Expression of Cone-like Properties by Chick Embryo Neural Retina Cells in Glial-free Monolayer Cultures

RUBEN ADLER, JAMES D. LINDSEY, and CYNTHIA L. ELSNER
The Michael M. Wynn Center for the Study of Retinal Degenerations, The Wilmer Ophthalmological Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT   We report here that cells present in embryonic chick retinal monolayer cultures express differentiated properties characteristic of chick cones developing in vivo. Cell suspensions from 8-d chick embryo retina (a stage when photoreceptor differentiation has not yet started) were cultured for up to 7 d in low density, glial-free monolayers. Under these conditions, monopolar cells represent ~40% of the total number of process-bearing neurons. After 6 d in vitro, most of these monopolar cells showed morphological features reminiscent of developing chick cones. These features could be detected with phase-contrast microscopy, lectin cytochemistry, and transmission and scanning electron microscopy. Characteristic cone traits expressed by cultured monopolar cells included the following: (a) a highly polarized organization; (b) a single, short, usually unbranched neurite; (c) the polarized position of the nucleus close to the origin of the neurite; (d) characteristic cone inner segment features such as abundant free ribosomes, a polarized Golgi apparatus, a cluster of mitochondria distal to the nucleus, a big, membrane-bound, pigment-containing vacuole reminiscent of the "lipid droplet" characteristic of chick cones, and at least in some cases, a well-developed paraboloid; (e) the presence of a complex of apical differentiations including abundant microvilli and in some cases also a cilium-like process; and (f) the staining of the apical region of the cell with peanut lectin, which has been shown to be selective for chick embryo cones (Blanks, J. C., and L. V. Johnson, 1983, J. Comp. Neurol., 221:31–41; and Blanks, J. C., and L. V. Johnson, 1984, Invest. Opthalmol. Visual Sci., 25:546–557). This pattern of differentiation achieved by 8-d chick retina cells after 6 d in vitro is similar to that shown by 14-d-old chick embryo cones in vivo. Outer segments are not present at this stage of development either in vivo or in vitro. This experimental system is now being used to search for cellular and molecular signals controlling survival and differentiation of cone cells.

One of the dominant principles in contemporary neurobiology is that the microenvironment surrounding the neurons controls their survival and differentiation. Deprivation experiments in vivo, for example, have demonstrated that neuronal pre- and postsynaptic connections are essential for neuronal survival and maturation (i.e., references 7, 8, and 12). In vitro studies, on the other hand, have been instrumental in demonstrating the existence of survival-promoting and/or differentiation-promoting macromolecular agents (for review, see references 2 and 15). The peripheral nervous system has been considerably more amenable than the central nervous system for the investigation of these molecules.

There is almost no information regarding regulatory factors controlling survival and differentiation of retinal photoreceptors. It is known, for example, that the retinal pigment epithelium can influence photoreceptor differentiation (10) and that genetically caused defects in the pigment epithelium can lead to photoreceptor degeneration (13). However, there is essentially no information regarding the molecular bases of these effects. In other tissues such as chick ciliary ganglion, factors regulating differentiation of the neurons have been characterized using monolayer cell cultures of the target cells (3, 15). Hence, deficits in our knowledge with regard to photoreceptors can be largely explained by the unavailability of adequate monolayer culture systems for this cell type. We described recently a protocol for the generation of glial-free, sparse, purified monolayers of chick embryo retina neurons (1). Monopolar cells were the most abundant cell type in the cultures (11). In the work reported here, phase-contrast microscopy, lectin cytochemistry, and scanning and transmission electron microscopy have been used to demonstrate that a vast majority of these monopolar cells exhibit many properties characteristic of developing chick cone cells.

MATERIALS AND METHODS

ical Co. (St. Louis, MO; linoleic acid-albumin; polyornithine, α-methylman-noside, D (+) galactose, glutaraldehyde); Polysciences Inc. (Warrington, PA; Gill's hematoxylin, eosin-light green, thiocarbohydrizide); Vector Laboratories, Inc. (Burlingame, CA; fluorescent lectins); J. T. Baker Chemical Co. (Phillis-burg, NJ; paraformaldehyde, sucrose); EM Science (Gibbstown, NJ; osmium tetroxide, cacodylate); Polaron Instruments Inc. (Hatfield, PA; Epon 812); and Ted Pella, Inc. (Tustin, CA; Araldite).

