Phenotypic Changes and Loss of N-CAM-mediated Adhesion in Transformed Embryonic Chicken Retinal Cells

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ABSTRACT Transformation of 6-d-old embryonic chicken retinal cells by Rous sarcoma virus (RSV) was found to cause significant changes in several cellular properties including adhesiveness, motility, and state of differentiation. The alterations in cell adhesivity were analyzed by means of specific antibodies to the calcium-independent neural cell adhesion molecule, N-CAM. In the RSV-transformed cells the amount of N-CAM present at the cell surface was significantly decreased relative to normal cells, as assessed by immunofluorescent staining, specific immunoprecipitation, and immunoblotting experiments. This decrease was reflected in a marked reduction in N-CAM-mediated adhesiveness measured in vitro. A different, calcium-dependent, adhesive system also present on neurons was not detectably altered by RSV transformation and, in contrast with previous studies on normal neurons, this adhesive system was detected without treatment by proteases.

In culture, the transformed cells formed fewer and less compact colonies than the normal retinal cells. Observation of the RSV-transformed retinal cells by time-lapse cinematography confirmed the reduction in adhesiveness and also revealed that the transformed cells were more highly motile than their normal counterparts. In addition, RSV transformation appeared to alter the differentiation of the cultured retinal cells. Immunofluorescent staining studies indicated that in contrast to mature neurons, transformed neural retinal cells expressed the 34,000-mol-wt tyrosine kinase substrate and reduced amounts of a neuron-specific ganglioside recognized by monoclonal antibody A2B5. These characteristics are shared by untransformed glial cells.

In double immunofluorescent staining experiments, many cells expressed both N-CAM and pp60src shortly after viral infection, which implies that the N-CAM-positive neuroepithelial cells were transformed by RSV. In addition, a highly purified population of N-CAM-positive neural retinal cells, selected using a fluorescence-activated cell sorter, was rapidly and extensively transformed by RSV at rates comparable to those of the unfractionated population. These results established that the transformed cells were largely derived from RSV-infected neuroepithelial cells rather than from a small population of retinal glial cells present in the primary culture. The findings suggest reconsideration of the possible origin of tumors classified by morphological criteria as derived from glia and raise the possibility that the normal homologue of pp60src may play a role in the commitment of neuroepithelial cells to neuronal or glial differentiation pathways.
cases it was increased (7-10). Moreover, it has not been possible until recently to ascribe any of the changes induced by transformation to alterations in a defined mechanism of cell-cell adhesion. Over the last several years, however, substantial progress has been made towards identifying and characterizing molecules that mediate cell-cell adhesion (reviewed in references 11 and 12) and it has therefore become feasible to correlate cellular transformation with definable molecular alterations occurring at the cell surface.

One particularly well-characterized adhesion molecule is the neural cell adhesion molecule N-CAM\(^1\) (11-15), which has been purified from embryonic (14) and adult (16) chicken brains and chemically analyzed. The embryonic or E-form of N-CAM consists of two closely related polypeptide chains of Mr = 160,000 and 130,000, each of which contains a significant carbohydrate component including an unusually large amount of sialic acid (14, 17). The E-form of N-CAM migrates on SDS polyacrylamide gels as a diffuse band ranging in molecular weight from 180,000 to 250,000 (14). Adult, or A-forms of N-CAM contain only one-third as much sialic acid but are otherwise closely similar or identical to the E-form (16, 18); A-forms of chicken N-CAM migrate on SDS polyacrylamide gels as two discrete species of Mr = 180,000 and 140,000 (16, 19, 20). N-CAM is present on all neurons (21) and mediates Ca\(^2+\)-independent adhesion (22). This adhesion occurs via the formation of second-order homophilic bonds (i.e., N-CAM to N-CAM) from cell to apposing cell, and recent studies on N-CAM in lipid vesicles have shown that the change from embryonic to adult forms (E-A conversion) results in a fourfold increase in rate of adhesion (23).

The development of precise assays to measure N-CAM-mediated adhesion, the isolation of specific monoclonal and polyclonal antibodies to N-CAM, and knowledge of its molecular forms and binding mechanisms together provide a basis for examining whether changes in the expression of this molecule are induced by transformation. In initial experiments (24), we found that N-CAM expression was temperature dependent in several rat cerebellar cell lines containing a mutant Rous sarcoma virus (RSV) that is temperature-sensitive for transformation. All of the lines examined contained substantial amounts of the Mr = 140,000 A-form of N-CAM when grown at the nonpermissive temperature, but expressed significantly reduced amounts of N-CAM when transformed by growth at the permissive temperature. The results also indicated that transformation by RSV caused dramatic concomitant changes in adhesion mediated by N-CAM.

