day 4 in culture, bound considerably less (Fig. 1*c* and *d*, Fig. 2*c* and *d*, and Table I). Differences in fluorescence intensities were judged on an arbitrary scale of 0 to ++++.

Another lectin, SBA, showed a striking difference in binding compared to Con A and WGA. The time-course of SBA binding closely followed the time-course of catecholamine appearance. SBA did not bind to any cells during the first 3 days in vitro. On the 4th day, coinciding with or slightly preceding the onset of catecholamine fluorescence (2), SBA bound to a few unpigmented cells located predominantly at the top of cell aggregates. The number of cells capable of binding SBA gradually increased and reached a maximum between 5 and 6 days in culture (Fig. 3 and Table I). By that time all adrenergic cells but no melanocytes bound SBA (Figs. 3 and 9). Cell processes resembling nerve fibers and cell aggregates bound the highest amount of SBA, ConA, and WGA (Figs. 8 and 9 and Table I). In addition, some unpigmented cells that did not contain catecholamines also bound SBA. Fluorescent SBA was often concentrated on cellular extensions and on connections between cells; this was usually seen most clearly on the large flat cells at the periphery of the cultures (Fig. 6*a*) but was also observed within the culture (i.e., Fig. 4*e* and *g*). This binding pattern persisted when SBA was adsorbed at 0°C or when cells were fixed with paraformaldehyde before SBA adsorption (Fig. 6*b*). Conversely, ConA and WGA did not show this distribution at either 37° or 0°C (compare Figs. 1, 2, and 5). Some unpigmented cells were

FIGURE 1 Binding of fluorescent WGA to neural crest cells during differentiation in vitro. Figures on the left show fluorescence of bound WGA and on the right show the same areas under phase optics. (*a*) A 3-day culture showing the clustered distribution of WGA-binding sites after lectin adsorption at 37°C. (*c*) A 6-day culture showing that unpigmented cells continue to bind WGA, whereas melanocytes (*m*) bind much less under the same conditions as in Fig. 1*a* and *b*. Bar, 10 μm. × 394.

FIGURE 2 Binding of fluorescent Con A to neural crest cells in vitro. Left side shows fluorescence and right side shows corresponding phase-contrast images. (*a*) At 3 days, Con A binding after adsorption of the lectin at 37°C results in a more uniform pattern than WGA, occasionally showing cap formation (*C*). (*c*) Con A binds both to unpigmented flattened cells and to aggregated (*agg*) cells at 6 days. Again, melanocytes (*m*) bind much less of the lectin. The small amounts of WGA (Fig. 1*c*) and Con A (Fig. 2*c*) that bind to melanocytes cluster above the nucleus but are not recorded in this photomicrograph. Bar, 10 μm. × 394.

