to synthesize and secrete the interphotoreceptor retinoid-binding protein (IRBP), a glycoprotein known to as into cells of pigment epithelial character, while dibutyryl cyclic AMP (db-cAMP) supports the development of cells more similar to conventional neurons.

Materials and Methods. Y-79 cells were cultured as previously described. Briefly, cells in suspension culture were centrifuged, dissociated by pipetting after addition of 5 ml of culture medium, and diluted to a final density of $4 \times 10^4$ cells/ml. The culture medium consisted of Eagle's Minimum Essential Medium (MEM) with Earle's salts, supplemented with 2 mM glutamine ($\text{Gibco}$, Grand Island, NY), 5 ng/ml putrescine, 4 µg/ml sodium selenite, 5 ng/ml progesterone, as described by Bottensteine. Laminin (10 µg/ml) was added when appropriate. Two milliliter portions of the cell suspension were seeded in 35 mm tissue culture plastic dishes (for electron microscopy), or tissue culture plastic chamber slides (Miles Scientific, Naperville, IL) (for immunohistochemistry). Three days after plating, floating cells were removed by replacement of the culture medium with fresh serum-free medium without laminin, containing 2 mM sodium butyrate or 4 mM dibutyryl-cAMP (Sigma, St. Louis, MO). This medium was changed every 3 days thereafter, and the cells were tested on the 12th day of culture.

For transmission electron microscopy, cells were scraped from the dishes, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 hr at 23°C, postfixed in O₃O₄, and embedded in Maraglas 655 (Ladd Research Industries, Burlington, VT). Sections

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References

Fig. 1. A, Phase contrast picture of 12-day-old butyrate-treated Y-79 cells. Cells were plated with serum-free medium containing 10 μg/ml laminin. Three days after plating, the laminin was removed, and fresh defined medium containing 2 mM sodium butyrate was added and replaced every 3 days. Note that most cells have a flat polygonal appearance. Several cells bearing long neurite-like processes can be observed as well (arrowheads) (×570). B, Cells growing as above, but treated with 4 mM db-cAMP. These cells exhibit neuronal differentiation (×570).

were stained with uranyl acetate–lead citrate and viewed in a 400 Phillips electron microscope.

Immunohistochemistry was carried out by employing an indirect immunofluorescent technique, as well as the avidin-biotin-peroxidase complex (ABC) method. Cells grown in monolayers for 12 days on chamber slides were fixed in −20°C methanol for 4 min followed by 2 additional minutes in −20°C acetone. For immunofluorescence, after 3 washes with phosphate-buffered saline (PBS), the first antibodies were applied for 45 min at 23°C. The dilutions of the antibodies were as follows: Neuron specific enolase (NSE) (1:100), glial fibrillary acidic protein (GFAP) (1:100), 68K neurofilament protein (NF-68) (4 μg/ml), choline acetyltransferase (Ch-AT) (4 μg/ml), interphotoreceptor retinoid-binding protein (IRBP) (150 μg/ml), and rhodopsin (Rh) (10 μg/ml). The sections then were washed in PBS and covered with the second antibodies specific for each first antibody and linked to fluorescein isothiocyanate (diluted 1:40, for 30 min) (Accurate, Westbury, NY). Cells were then rinsed with PBS, mounted in 50% glycerol, and viewed under a Zeiss standard microscope (Baltimore Instruments, Baltimore MD) equipped with epifluorescence. For the ABC method, cells were incubated in 30% H2O2 in methanol for 30 min after fixation, to block endogenous peroxidase activity. Subsequently, cells were washed in PBS and non-specific binding sites were blocked with 10% egg albumin in PBS for 30 min. After a brief wash in PBS, the first antibodies were then applied at the following concentrations: NSE (1:500), GFAP (1:200), S-100 (1:300), NF-68 (4 μg/ml), Ch-AT (4 μg/ml), IRBP (100 μg/ml), and Rh (10 μg/ml). After 1 hr at 23°C for all antibodies, except for NF-68 (1 hr at 37°C), the primary antibodies were washed off, and biotinylated second antibodies (1:100) were applied for 30 min at 23°C (Vector Lab, Burlingame, CA). Subsequently, cells were rinsed with PBS, the ABC applied for 30 min at 23°C, the reaction developed with 0.05% diaminobenzidine tetrahydrochloride in PBS with 0.01% H2O2 (Sigma Chemical Co, St. Louis, MO), and mounted in permount. The NSE and GFAP antibodies have been characterized elsewhere. The affinity purified IRBP antibody was as described previously, and the monoclonal anti-bovine rhodopsin antibody was a kind gift from Dr. P. Hargrave. The monoclonal antibodies against Ch-AT and NF-68 were purchased from Vector Labs. Both normal human brain and spinal cord sections were used as positive and negative controls for the commercially obtained antibodies to verify both antibody specificity and reactivity in our present experiments.