However, it is not known whether the expression of N-CAM in such established cell lines is representative of neural cells, it was desirable to test the effect of transformation on the expression of N-CAM in normal cells obtained directly from embryos. This approach offers the opportunity to examine other early consequences of RSV transformation that might affect neuron-neuron interactions. To study such changes, we transformed embryonic retinal cells with RSV as described by Calothy et al. (25). Because the retina is composed of various cell types, some of which do not express N-CAM, we carefully investigated the lineage of the transformed cells and correlated these results with the alterations induced by RSV.

**Materials and Methods**

**Cell Culture and Viruses:** Neural retinae were dissected from 6- or 7-d-old White Leghorn embryos (Spafas Poultry Farms, Norwich, CT) and the pigment epithelium was carefully removed. Cells were dispersed by trituration after treatment (20 min at 37°C, 70 rpm) with 0.25% trypsin (Gibco Laboratories, Grand Island, NY) containing 1 mM EDTA. After washing, the cells were suspended in Ham’s F10 medium containing 2% calf serum and plated at 5 x 10\(^5\) cells/35-mm tissue culture dish. Transformation of Schmidt-Ruppin RSV-strain A (SR-RSV-A) was performed as described (26). Control and transformed cultures were fed daily with Ham’s F10 containing 5% calf serum.

**Antibodies:** Antibodies to the rabbit IgG against immunofluorescence-purified chicken N-CAM and of Fab' fragments of these antibodies has been described (14, 27). Monoclonal antibody 492, which reacts well with v-src and poorly with c-src (28), was the kind gift of Dr. Joan Brugge and Dr. Leah Lipsch (State University of New York at Stony Brook). Aseites fluid containing monoclonal antibody AB25 (29) was harvested from mice injected with AB25 cells obtained from The American Type Culture Collection (Rockville, MD). Monoclonal antibodies to the 34,000-mol-wt tyrosine kinase substrate were prepared as described (30). Tumor-bearing rabbit (TBR) serum, which specifically recognizes pp60c-src, was also prepared as previously described (31).

**Immunoprecipitation and Immunoblot Analysis:** For analysis by immuno precipitation, normal and transformed cells (5 x 10\(^6\) cells in 100-mm dishes) were cultured for 5 d, incubated 16 h with 1 \(\mu\)g [\(^{32}\)P]JH]eucine (ICN Biomedicals, Nuclear, Boston, MA), rinsed, extracted, and centrifuged at 100,000 g for 30 min. The normal and transformed cells incorporated [\(^{32}\)P]JH[eucine to the same extent. The extracts were incubated with TBR serum, nonimmune serum, or anti-N-CAM antibodies followed by protein-A Sepharose (Pharmacia Inc., Piscataway, NJ). The immune precipitates were washed, solubilized, and separated on 8.5% SDS polyacrylamide gels (32) as described (30). After staining with Coomasie Brilliant Blue and destaining the gels, bands were imaged. Densitometric analysis of these proteins was visualized by fluorography at -70°C with Kodak SB-5 x-ray film. Immunoblot analyses were performed as described (34) using 4 \(\mu\)g/ml rabbit anti-N-CAM IgG. The relative amount of N-CAM in these experiments was quantified by densitometric scanning of the autoradiographs (35).

**pp60c-src Kinase Assay:** The ability of pp60c-src to catalyze transfer of \(^{32}\)PO-ATP to the heavy chain of TBR serum IgG was assayed as previously described (31).

**Immuno-fluorescent Staining:** Normal or transformed cells cultured in 35-mm tissue culture dishes were fixed for 5 min with 3.7% formaldehyde in PBS, quenched for 5 min with 0.1 M glycine in PBS, rinsed two times with PBS containing 1% normal goat serum, and blocked for 15 min with PBS containing 3% normal goat serum. When necessary, cells were permeabilized by treatment for 3 min at room temperature with 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in PBS. For staining of N-CAM, rabbit IgG was used at a final concentration of 0.1 mg/ml. Monoclonal anti-src 492 (culture supernatant concentrated 10 times by ammonium sulfate fractionation) was used at 1/10 dilution. Monoclonal AB25 ascites was used at 1/100 dilution. Aseites fluid containing monoclonal antibodies to the 34,000-mol-wt protein was diluted 1/1000.

Second antibodies used were: fluorescein-conjugated goat antibodies to rabbit IgG (Miles Laboratories, Elkhart, IN; 1/100 dilution); fluorescein-conjugated goat antibodies to mouse IgG (Cappel Laboratories, Inc., West Chester, PA; 1/100 dilution); and rhodamine-conjugated goat antibodies to rabbit IgG (Miles Laboratories; 1/100 dilution). All antibodies were diluted in PBS containing 3% goat serum. After washing, cells were mounted in 90% glycerol/10% PBS and observed by phase-contrast or fluorescence microscopy. All steps were performed at room temperature.