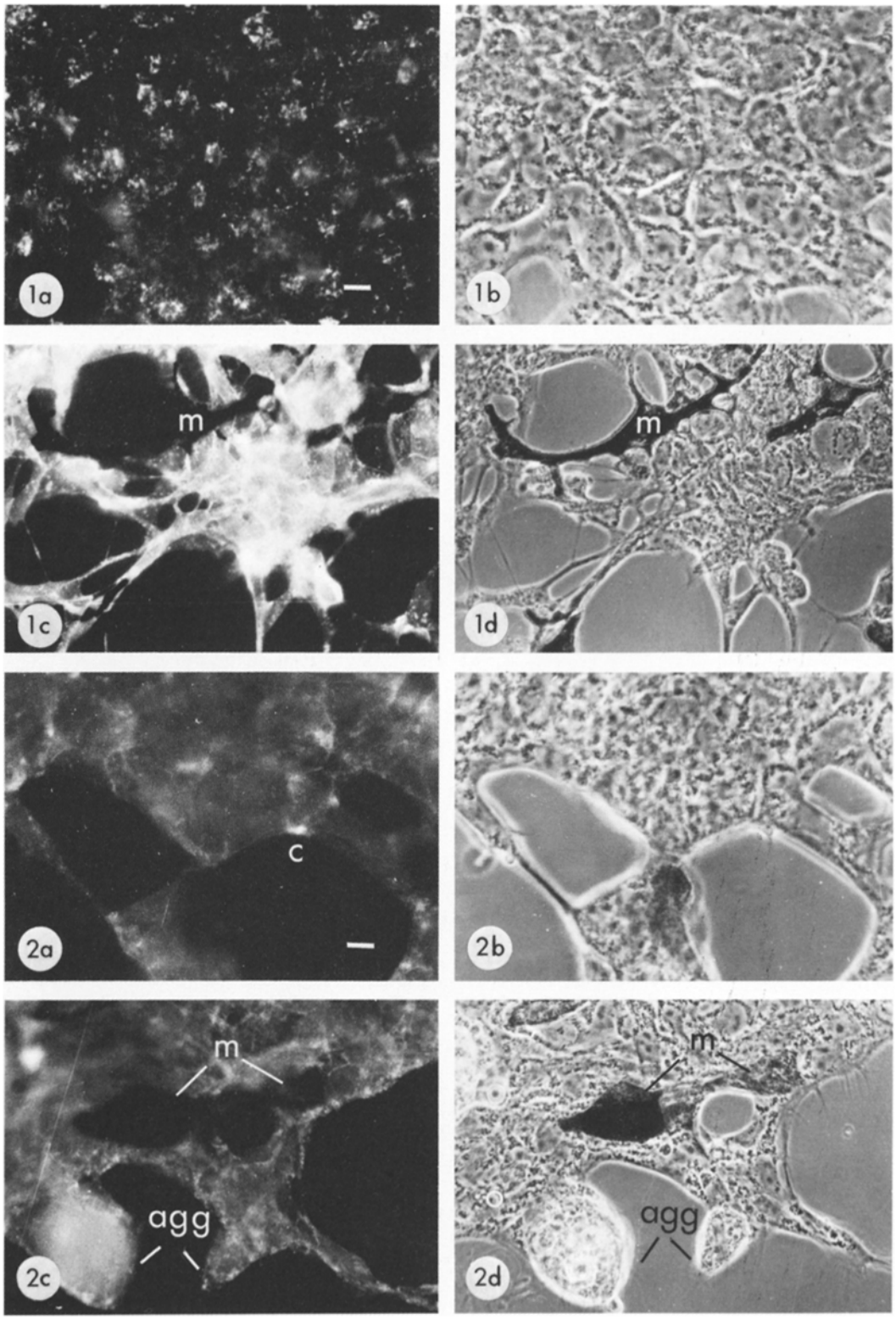

1a
1b
m
1c
m
1d
c
2a
2b
m
agg
2c
m
agg
2d

TABLE I

Binding of Lectins to Neural Crest Cells during Development In Vitro

Time period	Cell type	Fluorescein-labeled lectins*		
		WGA	Con A	SBA
Days 1–3	Undifferentiated cells	++-+++	++	0
	Undifferentiated cells + neuraminidase	++-+++	++	+++
Day 4	Unpigmented cells‡	++-+++	++	0
		++-+++	++	+
	Pigmented cells	+	+	0
	Cell aggregates	+++	++	+
Days 5 and 6	Unpigmented cells‡	+++	++	+++-++++
		+++	++	0
	Pigmented cells	+	+	0
	Cell aggregates	+++	++	+++-++++
	Adrenergic cells	+++	++	+++-++++
	Nerve-fiber-like cell processes	++++	++++	++++
	Unpigmented cells + neuraminidase	+++	++	++++
	Pigmented cells + neuraminidase	+	+	0

* The levels of fluorescent lectins that bind to cells during a 20-min incubation period range from none (0; compare Fig. 3*a*) to a maximum (++++; compare Fig. 3*g*).

‡ Unpigmented cells fall into two classes, positive and negative for SBA binding.

always present that neither bound SBA nor showed catecholamine fluorescence.

Pretreatment of cells with neuraminidase before day 4 induced binding of SBA even though the cells normally did not bind SBA then (Fig. 4). At later stages, neuraminidase induced all cells to bind SBA and enhanced the binding of the lectin to those cells already able to bind SBA. Neuraminidase treatment, however, did not induce melanocytes to bind noticeable amounts of SBA. It did not influence ConA or WGA fluorescence, nor did digestion with trypsin mimic the neuraminidase effect. Treatment of the culture with hyaluronidase did not affect the binding of ConA, WGA and SBA, although it slightly enhanced cluster formation of the binding sites of all three lectins. Neither lectin adsorption nor enzyme treatments were detrimental to neural crest cells. Viability typically exceeded 99% after these treatments.