Results. In the absence of laminin, plating Y-79 cells in uncoated tissue culture plastic dishes results in loose adhesion of less than 1% of the cells 3 days after seeding when either serum or serum-free culture medium is used. These cells show little tendency to differentiate and detach easily with each change of the culture medium. The inclusion of 10 μg/ml laminin in the defined, seeding medium, however, induces the attachment of 20–30% of cells. These cells, in the presence of 2 mM butyrate, continue to proliferate and differentiate into cells of flat, epithelial appearance which organize into clusters (Fig. 1A). Occasional cells bearing long neurite-like processes are also observed. In contrast, treatment of these cultures with 4 mM db-cAMP results in extensive neuronal-like differentiation of most (>90%) of the cells (Fig. 1B).

Transmission electron microscopy (TEM) of flat cells reveals that approximately 20% of the butyrate-treated cells bear cytoplasmic structures resembling melanosomes or premelanosomes (Fig. 2A, arrows). Higher
power shows that these structures are membrane-bound and, in the main, have a striated substructure (Fig. 2A, inset). Less than 1% tumor cells showed sparse neurosecretory granules in their cytoplasm. On the other hand, few, if any, melanosome-like organelles are observed in db-cAMP-treated cultures; rather, TEM of these cells confirms the differentiation of a subpopulation of them into a neuronal-like phenotype. These cells are more characteristic of conventional neurons, since they contain neurosecretory granules. These granules are seen dispersed in the cytoplasm, in processes, or in the apical portions of cytoplasm facing intercellular spaces in association with microvillous projections (Fig. 2B).

Immunofluorescent staining and staining with the avidin-biotin method of 12-day-old cells for various neuronal and glial markers were also employed (Table 1). Butyrate-treated cells showed the presence of glial fibrillary acidic protein (GFAP) (10% of cells), S-100 (40%), neuron-specific enolase (NSE) (5%), and 68K neurofilament protein (NF-68) (10-20%). Staining with antibodies against rhodopsin (Rh), choline acetyltransferase (Ch-AT), and interphotoreceptor retinoid-binding protein (IRBP) revealed no significant im-
The percentage of treated cells, and that rhodopsin is absent in both butyrate- and cyclic AMP-treated cells indicates that the present laminin-containing system does not support differentiation towards photoreceptor cells, as in our previous study in which butyrate treatment markedly induced IRBP (i.e., photoreceptor-like differentiation) in cultured Y-79 cells. The role of laminin in this process deserves further study.

Our results thus offer a new system for studying the differentiation of retinoblastoma and, possibly, other primitive neural tumors in vitro. Moreover, Y-79 cell differentiation can also be used as a model for studying normal and abnormal embryonic development of the retina and the central nervous system, since it parallels neural tube development at a very early stage, finally yielding all the major cell classes observed in the normal adult retina.

Key words: retinoblastoma, laminin, melanosome, differentiation, neuron, pigment epithelium, butyrate, cyclic AMP

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References


Table 1. Presence of neuronal and glial markers in Y-79 retinoblastoma cells

<table>
<thead>
<tr>
<th>Cellular Condition</th>
<th>NSE</th>
<th>GFAP</th>
<th>S-100</th>
<th>NF-68</th>
<th>Ch-AT</th>
<th>Rh</th>
<th>IRBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN + butyrate (2 mM)</td>
<td>+ (5%)</td>
<td>+ (10%)</td>
<td>+ (40%)</td>
<td>+ (10–20%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LN + db-cAMP (2 mM)</td>
<td>+ (10%)</td>
<td>+ (5–10%)</td>
<td>+ (5%)</td>
<td>+ (30%)</td>
<td>– (1–5%)</td>
<td>+ (1–5%)</td>
<td>–</td>
</tr>
</tbody>
</table>

For detection of various markers by immunohistochemistry, an indirect immunofluorescent technique, as well as the avidin-biotin-peroxidase complex (ABC) method, were employed. Cells were grown for 12 days prior to testing, as described in Figure 1 and fixed in −20°C methanol for 4 min, followed by 2 min in −20°C acetone. The numbers in parentheses represent the percentages of positive cells in each condition. NSE = neuron specific enolase; GFAP = glial fibrillary acidic protein; NF-68 = 68 kilodalton neurofilament protein; Ch-AT = choline acetyltransferase; Rh = Rhodopsin; IRBP = interphotoreceptor retinoid-binding protein; LN = laminin.