Cell Culture Techniques: Most aspects of the cell culture techniques used have already been described in detail (1, 4). Retina cells were obtained from 8-d White Leghorn chick embryos. At this stage, most neuronal types including photoreceptors are already postmitotic but photoreceptor differentia-tion has essentially not yet started (for review see reference 9). Neural retinas free of contamination from pigment epithelial or other cells were dissociated after brief trypsinization, and then suspended in culture medium (medium 199 supplemented with 10% fetal calf serum and 110 μg/ml of a linoleic acid-BSA preparation). Cell yields were 50 × 10^6 cells per retina. The cells were diluted to 300,000 cells/ml and 2 ml of this suspension was seeded in 35-mm dishes treated with polyornithine (1). Sometimes, the polyornithine-treated dishes were also treated with Schwannoma-conditioned medium which contains the neurite-promoting factor PNPF (1) before seeding. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air, examined daily using phase-contrast microscopy, and cultured for up to 7 d.

Light and Electron Microscopy: For light microscopical observa-tions, the cultures were fixed with 2% glutaraldehyde in PBS and stained with hematoxylin and eosin as described (1). For scanning electron microscopy, cultures grown in lined dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) were rinsed five times in Hanks' balanced salt solution and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 22°C. After three rinses in cacodylate buffer containing 10% (wt/vol) sucrose, the cells were postfixed for 1 h with 1% osmium tetroxide in 0.05 M cacodylate buffer containing 10% sucrose, rinsed in the same buffer, and treated for 30 min with 0.5% aqueous thiocarbohydrizide, followed by buffer rinses and a second fixation step in 1% osmium tetroxide. After dehydration in graded ethanols and critical-point drying, the specimens were coated with 40 nm of gold-palladium, in some cases preceded by a carbon layer. For transmission electron microscopy, cultures were fixed with 2% paraformaldehyde and 2% glutaral-dehyde in 0.15 M cacodylate buffer, pH 7.4. Fixation was carried out for 20 min at 22°C followed by 40 min at 4°C. The cells were rinsed in cold buffer and then postfixed for 7 min with cold 1% buffered osmium tetroxide. The cells were then dehydrated and embedded in Epon-Araldite. Ultrathin sections were gridstained with uranyl acetate and lead citrate before examination.

Lectin Cytochemistry: Cultures to be stained with fluorescent lectins were washed twice in HEPES-buffered Hank's balanced salt solution and incubated for 15–30 min at room temperature with 50–200 μg/ml of either rhodamine-labeled peanut lectin, or fluorescein isothiocyanate–labeled concan-avalin A, or a combination of the two. Other cultures were incubated in lectins in the presence of 0.1 M concentrations of the specific sugars α-methylman-noside (for concanavalin A) and β-galactose (for peanut lectin). At the end of the lectin incubation, the dishes were rinsed twice for 5 min in Hank's balanced salt solution and fixed for 30–45 min in 4% paraformaldehyde in PBS. The cultures were rinsed overnight in PBS and mounted with 95% glycerol in PBS.

Photography: Fluorescent photomicrographs were obtained with Ko-dak Tri-X film exposed for 45 s. The film was pushed to 800 ASA during development.