When adsorptions were carried out at 37°C, all cells which had bound lectins showed clustering (compare Figs. 1, 2, and 4) and, in rare instances, capping (Fig. 2*a*) of binding sites. Clustering was most prominent with WGA and SBA and less so with ConA. Clustering could be completely prevented either by prefixation of the cells with paraformaldehyde or by the adsorption of the lectin at 0°C, procedures which are known to restrict receptor mobility (20, 22). Pinocytosis of lectin-receptor complexes was suggested by the observation that fluorescent clusters which had been formed after an adsorption at 37°C could not be removed completely by haptenic sugars. When the adsorption had been carried out at 0°C, reducing pinocytosis, all fluorescence could be readily removed by hapten inhibitors. Haptenic sugars, however, failed to completely remove fluorescent SBA, WGA, and ConA from neurite-like cell processes even when the adsorption had been done at 0°C. This uniformly distributed residual fluorescence might reflect a high affinity of these three lectins to the surface of these processes rather than selective lectin uptake in this area.

Preincubation of lectins with their respective haptenic sugar prevented their adsorption by cells (Fig. 7), whereas incubation with sugars other than the correct hapten inhibitor did not interfere with binding of the lectins to the cells. When cells were incubated with fluorescent goat anti-guinea pig γ-globulin instead of lectins, no adsorption or uptake was noticed. These last two observations lend support to the idea that the described lectin-binding pattern for differentiating neural crest cells was neither a result of unspecific adsorption or uptake, nor the result of physical entrapment within the multilayer portion of the culture.

DISCUSSION

In the present communication we describe stage-specific changes of cell surface carbohydrates dur-