**Cell-Cell Aggregation Analysis:** For routine assays, cells were removed from culture dishes by treatment with 0.002% trypsin in Ca\(^2+\)-Mg\(^2+\)-free medium containing 1 mM EDTA (22). Under these conditions, the function and molecular form of N-CAM are not impaired (reference 22 and unpublished observations). Aggregation assays were carried out as described (27) except that the total particle number was determined with a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). In some assays, cells were removed from the dish with 1 mM EDTA in Ca\(^2+\)-Mg\(^2+\)-free medium without added trypsin.

**Time-lapse Cinematography:** Cells were dispersed from 6-d retinae, plated in 35-mm tissue culture dishes (Costar, Data Packaging, Cambridge, MA).
MA), and transformed as described previously. After 1 d, fresh medium was added and the dishes were gassed with 10% CO₂ and then sealed with parafilm. The cultures were observed by phase-contrast microscopy (x160) in a 37°C constant temperature room for the following 3 d. Exposures were taken at one frame per minute using Eastman 16 mm Plus-X Negative film 7231.

Fluorescence-activated Cell Sorter Analysis: A single cell suspension of 6-d embryonic retinal cells was prepared as described above and then cultured in suspension for 4 h to regenerate surface N-CAM (27). The cultured cells were harvested, incubated 15 min at 4°C with monoclonal anti-N-CAM No. 1 ascites fluid (1:2 dilution) (14) to reduce cell-cell aggregation, and then divided into two aliquots. One aliquot was stained with rabbit antibodies to N-CAM (0.1 mg/ml in Dulbecco's modified Eagle's medium containing 10 mM HEPES, pH 7.4, and 2% fetal calf serum); the other was stained with nonimmune IgG. Each aliquot was washed, incubated with a 1:100 dilution of fluorescein-coupled goat antibodies to rabbit IgG, and washed again. Small aliquots of these stained populations were analyzed for fluorescence intensity. Analyses of fluorescence intensity and cell sorting were performed with a FACS-II fluorescence-activated cell sorter (Becton Dickinson Immunocytometry Systems, Oxnard, CA) equipped with a 5-W argon-ion laser (Spectra-Physics Inc., Mountain View, CA). Exciting light, at 488 nm, was provided at an incident power of 300 mW. Emitted light was detected after optical filtration with 520 and 530 nm "cut-on" filters (Ditrich Optics, Marlboro MA) placed sequentially. The following instrument parameters were routinely employed: photomultiplier gain, 770 V; fluorescence preamplifier gain, 4/0.9; light scatter preamplifier gain, 2/1.0. The instrument was standardized daily with 10-μm "full bright" fluorescent microspheres (Coulter Electronics).

The unfractionated and selected cell populations were plated in 96-well dishes (Linbro Chemical Co., Hamden, CT) at approximately 1 x 10⁵ cells/well and, after attachment, some cells were transformed by addition of SR-RSV-A in Ham's F10/5% calf serum, while other cells were maintained as untransformed controls. For analysis of N-CAM expression, the transformed cells were passage twice and stained with specific antibodies, as described above. There were insufficient cells to perform immunofluorescence staining on the untransformed control cells inasmuch as these cells do not multiply in culture; however, these cells appeared healthy and rapidly formed typical cell aggregates, implying the continued expression of normal levels of N-CAM.

RESULTS

Transformation of Neural Retinal Cells by RSV

Dispersed neural retinal cells from 6- or 7-d chicken embryos were transformed by SR-RSV-A as described by Calothy et al. (25). Morphological differences between infected and control cultures were apparent between 12 and 24 h after virus addition, and fully transformed cultures were obtained after ~4 d. The extent of transformation in these cultures was assessed by the appearance of the transforming gene product of RSV, pp60src. Normal and RSV-transformed retinal cells were cultured for 5 d, labeled with [3H]leucine for 16 h, extracted and immunoprecipitated with nonimmune serum or TBR serum that recognizes pp60src (31) (Fig. 1A). A 60,000-mol-wt protein corresponding to pp60src was specifically precipitated from the extracts of transformed cells (Fig. 1A, lane 4) but not from uninfected cells (Fig. 1A, lane 3). The amount of pp60src was also measured by assaying its tyrosine-kinase activity (31). Significant levels of pp60src tyrosine-kinase activity were detected in the transformed cells when extracts were assayed for the transfer of phosphate to the IgG heavy chain of TBR serum (Fig. 1B, lane 2). A small amount of kinase activity ascribed to the normal cellular homologue of pp60src was present in the normal cell extract (Fig. 1B, lane 1). No transfer of 32P-O⁻ was detected when extracts of transformed cells were incubated with nonimmune serum (Fig. 1B, lane 3).

