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Analysis of the endocytic-lysosomal system (vacuolar apparatus) in astrocytes during proliferation and differentiation in primary culture

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ABSTRACT **The endocytic-lysosomal system of proliferating and differentiated astrocytes in primary culture was investigated using a combination of cytochemical, immunocytochemical and biochemical procedures. These included impregnation with osmium tetroxide and potassium iodide, phosphotungstic acid staining, cytochemical demonstration of acid phosphatase and thiamine pyrophosphatase activities and incorporation of cationized ferritin. The acid phosphatase activity was also analyzed using biochemical techniques. Our results indicate that while all astrocytes in primary culture have a developed endocytic-lysosomal system, this system is different in proliferating cells from that in differentiated astrocytes. Whereas in proliferating astrocytes it appears to be composed mainly of a variety of vacuoles and vesicles displaying a heterogeneous osmium tetroxide staining pattern, differentiated cells are characterized by the presence of small size vesicles showing an intense reaction. Both types of astrocyte showed abundant lysosomes, including multivesicular bodies, which presented an intense phosphatase acid activity. Biochemical analyses demonstrated that this activity increase during the proliferation period, reaching a maximum at 15 days of culture. Incorporation of cationized ferritin revealed that lysosomes and endosomes constitute separate systems. Finally, we have also found that the activity of thiamine pyrophosphatase, a marker for the Golgi complex, increases throughout the culture period. These results indicate that astrocytes could play an important role in regulating the macromolecular composition of the extracellular space.**

KEY WORDS: *astrocytes, lysosomes, endocytosis*

Introduction

Astrocytes play an important role in maintaining the composition of extracellular fluid in brain, regulating interactions among neurons and between neurons and the bloodstream. Thus, astrocytes are key to the homeostasis of potassium ions in interstitial fluid, in the control of interstitial pH and in the regulation of other constituents of the cerebral interstitium, in part by active uptake and temporary storage and in part by transport between brain tissue and the bloodstream (Bradford, 1986; Oldendorf, 1987; Somjen, 1987). Whereas the mechanisms involved in the regulation of potassium, other ions and small molecules have been extensively investigated (Bradford, 1986; Somjen, 1987; Reichenbach, 1991), little clear evidence exists as yet on the movement and regulation of macromolecules such as proteins in the extracellular space of the central nervous system (CNS).

It has recently been reported that rat cortical astrocytes, growing as multiple layers, can act as vehicles for the translocation of macromolecules such as transferrin and serum albumin. This suggests that one function of astrocytes may be to facilitate transport of macromolecules from one cell to another (Juurlink and Devon, 1990). These molecules appear to undergo receptor-mediated endocytosis followed by translocation and exocytosis (Juurlink and Devon, 1990). It has also been shown that insulin-like factors IGF-I and IGF-II are rapidly internalized after binding to their respective receptors, the IGF-I receptor and the man-6-P/ IGF-II receptor on the plasma membrane of rat astrocytes in primary culture (Auletta *et al.*, 1992). After endocytosis, significant proportions of both IGFs are degraded and released as amino acids. The endocytosis and degradation of IGF-I and IGF-II shows differences

Abbreviations used in this paper: AcPase, acid phosphatase; CF, cationized ferritin; CNS, central nervous system; GC, Golgi complex; GFAP, glial fibrillary acidic protein; IGF, insulin-like growth factor; MVB, multi-vesicular body; PTA, phosphotungstenic acid; TPPase, thiamine pyrophosphatase.

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suggesting separate pathways. Thus, IGF-II is internalized and degraded to a higher extent than IGF-I, and the processing of both factors shows differential sensitivity to protease and lysosomal inhibitors. Moreover, preliminary studies in our laboratory have demonstrated that cortical astrocytes in primary culture exhibit a large number of endocytic vesicles, a well developed Golgi complex (GC) and a complex lysosomal system (Mayordomo *et al.*, 1992). These studies strongly suggest that astrocytes have a developed endocytic system coupled with an intracellular system for transport, storage, exocytosis and/or degradation of ingested material. Both systems constitute the vacuolar apparatus, a term coined by De Duve and Wattiaux (1966). Therefore, the analysis of these systems in astrocytes could help to understand the mechanisms involved in the regulation of macromolecules in the cerebral interstitium.

Although endocytosis is a dynamic process, electron microscopy has been widely used to define the cellular components involved in this process (Hergoz and Farquhar, 1983; Juurlink and Devon, 1990).