RESULTS

As previously described, six different neuronal subcategories can be recognized in glia-free retinal monolayers (11). After 6 d in vitro, our cultures contained approximately 100,000 neurons, and 20% of these cells expressed differentiated prop-erties typical of developing chick cones. Cone-like monopolar cells appeared highly polarized, with a short, single neurite frequently showing a growth cone with abundant filopodial processes (Fig. 1). In the description that follows, different regions of the cell body will be referred to as "proximal" or "distal" using the origin of the neurite as a reference point. The cell nucleus occupies almost completely the region of the perikaryon closer to the origin of the neuritic process. Under the phase-contrast microscope, the cytoplasm distal to the nucleus showed attributes typical of a cone inner segment. For example, many of these monopolar cells showed a single,
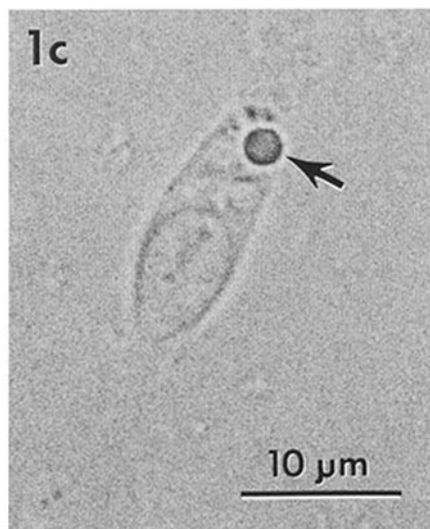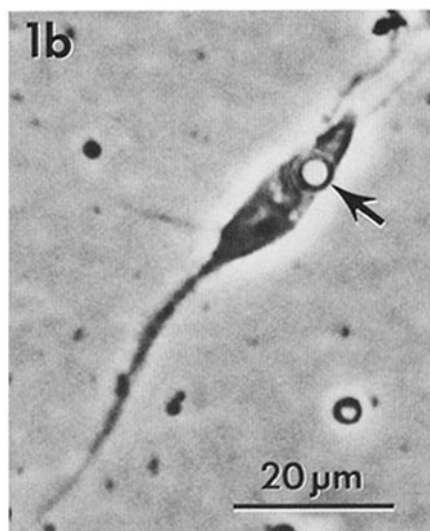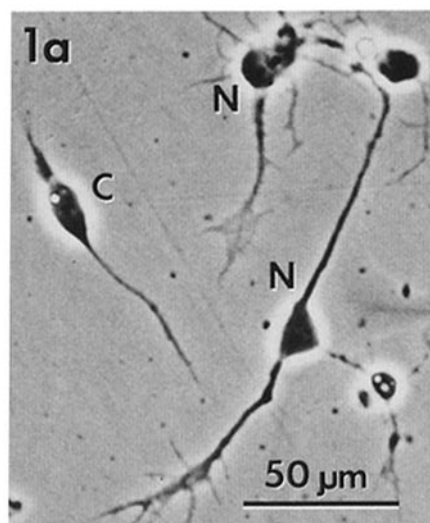