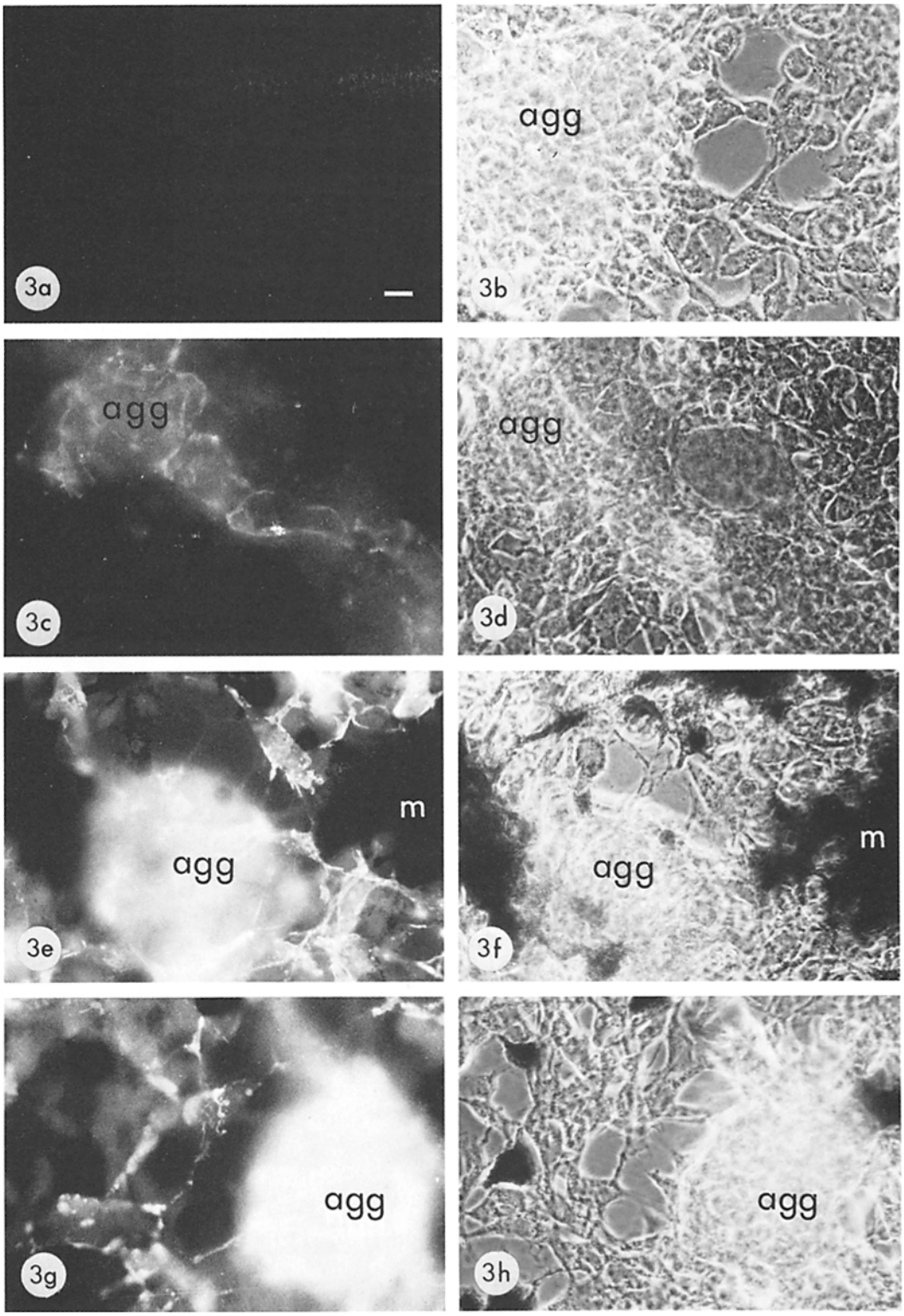
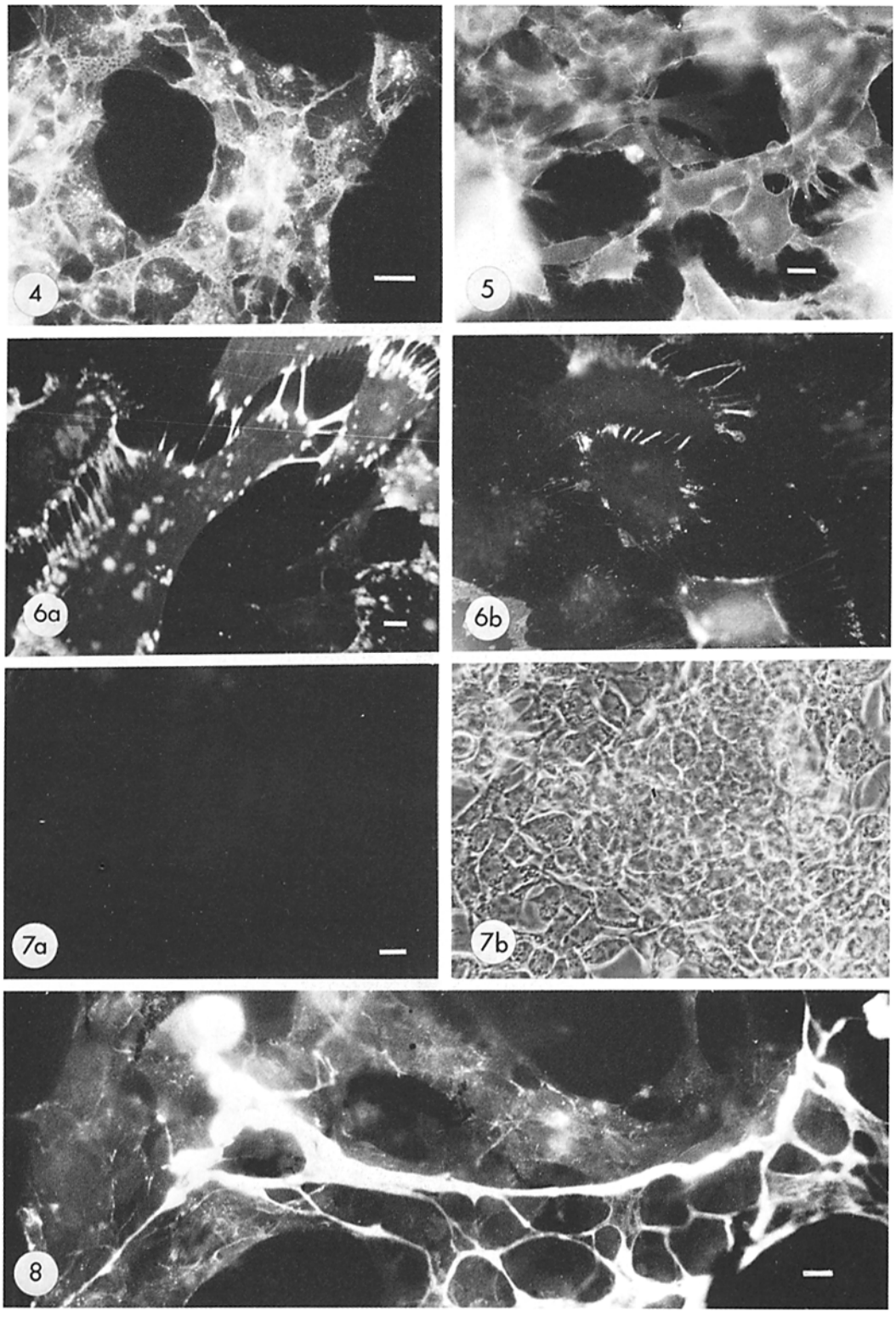