Lineage of the Transformed Cell Population

A prerequisite for investigating the changes induced by RSV was establishing the lineage of the transformed cell population. When cultured, the neural retina from 6–7-d embryos consists of a large number of rapidly dividing, immature neuroepithelial cells (36, 37) which are N-CAM-positive (38), and a small number of N-CAM-negative (38) non-neuronal cells that appear to correspond to glial cells (36, 37, 39). Although the rate and extent of transformation by RSV suggested that the transformed cell population arose from the neuroepithelial cells, it remained possible that the population arose by transformation and rapid proliferation of the N-CAM-negative glial cells. It became particularly important to investigate this lineage when it was found that the transformed cells did not express N-CAM (see below).

To test whether N-CAM-positive neuroepithelial cells were being infected and transformed by RSV, we performed double-immunofluorescence staining experiments to detect any cells that simultaneously expressed N-CAM and pp60src shortly after infection. Freshly plated cells were incubated with SR-RSV-A for 2–3 d, then fixed, permeabilized, and stained with a mixture of rabbit anti-N-CAM and mouse monoclonal antibodies to pp60src. Fig. 2 shows two representative fields from one such double staining experiment in which the N-CAM staining was visualized by means of rhodamine-coupled goat antibodies against rabbit IgG and the pp60src staining was visualized by fluorescein-labeled goat antibodies against mouse IgG. In addition to a large number of normal cells that express N-CAM, both pairs of photographs also show cells that simultaneously expressed N-CAM and pp60src, which establishes that N-CAM-positive neuro-
epithelial cells were transformed by RSV. This conclusion was indicative, but was neither definitive nor quantitative. We therefore turned to a method for analyzing the rate of transformation of a purified population of neuroepithelial cells.

Transformation of a Purified Population of N-CAM-positive Cells by RSV

If the transformed cell population arose solely by transformation and differential proliferation of glial cells, then a population of retinal cells from which the glial cells had been removed would not be transformed by RSV. Conversely, if the transformed cells were derived by transformation of the neuronal cells, a population of retinal cells depleted of glial cells would transform in a fashion similar or identical to that of an unfractionated population. To assess these possibilities, we examined the rate of transformation of a pure population of N-CAM-positive cells selected by use of a fluorescence-activated cell sorter (Fig. 3 and Table I). Freshly trypsinized cells from 6-d retinae were cultured for 4 h in suspension to regenerate surface proteins and were then stained with nonimmune serum or rabbit anti-N-CAM, followed by fluorescein-labeled goat antibodies to rabbit IgG. Aliquots of these cells were analyzed for fluorescence intensity and light scattering on a Becton Dickinson FACS-II. A summary histogram showing the fluorescence profiles of cells stained by anti-N-CAM and control sera is reproduced in Fig. 3. Cells incubated with nonimmune serum displayed low levels of fluorescence. To select a population that would be virtually devoid of N-CAM-negative cells, a window for cell sorting was chosen with a high lower-limit of fluorescence (Fig. 3). The unfractiated and fractionated populations were examined microscopically and visually scored for N-CAM-positive and -negative cells. The results of one experiment, summarized in Table I, indicated that the fluorescence-activated cell sorter
purity in an 18.8-fold reduction in the number of N-CAM-negative cells.

The unfractionated and fractionated cells were plated in 96-well dishes, SR-RSV-A was added, and the cultures were monitored microscopically for morphological transformation. The rate of transformation was very similar (4.0 versus 4.0-4.5 d for complete transformation) in the unfractionated and purified N-CAM-positive populations, respectively (Table I). Both populations transformed only slightly more slowly than unstained, untreated cells transformed by the usual protocol. Subsequent immunofluorescence staining experiments (not shown) established that, like the unfractionated population (see below), transformation of the purified population of N-CAM-containing cells resulted in loss of N-CAM. These findings indicate that the vast majority of the transformed cell population was derived from N-CAM-positive neuroepithelial cells that were transformed by RSV. With this assurance, all subsequent experiments were performed with unfractionated cell populations.

Expression of N-CAM by RSV-transformed Neural Retinal Cells

The expression of N-CAM in normal and transformed neural cells was analyzed by several different methods. The amount and distribution of N-CAM on the surface of normal and RSV-transformed retinal cells was visualized by indirect immunofluorescence staining (Fig. 4). Freshly dispersed embryonic retinal cells rapidly collected into large, compact aggregates that were occasionally interconnected by neurite processes. Virtually all of these small, round cells were brightly stained with antibodies to N-CAM (Fig. 4B) as previously reported (38). These aggregates rested on top of a few flat cells that continued to divide in culture and apparently corresponded to retinal glial cells (36, 37, 39). These cells were not stained (not shown). In contrast to the normal cells, the RSV-transformed cells had spread into more flattened aggregates and many isolated cells were present between aggregates. Individual cells were much larger and were either round and highly refractile or flattened against the substratum and non-refractile (Fig. 4D). The morphologically transformed cells exhibited significantly decreased levels of N-CAM staining (Fig. 4E), while a few small, morphologically normal neurons, that were presumably postmitotic at the time of viral infection, retained high levels of N-CAM. Nonimmune sera gave very low levels of nonspecific staining (Fig. 4, C and F).