The fact that melanogenesis is very rarely observed in retinoblastomas in vivo in conjunction with the formation of pigment granules in vitro may indicate that these cells carry the genetic message, but lack the appropriate phenotype in vivo, as it has been reported in a case of an amelanotic malignant melanoma. It thus appears that retinoblastoma may involve not only the inner portion of the optic vesicle, which gives rise to neuronal and glial cells of the human retina, but also the outer layer, which provides the retinal pigment epithelium. Thus, the origin of retinoblastoma may be from an embryonic retinoblast arising before the formation of the outer and inner layers of the optic cup. In culture, these tumors have the potential of partial differentiation into various cell types, which varyably express differentiated characteristics, depending on substratum, treatment, etc (Table 1). The fact that IRBP, for example, is present only in a very small percentage of treated cells, and that rhodopsin is absent in both butyrate- and cyclic AMP-treated cells indicates that the present laminin-containing system does not support differentiation towards photoreceptor cells, as in our previous study in which butyrate treatment markedly induced IRBP (i.e., photoreceptor-like differentiation) in cultured Y-79 cells. The role of laminin in this process deserves further study.

Immunoferritin Localization of Actin in Retinal Photoreceptors

Michael H. Chaitin* and Deon Bok

The contractile protein actin was recently localized to the distal portion of the connecting cilium in frog photoreceptors (Chaitin et al J Cell Biol 99:239–247, 1984). This is the site where the ciliary plasma membrane evaginates to form new outer segment disks (Steinberg et al J Comp Neurol 190:501–518, 1980). In the present study, aldehyde fixed mammalian retinas were embedded in Lowicryl K4M, and thin tissue sections were reacted with antiactin antibodies using indirect immunoferritin labeling. Utilizing this technique, actin has been localized to the distal cilium in rat, cow, monkey, and human photoreceptors. These results provide additional evidence that an actin mediated contractile mechanism may regulate outer segment disk morphogenesis in vertebrate photoreceptors. As previously noted in frog retina, antiactin also labeled the bundle of filaments within photoreceptor calycal processes, and this label extended into the inner segment, subjacent to the plasma membrane. Within the inner segment, however, the striated rootlet was unlabeled. Invest Ophthalmol Vis Sci 27:1764–1767, 1986

The photoreceptor outer segment (OS) is a specialized organelle which arises from a modified cilium. It consists of a stack of membranous disks, within which the visual pigments exist as integral membrane proteins. The OS membrane system has been shown to undergo a continuing renewal whereby new disks are added at the base of the OS and older disks are intermittently shed from the tip.1 This renewal process enables the OS to maintain its molecular integrity, as well as a relatively constant length, throughout life.

In a recent immunocytochemical study, actin was localized to the distal portion of the connecting cilium (CC) in frog photoreceptors.2 Since this is the site where the CC plasma membrane evaginates to form new OS disks,3 the result suggested that an actin mediated contractile mechanism may underlie OS disk morphogenesis. The purpose of the current study was to determine if actin is also concentrated in the distal CC of other vertebrate photoreceptors. For this study, aldehyde fixed mammalian retinas were embedded in Lowicryl K4M,4 and thin tissue sections were reacted with antiactin antibodies using indirect immunoferritin labeling.

Also of interest in this study were two filamentous structures within the photoreceptor inner segment. These included the striated rootlet, as well as the filament bundles which extend from calycal processes.

Materials and Methods. Tissue: Eyes were enucleated from a Fischer rat and from a Rhesus monkey that were sacrificed during the late morning hours. A cow eye was obtained during the morning from a local slaughterhouse, and a human eye was received through the Jules Stein Eye Institute donor eye program from an individual who had expired in the early morning hours. After removal of the anterior segments and the vitreous, each eyecup was put into fixative. This investigation adhered to the ARVO Resolution on the Use of Animals in Research.

Tissue processing: In the previous study of actin in photoreceptors,7 retinas were embedded in aldehyde cross-linked bovine serum albumin. For the current study, Lowicryl K4M4 has been used. With Lowicryl embedded tissue, the cellular fine structure can be much better visualized, and good preservation of protein antigenicity has been reported.4

Eyecups were fixed either at room temperature, or at 4°C for 2 hr in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4. The tissue was subsequently rinsed with 0.1 M sodium cacodylate, pH 7.4, and then placed in a storage fixative of 2% paraformaldehyde, 0.1 M sodium cacodylate, pH 7.4 at 4°C for several days before being processed further. Preliminary experiments indicated that the latter step led to increased antigenicity of actin within the tissue.

For immunocytochemistry, tissue strips were rinsed with buffer, and then dehydrated in a series of dimethyl formamide (DMF) washes and embedded in Lowicryl K4M (Polysciences Inc., Warrington, PA) as follows: 50% DMF (30 min), 75% DMF (30 min), 90% DMF (60 min), 2:1 DMF:Lowicryl (60 min), 1:1 DMF:Low-