FIGURE 1   Retinal cells expressing differentiated characteristics in culture. (a) Phase-contrast microscopy image of a cone-like cell (C) and two multipolar retinal neurons (N). 6-d culture; × 410. (b) Enlarged phase-contrast microscopy image of a cone-like cell illus-trating the characteristic lipid droplet (arrow). 6-d culture; × 1,100. (c) Bright-field microscopy image of a cone-like cell illustrating the pigmented lipid droplet (arrow). 7-d culture; × 2,100.
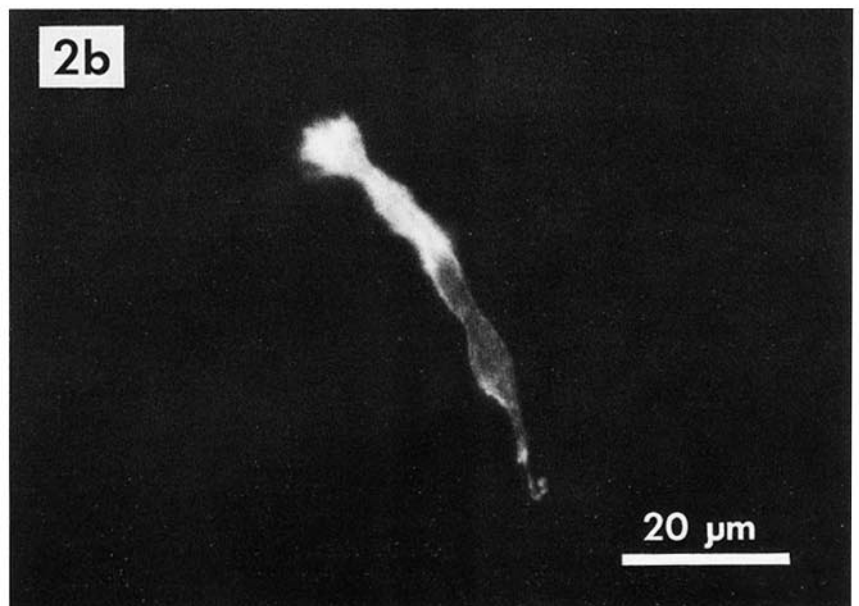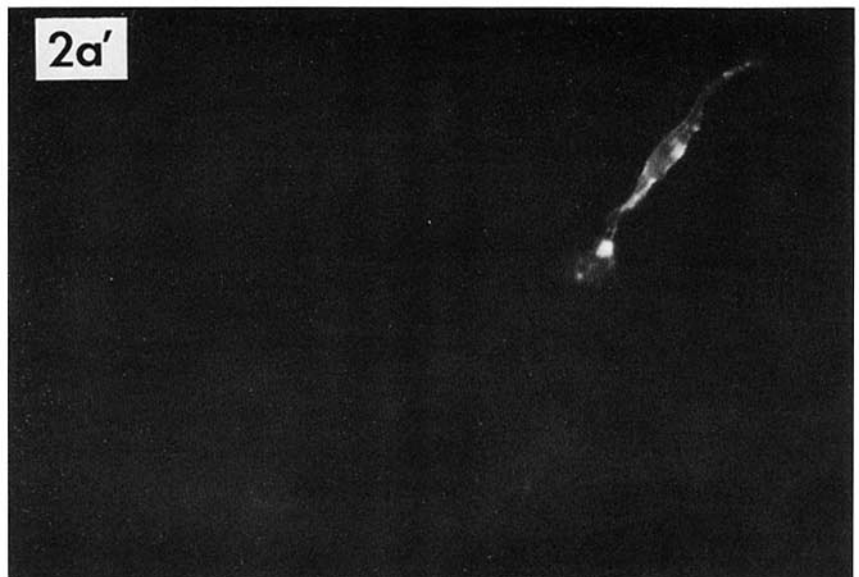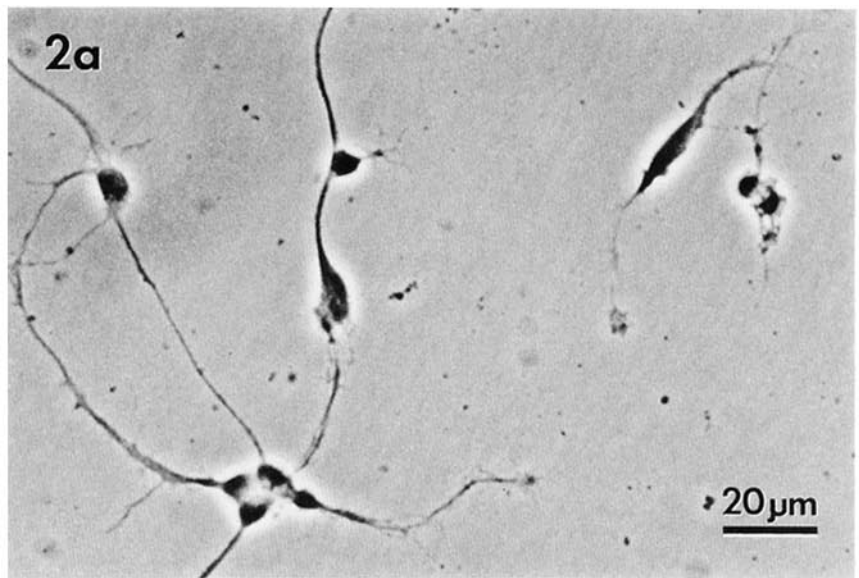
FIGURE 2 Cone-like cells cytochemically stained with rhodamine-labeled peanut lectin. (a) Phase-contrast image of a 6-d culture illustrating both cone-like cells and multipolar neurons within the field. (a') Fluorescence image of the same field illustrating that peanut lectin only binds to the cone-like cells. (b) Enlargement of a cone-like cell demonstrating, by fluorescence microscopy, peanut lectin staining localized largely to the "inner segment" region and its associated apical membranous expansion. 6-d culture. (a and a') × 630; (b) × 1,100.