As a first approach to study the components of the vacuolar system in astrocytes and to analyze possible changes in this system during astrocyte differentiation in primary culture, in the present work we have used cytochemical, immunocytochemical and biochemical techniques that have been widely used in other cell types to characterize the different components of the endocytic and lysosomal systems.

Results

Purity of astrocyte cultures

In the cultures, the proportion of GFAP-positive cells ranged from 85-90%. A small portion of oligodendrocytes (2-5%) was also observed and the absence of neurons in the cultures was assessed using a monoclonal anti-neurofilament 68-kDa antibody. Cell viabilities averaged 90% as determined by trypan blue check.

Osmium tetroxide impregnation

We have compared the results obtained using Friend's procedure (1969) with those obtained with the method of Rambourg *et al.* (1987). Although both procedures result in a similar staining pattern of the vacuolar system, the Rambourg method gave better information than did Friend's (1969). The main advantages of the procedure of the former were the better ultrastructural preservation, improved staining quality and reduction in the reaction product deposition at the edge of the structures showing reaction product.

Proliferating and differentiated astrocytes in primary culture showed striking differences in the osmium staining. Proliferating cells displayed a complex vacuolar system composed mainly of a large number of stained vacuoles and vesicles of different size and shape, multivesicular bodies (MVBs) and a well developed GC (Figs. 1, 2). However, the osmium staining was not uniform. Thus, according to the staining pattern, several subpopulations of vacuoles and vesicles can be described. In some cases, these elements are densely stained, showing a variable amount of granular material. In other cases, the reaction product is missing in the center of the vesicles. Finally, some vesicular or vacuolar elements had no reaction product (Fig. 1B). In many cells, several densely stained vesicles appeared connected by small bridges that also showed positive staining (Fig. 1C,G). Some of these vesicles displayed a diffuse profile. These complexes were observed both in the cellular body and in the processes, near the plasma membrane. In most of the cells, all the cisternae of GC and associated vesicles appeared densely stained (Fig. 1E) and it was therefore difficult to distinguish between the cis- and trans- portions of GC. In both the nuclear envelope and MVB matrix, the reaction product was particulate. Whereas most MVBs showed labeling (Fig. 1F), the nuclear envelope staining was found only in some cells (Fig. 1A). Lipid droplets showed, in many cases, a rim of reaction product (Fig. 1D). Finally, structures resembling endosomes containing a fine reaction product were frequently found (Fig. 1C). Although this pattern of osmium staining was seen in most of the cells, a small population of astrocytes with very little or no reaction product was also found.

Differentiated astrocytes displayed, after osmium impregnation, a staining pattern which greatly differed from that of proliferating cells. Thus, in differentiated astrocytes, staining was restricted to GC and to small size vesicles which, as in proliferating cells, appeared connected in many cells by filaments (Fig. 2A,B). These structures were seen mainly in the cellular periphery and in the processes, between intermediate filaments and plasma membrane, and correspond to the so-called «osmiophilic network» previously described in spinal ganglion cells (Rambourg and Clermont, 1984).

OsKI staining

When astrocytes were processed for OsKI staining according to Locke and Huie (1983), a weak reaction was observed in both proliferating and differentiated astrocytes. The reaction product appeared as a fine precipitate located mainly in the nuclear envelope and in the periphery of some clear vacuoles (Fig. 3A). The remaining vacuolar system elements lacked significant reaction product.

PTA staining

Proliferating and differentiated astrocytes showed a PTA staining pattern which was to some extent similar to that found after OsO_4 impregnation. In non-differentiated astrocytes, staining was located as a diffuse precipitate in the periphery of some vesicular and vacuolar elements and filled some vesicles (Fig. 3B,C). Reaction in the GC was very scarce. Some cells showed reaction product in the plasma membrane. In differentiated cells the PTA staining pattern was similar to that of immature cells although the number of labeled elements was diminished with respect to proliferating astrocytes.

Cytochemistry

Proliferating astrocytes showed a large number of lysosomes, including dense bodies, MVBs, primary lysosomes, autophagic vacuoles and lysosomes containing myelinic figures, which consistently displayed AcPase activity (Fig. 5H). This staining was intense, the lysosomes being filled with a fine-amorphous reaction product. In contrast, trans-cisternae of GC lacked reaction product. In differentiated astrocytes, the AcPase activity was also restricted to lysosomes and no differences in this activity per individual lysosome were found as compared with proliferating cells. However, as revealed by stereological techniques, these cells showed an increased number of lysosomes as compared with proliferating astrocytes. Thus, the volume density of lysosomes in proliferating and differentiated cells was 2.5% and 4.4%, respectively.