The form and content of N-CAM expressed by the normal and transformed retinal cultures were compared by specific immunoprecipitation. Normal and RSV-transformed cells that had been cultured for 5 d were labeled for 16 h with [3H]leucine, extracted with detergent, and immunoprecipitated with rabbit anti-N-CAM (Fig. 5A, lanes 1 and 2). Very small amounts of [3H]leucine-labeled N-CAM were immunoprecipitated from extracts of transformed neural retinal cells (Fig. 5A, lane 2, and Table II). In contrast, significant amounts of radiolabeled N-CAM were precipitated from the normal cell extract and migrated on SDS polyacrylamide gels as two discrete bands of Mr = 180,000 and 140,000 together with a small amount of material that migrated as a broad diffuse band with Mr = 150,000-200,000 (Fig. 5A, lane 1). Quantitative densitometric scanning of the autoradiograph (Table II) revealed that about six times as much radiolabeled N-CAM was precipitated from the normal extract as from the transformed extract.

We verified this difference in N-CAM content by immunoblotting unlabeled extracts of normal and transformed cells (Fig. 5B and Table II). Anti-N-CAM bound to components with Mr = 180,000 and 140,000 in the SDS gel fractionated extract of normal cells (Fig. 5B, lane 1) or of cells infected with the transformation-defective mutant td 107 (lane 2), whereas little anti-N-CAM bound to the fractionated extract of transformed cells (Fig. 5B, lane 3). Quantitative scanning of this autoradiograph indicated that the normal cell extract contained over 10 times as much N-CAM as did the transformed cell extract (Table II). These results indicate that
FIGURE 4 Indirect immunofluorescent visualization of N-CAM on normal and RSV-transformed retinal cells. Normal (A–C) or transformed (D–F) retinal cells from 6-d embryos were cultured for 5 d and then stained with rabbit antibodies to immunoaffinity-purified N-CAM (B and E) or nonimmune serum (C and F) as described in Materials and Methods. Cells were visualized by phase-contrast (A and D) or fluorescence (B, C, E, F) microscopy. Bar, 50 μm.

functional pp60^src is necessary for the change in N-CAM expression. Moreover, N-CAM expression was unaffected by the processes of infection and viral replication that occurred in the td 107-infected cells as determined by immunoprecipitation of viral structural components in parallel experiments (not shown). Preliminary studies using temperature-sensitive transformation-defective viruses also support this conclusion.

Evaluation of the Adhesiveness of Normal and Transformed Cells

Both the decrease in N-CAM content and the altered colony morphology of the RSV-transformed cells suggested that their adhesive interactions might have been altered by transformation. To test this possibility, monolayer cultures of normal or RSV-transformed neural retinal cells were dispersed under conditions that preserve substantial amounts of N-CAM on the cell surface (22). The normal cells aggregated rapidly and extensively (Fig. 6). This aggregation is inhibited ~90% by Fab' fragments of anti-N-CAM (not shown). In contrast, the cells transformed by RSV aggregated more slowly and to a much lesser extent (Fig. 6). Microscopic examination revealed that any aggregates that were present in the transformed cell sample consisted almost entirely of small, presumably untransformed cells; the large (transformed) cells were present as isolated, unaggregated cells.

This observation reflected the mixed character of the primary culture treated with RSV: although the culture consisted mainly of large transformed cells, there was an appreciable proportion of small, nontransformed cells. To enrich the transformed cell population, the culture was trypsinized and replated. Although the plating efficiency of the RSV-infected cells was reduced, after several days of growth the plate was covered with cells displaying either a large, flat, nonrefractile morphology or a large, round, highly refractile appearance. Very few small, round (nontransformed) cells were evident. Virtually all of these secondary RSV-transformed cells were positively stained by TBR serum but were not stained with antibodies to N-CAM (not shown).