FIGURE 3 Scanning electron micrographs illustrating the different morphologies of multipolar retinal neurons (a) and cone-like cells (b). Note the apical membranous expansion of the cone-like cells with numerous microvilli (arrowhead in b). Sometimes a stout process suggestive of a cilium arose from the apical region (arrowhead in inset). (a and b) 6-d culture. (a) × 1,300; (b) × 2,100. (Inset) 7-d culture; × 3,000.
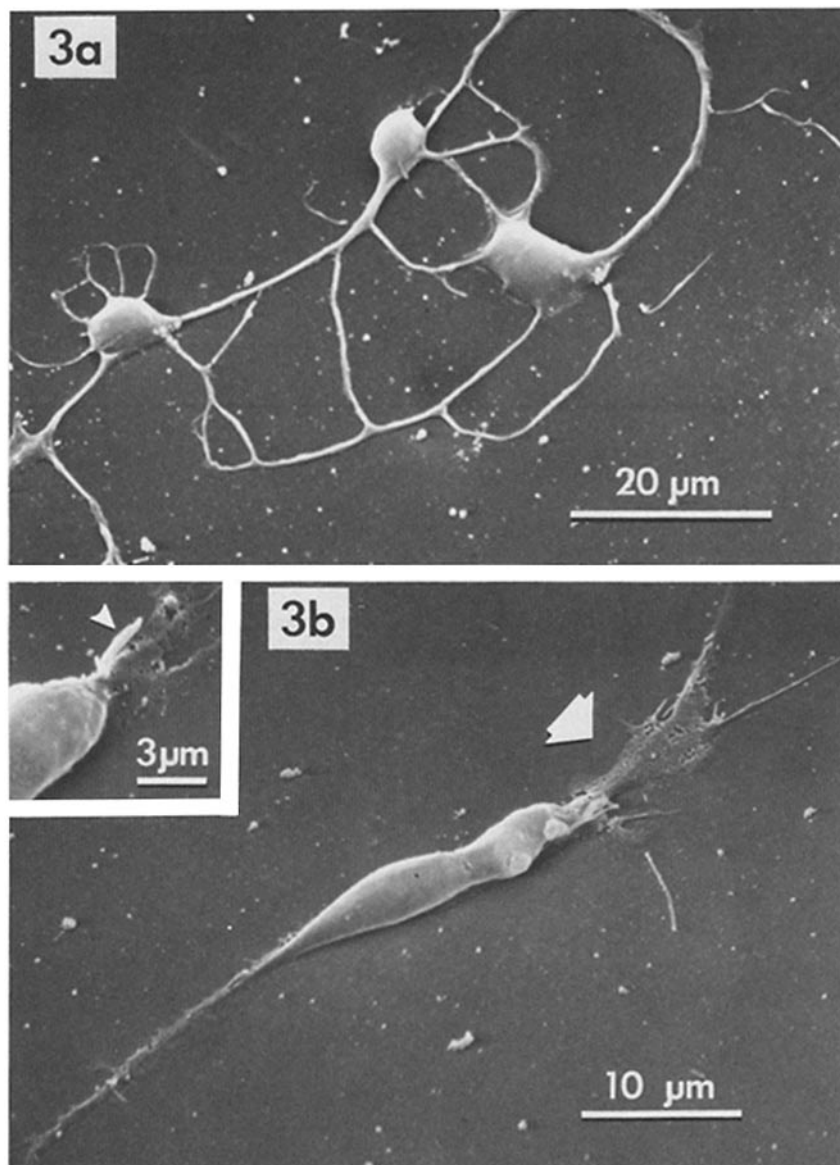
refractile vacuole reminiscent of the lipid droplet characteristic of chick cones (Fig. 1). In unstained cultures examined with bright-field microscopy, many of these lipid droplets showed conspicuous pigmentation (Fig. 1c). This pigment was yellow-green in all cases observed. These vacuoles were not evident in other types of process-bearing neurons (Fig. 1). The "inner segment" region also showed an accumulation of phase-dark bodies resembling mitochondria (see below). No evidence of outer segment development was observed under the phase-contrast microscope. In most cone-like cells the distalmost region of the "inner segment" appeared to taper into a thin, narrow membranous expansion (Fig. 1). This structure will be described in more detail in the following subsections. The degree of differentiation observed by phase-contrast microscopy did not appear to change when cultures were allowed to develop for an additional 5 d.