In both proliferating and differentiated astrocytes, TPPase activity was located in GC where the reaction product was found mainly in the trans-cisternae (Fig. 4A) and no activity was found in GC associated vesicles. The labeling was more pronounced in differentiated astrocytes than in proliferating ones.

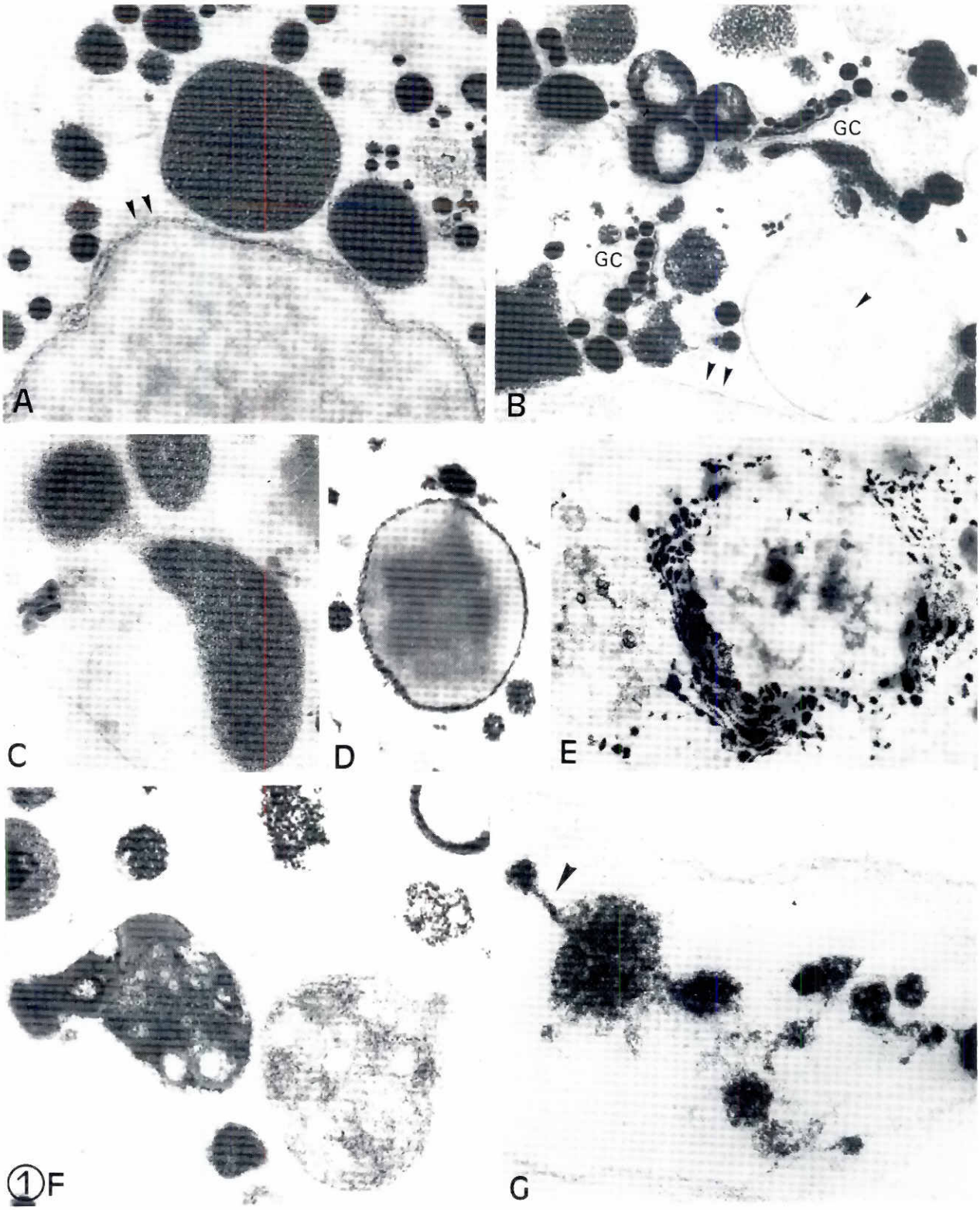

Fig. 1. Osmium tetroxide impregnation. Proliferating astrocytes. (A) *Several vacuoles and vesicles containing a granular reaction product are shown. The nuclear envelope displays a fine precipitate (arrowheads).* **(B)** *Corresponds to a Golgi complex (GC) area, where several Golgi complexes showed positive reaction. Vesicles and vacuoles displaying different staining patterns are also shown. The nuclear envelope and mitochondria lacked reaction product (arrowheads).* **(C)** *Correspond to possible endosome fusions.* **(D)** *A lipid droplet showing a rim of precipitate in the periphery is seen.* **(E)** *A Golgi complex displaying an intense labeling in all cisternae and in some associated vesicles is shown.* **(F)** *Corresponds to two MVBs.* **(G)** *A process containing several vesicles connected by fine bridges (arrowhead) are shown. A x27,400; B x21,300; C x57,000; D x30,000; E x13,600; F x30,000; G x65,400.*

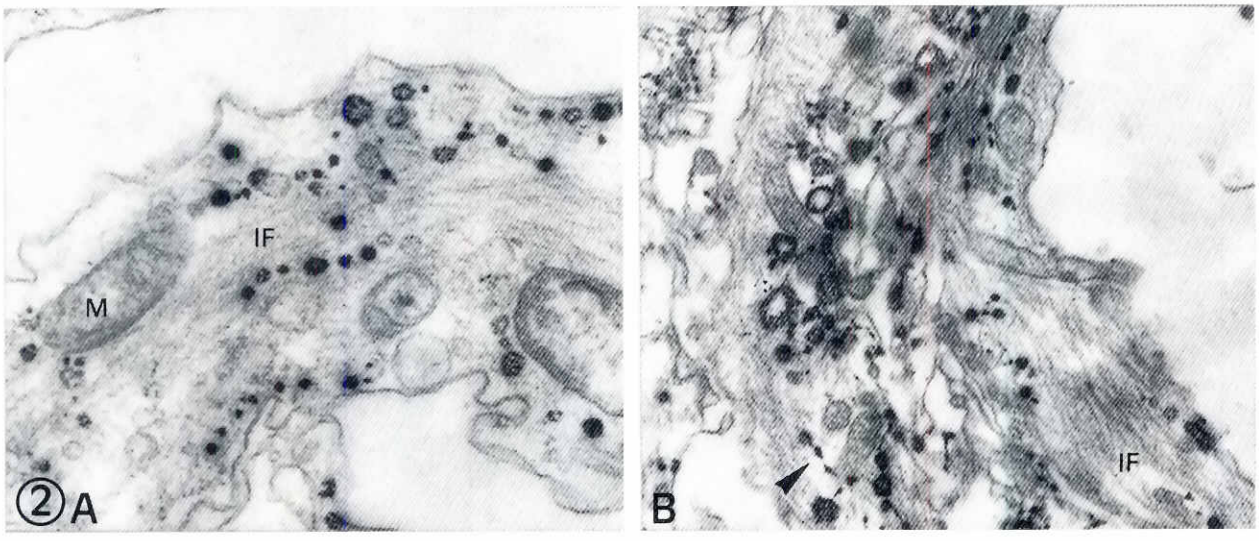