Adhesion mediated by N-CAM is Ca²⁺ independent (22), and as expected, these secondary RSV-transformed cells did not aggregate in the absence of Ca²⁺ (Table III). We also tested whether the transformed cells could aggregate via a second, Ca²⁺-dependent, adhesion system (22, 40–43). In normal neuronal cells, aggregation due to this system can only be detected after inactivating the N-CAM system by treatment with trypsin in the presence of Ca²⁺. RSV-transformed cells prepared in this manner aggregated (30%) in the presence of Ca²⁺, but not its absence (Table III). This aggregation was not inhibited by antibodies to N-CAM, but was effectively reduced by antibodies (anti-CD-cell) selective for the Ca²⁺-dependent system. It was also possible, because of the reduction in N-CAM that occurs after RSV transformation, to test the Ca²⁺-dependent aggregation of these cells without any exogenous proteolytic treatment. When released from the dish
Figure 5  Effect of RSV replication and transformation on N-CAM expression. (A) The amount and molecular form of [3H]leucine-labeled N-CAM that was specifically immunoprecipitated from aliquots of the same extracts of normal (lane 1) and RSV-transformed (lane 2) cells described in Fig. 1. (B) Embryonic retinal cells were dispersed, plated, and incubated in the absence of virus (lane 1), incubated with a transformation-defective mutant (td 107) of RSV (lane 2), or with the transforming SR-RSV-A strain (lane 3). After 4 d, detergent extracts were prepared and fractionated on an 8.5% SDS polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with rabbit antibodies to affinity-purified N-CAM followed by 125I-protein A as described in Materials and Methods. N-CAM antigens, with molecular weights of 140,000 and 180,000, were visualized by autoradiography.

Table II  Quantification of N-CAM in Normal and RSV-transformed Retinal Cells

| Experiment* | Amount of N-CAM* | Ratio (normal/ lane 107 SR-RSV-A) | ND | 388 | 6.2 |
|-------------|------------------|----------------------------------|----|-----|----|
| Immunoprecipitation of labeled N-CAM | 2,415 | ND | 388 | 6.2 |
| Immunoblot of cell lysate | 7,102 | 6,430 | 676 | 10.5 |

* Data for the immunoprecipitation experiment are derived from Fig. 5A, lanes 1 and 2. Data for the immunoblot experiment are derived from Fig. 5B, lanes 1–3.

* ND, not determined.

Calculated as percent decrease in total particles during a 25-min incubation.

Table III  Aggregation of Secondary RSV-transformed Retinal Cells

| Method of preparation* | Ca2+ | Fab* | Aggregation |
|------------------------|------|------|-------------|
| 0.002% Trypsin/1 mM EDTA | – | Nonimmune | 0 |
| + Nonimmune | 0 |
| 0.02% Trypsin/10 mM CaCl2 | – | Nonimmune | 0 |
| + Nonimmune | 0 |
| + Anti-N-CAM | 30 |
| + Anti-CD-cell | 2 |
| + Anti-N-CAM | 28 |
| + Anti-CD-cell | 2 |
| + Nonimmune | 14 |
| + Anti-N-CAM | 49 |
| + Anti-CD-cell | 19 |

* Primary cultures of retinal cells that had been fully transformed by RSV were trypsinized, replated, and allowed to grow to confluence.

* Cells were removed from culture dishes by treatment for 15 min at 37°C under the indicated conditions.

* Cells were preincubated with 1.2 mg Fab' fragments of IgG prepared from nonimmune sera, specific anti-N-CAM antibodies, or antibodies to retinal cells (anti-CD-cell) that were prepared using conditions selective for the Ca2+-dependent adhesion system (22).

* Computed as percent decrease in total particles during a 25-min incubation.

It has previously been suggested (42) that the Ca2+-dependent aggregation system must be proteolytically activated. The results shown in Table III provide the first indication that the neuronal Ca2+-dependent system may function without exogenous proteolytic treatment.

Observations by Time-lapse Cinematography

To compare the transformation-induced alterations in cell morphology, adhesion, and motility more closely, the normal and RSV-transformed cells were observed by time-lapse cinematography. The retinal cells were first allowed to attach to culture dishes as a dispersed monolayer and then to collect into aggregates. For the next 2 d, control and RSV-transformed cells in sealed dishes were observed by time-lapse cinematography. In the control cultures, the cell aggregates were stable and relatively static. Although some motion was...
apparent, the neuronal cells remained in their original position in the aggregate, the overall shape and location of the aggregates did not change, and little cell division was observed. In contrast, flat cells in the culture showed very different behavior. They migrated actively over the surface of the dish, occasionally passing under clusters of neuronal cells. Rapid membrane changes, including ruffling and blebbing, occurred frequently in these flat cells.

In the transformed cultures several striking differences were observed by time-lapse cinematography. Although the neuronal cells initially formed aggregates, the aggregates progressively flattened out into a more nearly single cell layer, and individual cells eventually broke free of the aggregates. The associations between neuronal cells in aggregates became looser, and the position of individual cells relative to surrounding cells continually changed. Cells that had detached from an aggregate underwent changes in morphology: the cells spread on the surface, becoming elongated rather than round. The isolated cells also appeared to become larger. Cells with this morphology are evident in Fig. 4D. These cells occasionally detached from the surface and re-entered aggregates, but as transformation progressed, more of these cells left the aggregates and began to migrate over the substratum. Although it was not possible to follow individual cells over the whole course of infection, there appeared to be a continuity in the morphological changes inasmuch as many initially flattened cells were eventually observed to round up and appear highly refractile. These round cells were substantially larger than the original neuroepithelial cells, and this was the predominant morphology (Fig. 4D) in fully transformed cultures. The rounded cells showed intense surface blebbing activity and both the large flat and round cells were highly motile, moving underneath aggregates of neuronal cells and emerging at the far side, or migrating between other cells.