In the chick retina in vivo most neuronal types can be stained with concanavalin A, whereas peanut lectin is specific for cone cells (5, 6). A similar lectin staining pattern was observed in the retinal monolayer cultures. Most of the neuronal elements present in chick retina monolayers, including the cone-like cells, were brightly stained with fluoroscein

isothiocyanate–labeled concanavalin A (not shown). This was blocked by co-incubation with α-methylmannoside. Cone-like cells, however, were the only cells in the cultures that could be stained with rhodamine-linked peanut lectin (Fig. 2a). The cone neurite and nuclear region showed only background level of staining. Bright fluorescence, however, was seen in the inner segment as well as in its distal membranous expansion (Fig. 2b). In fact, this expansion was more easily visualized in lectin-stained preparations than in unstained cultures examined with phase-contrast microscopy. Control preparations, in which the peanut lectin treatment was done in the presence of β-galactose, showed no staining of any cell structures.

Fig. 3 shows a low magnification scanning electron microscopic picture illustrating the differences in structure between cone-like cells and other neuronal types. Most of the multipolar process-bearing cells show several thin neurites with abundant ramifications and no membranous expansions other than neurite growth cones. Cone-like cells, in contrast, appear highly polarized, and showed a characteristic structure in the distal portion of the inner segment. A membranous expansion which can be seen in this region probably corre-
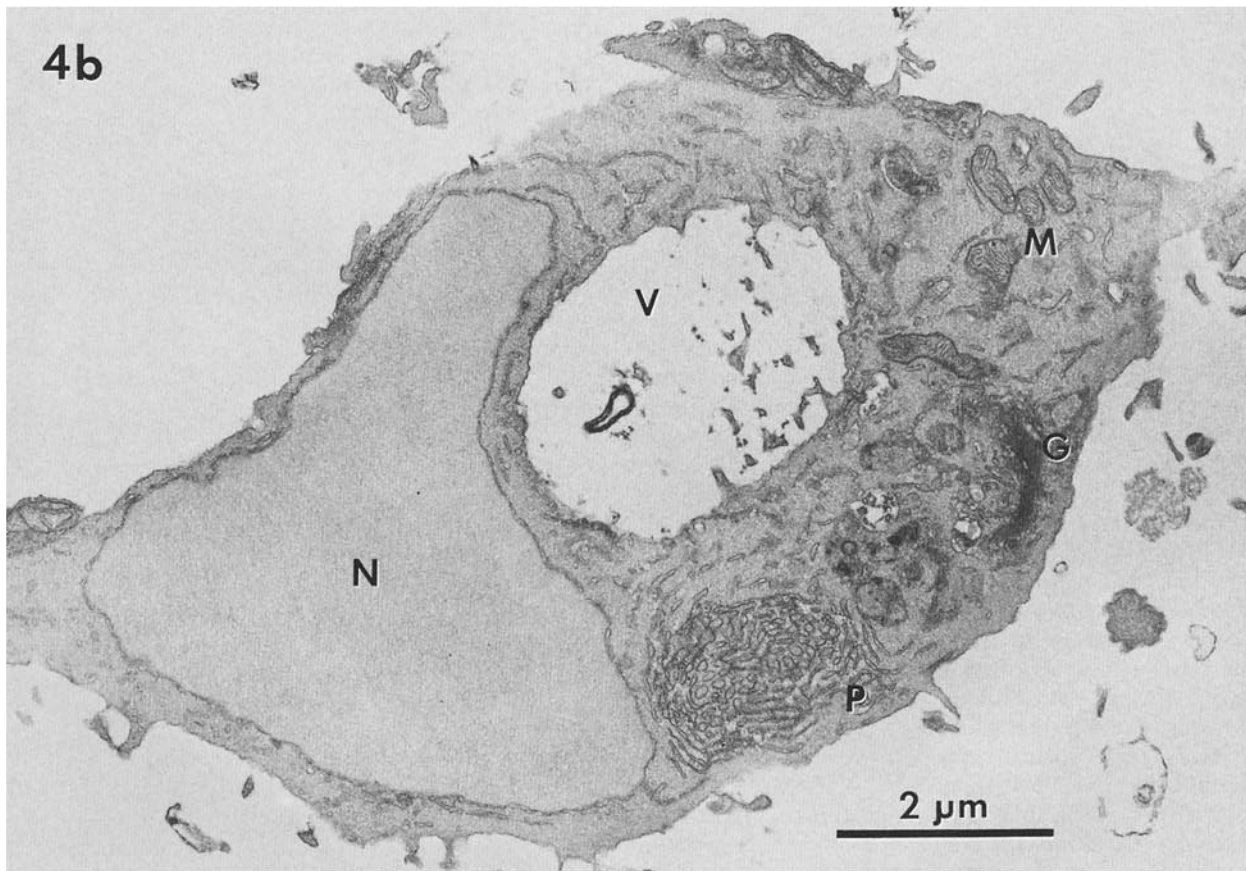
FIGURE 4   Transmission electron micrographs of 6-d cone-like cells illustrating the polarized positioning of the nuclei (N) and mitochondria (M), as well as the large vacuole (V) corresponding to the lipid droplet. Some cells (b) also contained Golgi apparatus (G) and a parabaloid (P). (a) × 10,700; (b) × 14,200.