FIGURE 3 SBA binding to neural crest cells at different stages of development (lectin adsorption at 37°C). The left column shows fluorescence photomicrographs whereas the right side illustrates the same areas with phase-contrast optics. (*a*) Day 3, no SBA binding. (*c*) Day 4, weak binding is located primarily at the top of cell aggregates (*agg*). (*e*) Day 5, strong SBA fluorescence on cell aggregates and on some flattened unpigmented cells but not on melanocytes (*m*). (*g*) Day 6, strong binding of SBA on cell aggregates and some but not all flattened cells. Bar, 10 μm. × 394.


4
5
6a
6b
7a
7b
8

ing the in vitro differentiation of quail neural crest cells into melanocytes and adrenergic cells. Clustering and the failure of hyaluronidase to interfere with the binding pattern of ConA, WGA, and SBA suggest that lectins bound to the cell surface rather than to components of the extracellular matrix. Only the correct haptenic sugars prevented binding of lectins, demonstrating that fluorescence was not due to nonspecific binding or uptake. In addition, physical entrapment of fluorescent lectins was ruled out because inert fluorescein-labeled protein was neither bound nor taken up by the neural crest cells. Differences in efficacy of haptenic sugars to dissociate lectins bound to cells at 37°C compared to 0°C indicated that at 37°C some lectin-receptor complexes had been internalized, a phenomenon that has been described also for other cell types and multivalent ligands (13).

Crest cells that had just migrated out of the neural tube were homogeneous with respect to the binding of ConA, WGA, and SBA. If different crest derivatives were already determined at that early stage, then determination did not result in a modification of cell surface carbohydrates detectable with lectins. This, however, does not preclude the possibility that crest cells are determined before migration. Events responsible for determination may be too subtle to be detected by the techniques used here or they may be of a different nature.

Changes in the lectin-binding pattern during differentiation of neural crest cells were most striking when SBA was used as a probe. SBA is known to bind to terminal galactosyl residues or *N*-acetyl-galactosyl residues which are often penultimate to sialic acid (7, 19). Experiments with exogenous neuraminidase demonstrated "masked" SBA-binding sites in young cultures and, to a much lesser degree, also in older unpigmented cells. Some of these cryptic sites might be exposed during the differentiation of neural crest cells into adrenergic cells, whereas the same sites seem to be deleted during differentiation into melanocytes. It is therefore conceivable that the onset of SBA binding by adrenergic cells on the 4th day in culture reflects a gradual loss of cell surface sialic acid residues. This loss might be caused either by a reduction of sialyltransferase activity or by an enhancement of cell surface neuraminidase activity. Initiation of SBA binding could also indicate the appearance of novel cell surface glycoproteins or glycolipids containing terminal galactosyl residues or *N*-acetyl-galactosyl residues or combinations of such mechanisms. Assembly of unpigmented cells into loose aggregates clearly preceded SBA binding. Condensation of these aggregates into dense clumps coincided with the appearance of SBA-binding sites. It is conceivable that removal of cell surface sialic acid and, thus, negative charge might facilitate the formation of close cell-cell contacts and denser aggregates. This was supported by our observation that high amounts of SBA often were bound to cellular processes which established intercellular contacts.