Antigenic Markers Expressed by the Transformed Cells

In terms of cell size, motility, and absence of N-CAM, the flat nonrefractile transformed cells were superficially similar to cultured glial cells. To characterize further the differentiation properties of these transformed cells, we examined two markers specific for neurons or glia. Monoclonal antibody A2B5 recognizes ganglioside G_{0,2} (44) which is expressed by neurons, but not retinal glial cells (29). In contrast, we have shown that antibodies to the 34,000-mol-wt tyrosine kinase substrate bind to cultured glial cells but do not stain cultured neurons (45). To test for expression of these antigens, normal and RSV-transformed retinal cells that had been cultured for 5 d were fixed, permeabilized (in the case of the 34,000-mol-wt antigen), and stained with either monoclonal antibody A2B5 or monoclonal antibodies to the 34,000-mol-wt tyrosine kinase substrate. After incubation with fluorescein-conjugated second antibodies, the cells were visualized by fluorescence microscopy. The results are shown in Figs. 7 and 8.

In culture, most normal retinal cells, but not glial cells,
Figure 8  Indirect immunofluorescent visualization of the 34,000-mol-wt tyrosine kinase substrate in normal and RSV-transformed retinal cells. Cultured normal (A and B) and transformed (C and D) retinal cells were fixed, permeabilized, and stained with monoclonal antibodies to the 34,000-mol-wt tyrosine kinase substrate followed by fluorescein-conjugated goat antibodies to mouse IgG. Cells were photographed under phase-contrast illumination (panels A and C) or fluorescence microscopy (panels B and D). Bar, 20 μm.

were stained by monoclonal antibody A2B5 (Fig. 7B) as previously described by Eisenbarth et al. (29). Strikingly, nearly all of the cells that were morphologically transformed were also unstained by monoclonal antibody A2B5 (Fig. 7E). Of the few cells that were positively stained, most had small round cell bodies and extensive processes, suggesting that they were residual untransformed cells. However, a small proportion of the positively stained cells was morphologically transformed (not shown). These cells may be incompletely transformed or may represent a small proportion of fully transformed cells that retain some neuronal characteristics. Very low levels of nonspecific staining were observed when the normal and transformed cells were incubated with an irrelevant monoclonal antibody (Fig. 7, C and F).

Specific antibodies to the 34,000-mol-wt tyrosine kinase substrate stained most of the transformed cells brightly (Fig. 8D) whereas normal neurons (Fig. 8B) were unstained, as previously reported (45). This result is in agreement with the work of Poirier et al. (46) who have previously shown that multiple passage RSV-transformed retinal cells contained the 34,000-mol-wt protein. Glial cells present in the culture of normal 6-d embryonic retina were positively stained (Fig. 8B) although not as brightly as glial cells derived from older embryos (45).
DISCUSSION

In the present study, we have found that embryonic retinal cells transformed by RSV undergo alterations in several processes of surface molecular expression and differentiation crucial to cell-cell interactions. A pivotal finding was that the transformed cells expressed significantly reduced amounts of N-CAM. The present experiments indicate that the alteration of N-CAM expression by RSV transformation occurs in normal cells taken directly from embryos and is not simply an artifact of established cell lines. Although the cells that were transformed were N-CAM-positive cells that ordinarily give rise primarily to neurons, the transformed cells shared several properties with normal glia.

To evaluate the changes induced by RSV, it was essential to establish the lineage of the transformed cell population. The retina of a 6-7-d chicken embryo consists primarily of N-CAM-positive, immature neuroepithelial cells but also contains a small number of glial cells, which do not express N-CAM (36-39). Because the transformed cells lacked N-CAM and displayed other characteristics similar to the glial cells, they could have arisen by transformation and differential proliferation of the N-CAM-negative glial cells. However, the evidence supported an alternative explanation: the transformed cells originated by morphological and biochemical alteration of the N-CAM-positive, immature neuronal cells. Conclusive evidence for this was provided by experiments in which a purified population of N-CAM-positive cells was found to transform at essentially the same rate and to the same extent as the population that contained glial cells. When fully transformed, this purified population of cells expressed low levels of N-CAM.