sponds to the peanut lectin-stained structure described with fluorescence microscopy. This distal region also shows many thin, long microvilli-like processes (Fig. 3b). In addition, some cells show a thick distal process tentatively identified as a cilium (Fig. 3b, inset). No evidence of structures suggestive of outer segment formation were observed.

As shown in Fig. 4, in transmission electron micrographs the perikaryon of cone-like cells showed a polarized nucleus that exhibits a largely dispersed pattern of heterochromatin. Some small clumps of heterochromatin were regularly seen near the nuclear envelope and occasionally throughout the nucleoplasm. The distal region of the cell body showed abundant free ribosomes. The inner segment region showed a big vacuole corresponding to the lipid droplet described with light microscopy. This vacuole is membrane bound and is largely devoid of cell components that are preserved by conventional transmission electron microscopic preparative procedures. The inner segment region also showed a large accumulation of mitochondria neighboring the membrane-bound big vacuole. A conspicuous but relatively immature Golgi complex was also occasionally seen in this region. Some cultured cone-like cells showed, in addition, a fairly well-developed paraboloid consisting of smooth endoplasmic reticulum membranes accompanied by particles that probably correspond to glycogen deposits (Fig. 4b). Cilia and/or basal bodies were also occasionally observed (not shown). No evidence of outer segment organization has been observed in these preparations.

DISCUSSION

It seems reasonable to propose that many of the monopolar cells present in glial-free retina neuronal monolayers are in fact developing cones. The discussion of this identification must consider that the embryonic chick retina can be dissected out of the embryo free of contaminations with any other tissue including pigment epithelium, connective tissue, and blood vessels. Thus, these cells types are not likely to be found in the present cultures. Moreover, previous work from this laboratory showed that glial cells failed to develop in chick embryo retina monolayers cultured at low densities on highly adhesive substrata such as polyornithine (4). This study also showed that practically 100% of the process-bearing cells present in these cultures could be stained with tetanus toxin, corroborating their neuronal identity. The issue, then, is whether monopolar neurons present in the cultures can be recognized as cones rather than as any other neuronal type.

The evidence presented here indicates that many of these cells do indeed express phenotypic features typical of a developing chick cone. This pattern of differentiation observed in vitro must be compared with the developmental timetable for cone and other retina cells in vivo. The description that follows is based on thymidine incorporation and electron microscopic studies (reviewed in reference 9). At 8 d of development, most retinal neurons including photoreceptors are already postmitotic (i.e., have already been "born"). Some cone cell differentiation can be observed between embryonic days 8 and 14 of in vivo development, a period equivalent to the time covered by our in vitro studies. During that time, however, cone cell differentiation can be recognized both at the synaptic end and in the inner segment, but no indication of outer segment development had been reported before embryonic day 15 (14). Inner segment development between days 8 and 14 includes the appearance of a big and conspicuous vacuole ("lipid droplet"), the development of a polarized Golgi apparatus, the accumulation of mitochondria in the