At least two other cellular systems have been described, so far, in which cell surface sialic acid is thought to play a role in differentiation. Treatment of mouse bone marrow cells with neuraminidase affects development of erythroid cells grown both in vitro and in vivo. When such neuraminidase-treated cells were injected into irradiated mice, they produced 50% fewer colonies in the spleen (21). In vitro, the number of erythroid

FIGURE 4 SBA binding to neuraminidase-treated cells 18 h after explanation of the neural tube. Bar, 10 μm. × 631.

FIGURE 5 Adsorption of lectins to cells at reduced temperature inhibits cluster formation. This is shown here with WGA bound to day-5 cells at 0°C. Bar, 10 μm. × 394.

FIGURE 6 SBA often concentrates on cellular extensions and points of close contact between neighboring cells after adsorption of the lectin at 37°C (Fig. 6*a*) and after adsorption of SBA to prefixed cells (Fig. 6*b*). Bar, 10 μm. × 316.

FIGURE 7 Control for specificity of lectin binding, day-5 cultures. (*a*) WGA preincubated with 0.1 M *N*-acetyl-D-glucosamine failed to bind to crest cells. (*b*) Same area as Fig. 7*a* under phase optics. Bar, 10 μm. × 394.

FIGURE 8 Nerve-fiber-like cell processes bind particularly high amounts of all three lectins without clustering at 37°C. Here, binding of SBA at day 6 is illustrated. Bar, 10 μm. × 420.