The loss of N-CAM in embryonic retinal cells transformed by RSV is in accord with similar observations on the effect of RSV transformation in established cell lines from rat cerebellum (24). In both cases, the reduction in N-CAM expression was reflected in a large decrease in the adhesiveness of the cells measured in vitro and, in the present study, in culture as well. As in the rat cell lines, the change in N-CAM expression in embryonic chicken neurons appears to occur more slowly than changes in other transformation parameters, supporting the suggestion (24) that the effect of pp60^src on N-CAM expression is most likely indirect. Together with the previous results (24), the present experiments suggest that the effect on N-CAM expression is a general one, in two senses: it occurs in neuronal cells from both retina and cerebellum, and it occurs in an avian as well as in rodent species. However, a complete understanding of the relationship between transformation and N-CAM expression will require further studies with a variety of cells transformed by chemical carcinogens as well as by viruses containing transforming genes other than src.

Transformation by RSV clearly appeared to alter the differentiation of the neuroepithelial cells. During normal development, the retinal neuroepithelial cells primarily differentiate into mature neurons, although at early stages these cells may also serve as precursors to the Müller glial cells. In contrast to mature neurons, both the transformed cells and cultured glial cells were found to be large, highly motile, and actively proliferating, and both expressed the 34,000-mol-wt tyrosine kinase substrate, low amounts of N-CAM, and low amounts of a ganglioside recognized by monoclonal antibody A2B5. Thus, while it would be premature to say that the transformed cells had the characteristics of glial cells, they did share several properties with these cells. These considerations suggest that many of the changes seen during RSV transformation are not caused directly by the RSV-transforming protein, but rather may be secondary consequences of a primary effect on the overall differentiation program of the cell. Similar global effects of RSV on cytodifferentiation have previously been observed in chondroblasts (47), pigmented epithelial cells (48, 49), and myoblasts (50, 51). These results also raise the possibility that the normal homologue of pp60^src, which is present at high levels in neural tissue (52), may play a role in the commitment of neuroepithelial cells to neuronal or glial differentiation pathways.

When RSV is injected intracranially in several species, the tumors that form are classified as gliomas (53). The similarities seen here between the transformed neuroepithelial cells and cultured glial cells provoked the conjecture that some of these tumors might actually be derived from neuronal precursor cells rather than glial cells. Roughly 50% of all spontaneous tumors that originate in neural tissue are classified histologically as gliomas (54), and it is possible that some of these tumors also may have arisen from neuroepithelial, rather than glial cells. Further characterization of neuronal precursor and glial cells transformed in vitro may suggest clinically useful criteria for more precise diagnosis of spontaneous neural tumors.

In addition to the decrease in surface N-CAM, another change likely to result in altered cell-cell interaction after RSV transformation was the increase in cell motility. Together, these observations may be relevant to some aspects of tumor cell metastasis. The loss of a major, tissue-specific cell-cell adhesion system such as N-CAM accompanied by an increase in motility might significantly enhance the probability that a tumor cell would detach from its normal tissue framework and invade surrounding tissue. After having detached from the original tumor, cells that penetrate the blood or lymph systems may eventually lodge in distant organs and proliferate there. The adhesive properties of the tumor cells may figure in these later steps as well. Clinical observations and studies of metastasizing cell lines have established that certain tumors and cell lines preferentially metastasize to particular tissues (reviewed in reference 55). The specificity of the cell adhesion molecules expressed by given transformed cells could be one factor that influences these patterns of metastasis (see also references 56 and 57).

In the absence of a major cell adhesion system such as N-CAM, the properties of secondary systems or new adhesive systems induced by the process of transformation might assume greater significance. In the experiments reported here, for example, the transformed retinal cells lost their ability to aggregate via the N-CAM system but retained the ability to aggregate via a Ca^{2+}-dependent system, which is clearly present on neurons, but has not yet been fully characterized at the molecular level (22, 40-43). Furthermore, in addition to N-CAM, normal neurons also express a molecule, called Ng-CAM, that appears to be involved in the adhesion between neurons and glia (58, 59). The effects of RSV transformation on this adhesion system have not yet been examined. In addition, it will be important to determine whether transformation causes changes in the interaction of neural cells with the extracellular matrix, such as those observed in non-neuronal cells (60). The recent identification of molecules involved in the substrate interactions of neurons (61, 62) may eventually allow a full analysis of the effects of transformation on the motility, cell-cell, and cell-substrate adhesions of neural
cells.

To relate all of these findings in vitro to events occurring in vivo, the changes in adhesiveness, motility, and differentiation that we have observed in transformed retinal cells must be evaluated with appropriate assays for each of the steps in tumor cell invasion and metastasis. Nonetheless, the sharp correlations observed in the present study provide some support for the hypothesis that specific changes in cell-cell adhesiveness mediated via the expression of cell adhesion molecules are a significant feature of the metastatic process.

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