elipsoidal region, and the presence of a cilium at the apical end of the cells. Selective staining with peanut lectin can also be recognized in the inner segment region (5, 6). It is very striking that cells that have been separated from their normal environment and allowed to develop in dispersed culture can express a pattern of phenotypic differentiation so similar to the one they show in the in vivo situation, where they develop in close association with other retina neurons, glial cells, and the pigmented epithelium. Also noteworthy is that the culture medium used in these experiments was supplemented with 10% fetal calf serum—a complicating factor in a culture system. The fact that retinal cell responses to the neurite-promoting factor PNPF are inhibited by serum (1), for example, may perhaps explain our failure to observe any response of cultured cone cells to this factor (data not shown). Work is now in progress in this laboratory to develop a chemically defined medium capable of supporting similar cone maturation.

We have not observed any evidence of outer segment differentiation in cultured retinal cones. Rather, the cells show a substratum-bound membranous expansion which can be stained with peanut lectin. It would be tempting to speculate that this membranous expansion is made up of materials that in the in vivo situation would be incorporated into the outer segment but that in the in vitro conditions failed to become organized into the characteristic outer segment structure. In this context, Hollyfield and Witkovsky (10) observed that when amphibian neural retinas were separated from the pigment epithelium, photoreceptor development progressed through the differentiation of the inner segment, but outer segment formation failed to occur. The experimental system presented here offers a very suitable model for the investigation of influences from the pigment epithelium and other cell types on photoreceptor maturation.

REFERENCES

1. Adler, R. 1982. Regulation of neurite growth in purified retina cultures. Effects of PNPF, a substratum-bound neurite-promoting factor. J. Neurosci. Res. 8:165–177.
2. Adler, R. 1982. Trophic and neurite-promoting factors in eye development. In The Structure of the Eye. J. G. Hollyfield, editor. Elsevier/North Holland Biomedical Press, New York. 215–228.
3. Adler, R., K. B. Landa, M. Manthorpe, and S. Varon. 1979. Cholinergic neuronotrophic factors. II. Selective intraocular distribution of soluble tropic activity for ciliary ganglionic neurons. Science (Wash. DC). 204:1434–1436.
4. Adler, R., P. J. Magistretti, A. G. Hyndman, and W. J. Shoemaker. 1982. Purification and cytochemical identification of neuronal and nonneuronal cells in chick embryo retina cultures. Dev. Neurosci. 5:27–39.
5. Blanks, J. C., and L. V. Johnson. 1983. Selective lectin binding of the developing mouse retina. J. Comp. Neurol. 221:31–41.
6. Blanks, J. C., and L. V. Johnson. 1984. Specific binding of peanut lectin to a class of retinal photoreceptor cells: a species comparison. Invest. Ophthalmol. Visual Sci. 25:546–577.
7. Black, I. 1978. Regulation of autonomic development. Annu. Rev. Neurosci. 1:183–214.
8. Cowan, W. M. 1973. Neuronal death as a regulative mechanism in the control of cell number in the nervous system. In Development and Aging of the Nervous System. M. Rockstein and M. C. Sussman, editors. Academic Press, Inc., New York. 19–41.
9. Grün, G. 1982. The development of the vertebrate retina: a comparative study. Adv. Anat. Embryol. Cell Biol. 78:7–85.
10. Hollyfield, J. E., and P. Witkovsky. 1974. Pigmented retinal epithelium involvement in photoreceptor development and function. J. Exp. Zool. 189:357–378.
11. Hyndman, A. G., and R. Adler. 1982. Neural retina development in vitro. Effects of tissue extracts on survival and neuritic development in purified neuronal cultures. Dev. Neurosci. 5:40–53.
12. Landmesser, L., and G. Pilar. 1978. Interactions between neurons and their targets during in vivo synaptogenesis. Fed. Proc. Am. Soc. Exp. Biol. 37:2016–2022.
13. LaVail, M. M. 1981. Analysis of neurological mutants with inherited retinal degeneration. Invest. Ophthal. Visual Sci. 21:630–657.
14. Meller, K., and W. Tetzlaff. 1976. Scanning electron microscopic studies on the development of the chick retina. Cell Tissue Res. 170:145–160.
15. Varon, S., and R. Adler. 1981. Trophic and specifying factors directed to neuronal cells. Adv. Cell. Neurobiol. 2:115–163.