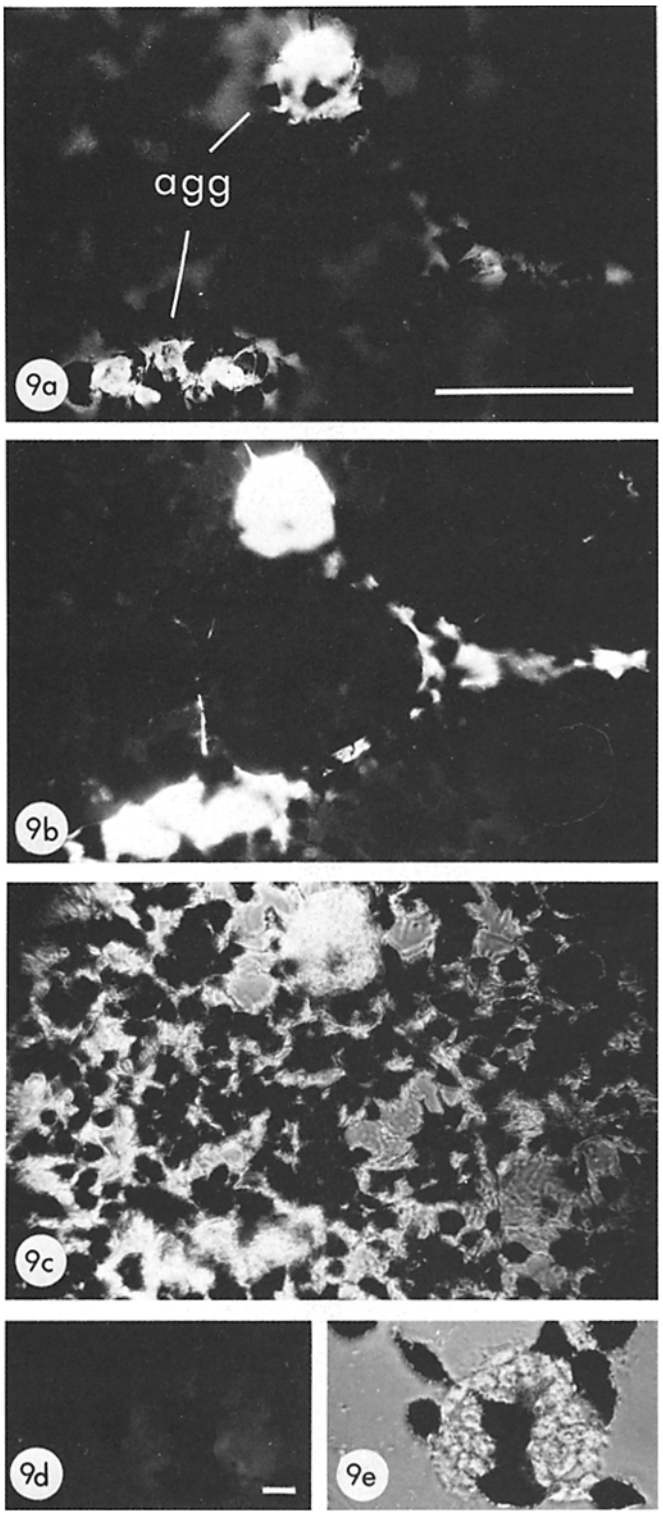


FIGURE 9 Photomicrographs (*a*-*c*) of the same field illustrating that cell aggregates at 6 days both bind SBA and contain catecholamines. (*a*) Conditions to show SBA-binding. (*b*) Conditions to show catecholamine fluorescence. (*c*) Same field under phase-contrast optics. (*d*) A 6-day culture treated with fluorescent SBA, fixed and heated without paraformaldehyde, does not induce catecholamine fluorescence and demonstrates that fluorescein fluorescence is destroyed during the heating procedure. Therefore, fluorescent SBA binding does not contribute to fluorescence in the catecholamine test. (*e*) Phase-contrast presentation of Fig. 9*d*. (*a*-*c*) Bar, 100 μm. × 262. (*d*-*e*) Bar, 10 μm. × 394.

burst-forming units which are believed to be the most primitive committed members of the erythroid pathway increased after bone marrow was treated with neuraminidase. On the other hand, more mature erythroid progenitors were no longer affected by neuraminidase (17). In addition, Skutelsky and Farquhar (18) reported changes in sialic acid content of the surface of maturing erythrocytes in the transition between erythroblast and mature erythrocyte. They noticed a gradual decrease in sialic acid at early stages followed by a sharp increase at the time of nuclear expulsion. This pronounced change of negative surface charge could be explained either by a local modification of glycosylated groups or by a selective segregation of membrane sialoproteins (glycophorin). Dreyfus et al. (6) described a membrane-bound neuraminidase in chick embryo retina, preferentially using gangliosides as a substrate. Its activity increased during embryonal development and reached a maximum by the time the retina was morphologically differentiated (5).

Several possibilities have been considered for the identity of the nonadrenergic, unpigmented cells observed in older primary cultures. Some of these cells bound SBA and others did not; SBA binding, however, could be induced in the latter by neuraminidase treatment. These cells might represent early stages in the adrenergic line arrested before morphological differentiation. Alternatively, they could also represent other derivatives of the neural crest. Work in progress focuses on resolving this question as well as allowing identification and biochemical characterization of the lectin receptors.

We wish to acknowledge Miss Linda Chandlee for her technical assistance.

This project was supported by a grant from the U. S. Public Health Service, HD-07389, and a Basil O'Connor Starter research grant from the National Foundation March of Dimes to A. M. Cohen, and a postdoctoral fellowship of the Swiss National Science Foundation awarded to M. Sieber-Blum.

Part of this work has been presented at the Fifth International Conference on Birth Defects, Montreal, Canada, 21–27 August 1977.

Received for publication 9 June 1977, and in revised form 17 October 1977.

REFERENCES

1. CHAN, L.-N. 1977. Changes in the composition of plasma membrane proteins during differentiation of embryonic chick erythroid cells. *Proc. Natl. Acad. Sci. U. S. A.* **74:**1062–1066
2. COHEN, A. M. 1977. Independent expression of the adrenergic phenotype by neural crest cells *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* **74:**2899–2903
3. COHEN, A. M., and I. R. KONIGSBERG. 1975. A clonal approach to the problem of neural crest determination. *Dev. Biol.* **46:**262–280
4. DERBY, M. A., J. E. PINTAR, and J. A. WESTON. 1976. Glycosaminoglycans and the development of the trunk neural crest. *J. Gen. Physiol.* **68:**4a
5. DREYFUS, H., S. HARTH, A. PETRI, P. F. URBAN, and P. MANDEL. 1977. Action of endogenous neuraminidase on sialo-compounds in the developing chick retina. *Biochem. Soc. Trans.* **5:**198–200
6. DREYFUS, H., S. HARTH, P. F. URBAN, and P. MANDEL. 1976. On the presence of a "particle-bound" neuraminidase in retina – A developmental study. *Life Sci.* **18:**1057–1064
7. GOLDMAN, R., N. SHARON, and R. LOTAN. 1976. A differential response elicited in macrophages on interaction with lectins. *Exp. Cell Res.* **99:**408–422
8. LIS, H., B.-A. SELA, L. SACHS, and N. SHARON. 1970. Specific inhibition of *N*-acetyl-D-galactosamine of the interaction between soy bean agglutinin and animal cell surfaces. *Biochim. Biophys. Acta.* **211:**582–585
9. LORCH, E., and G. RENTSCH. 1969. A simple method for staining and counting adipose tissue fat cells. *Diabetologia.* **5:**356–357
10. LORÉN, I., A. BJÖRKLUND, B. FALCK, and O. LINDVALL. 1976. An improved histofluorescence procedure for freeze-dried paraffin-embedded tissue based on combined formaldehyde-glyoxilic acid perfusion with high magnesium content and acid pH. *Histochemistry.* **49:**177–192
11. MCCLAY, D. R., H. F. CHAMBERS, and R. H. WARREN. 1977. Specificity of cell-cell interactions in sea urchin embryos – Appearance of new cell-surface determinants at gastrulation. *Dev. Biol.* **56:**343–355
12. NAGATA, Y., and M. M. BURGER. 1974. Wheat germ agglutinin. Molecular characterization and specificity of sugar binding. *J. Biol. Chem.* **249:** 3116–3122
13. NICOLSON, G. L. 1976. Transmembrane control of the receptors on normal and tumor cells. I. Cytoplasmic influence over cell surface components. *Biochim. Biophys. Acta.* **457:**57–108
14. ROSEMAN, S. 1970. The synthesis of complex carbohydrates by multiglycosyltransferase systems and their potential function in intercellular adhesion. *Chem. Phys. Lipids.* **5:**270–297
15. SHARON, N., and H. LIS. 1972. Lectins: cell-agglutinating and sugar-specific proteins. *Science (Wash. D. C.).* **177:**949–959
16. SHUR, B. D. 1977. Cell-surface glycosyltransferases in gastrulating chick embryos. I. Temporally and

spatially specific patterns of four endogenous glycosyltransferase activities. *Dev. Biol.* **58**:23-39

17. SIEBER, F. 1976. Erythroid colony growth in culture: effects of desialated erythropoietin, neuraminidase, dimethyl sulfoxide, and amphotericin B. *In* Progress in Differentiation Research. Müller-Bérat, editor. North-Holland Publishing Co, Amsterdam. 521-528.
18. SKUTELSKY, E., and M. FARQUHAR. 1976. Variations in distribution of ConA receptor sites and anionic groups during red blood cell differentiation in the rat. *J. Cell Biol.* **71**:218-231
19. SPIRO, R. G. 1969. Glycoproteins: their biochemistry, biology and role in human disease. *N. Engl. J. Med.* **281**:991-1001
20. TAYLOR, R. B., P. H. DUFFUS, M. C. RAFF, and S. DE PETRIS. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nat. New Biol.* **233**:225-229
21. TONELLI, O., and R. H. MEINTS. 1977. Surface molecules of hematopoietic stem cells: requirement of sialic acid in spleen colony formation. *Science (Wash. D. C.)*. **195**:897-898
22. UNANUE, E. R., W. D. PERKINS, and M. J. KARNOVSKY. 1972. Ligand-induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural autoradiography. *J. Exp. Med.* **136**:885-906
23. WESTON, J. A. 1970. The migration and differentiation of neural crest cells. *In* Advances in Morphogenesis. M. Abercrombie, J. Brachet, and T. King, editors. Academic Press, Inc., New York **8**:41-114.
24. WESTON, J. A., and S. L. BUTLER. 1966. Temporal factors affecting localization of neural crest cells in the chicken embryo. *Dev. Biol.* **14**:246-266