Fig. 2. Osmium tetroxide impregnation. Differentiated astrocytes. (A,B) *Two processes corresponding to differentiated astrocytes containing intermediate filaments (IF), mitochondria (M), and two types of small size vesicles containing reaction product. Many of these vesicles are connected by stained bridges (arrowhead). A x35,600; B x16,000.*

Labeling with CF

When proliferating and differentiated astrocytes were incubated with CF for 30 min at 37°C, a very uneven surface labeling of the cells was seen. Most of microvilli lacked a labeling with the tracer, which was often seen between these structures (Fig. 5B). In many cases CF filled the extracellular space between adjacent astrocytes. In some cells, a preferential labeling of the basal portion of the cilia was also observed (Fig. 5C). Finally, CF was found in both the coated and uncoated pits and nascent endocytic vesicles (Fig. 5A).

Intracellularly, CF was seen in some tubular elements and in several types of vacuoles and vesicles (Fig. 5D,E,F,G): a) large vacuoles with an electron-lucent content where CF preferentially binds to the inner aspect of the limiting membrane; b) vacuoles completely filled by CF; c) structures resembling early endosomes; d) small size vesicles; and e) some MVBs. After the incubation period, CF did not appear to be bound to the cisternae membrane of GC although some vesicles containing CF were seen close to the GC region. An interesting finding was that most of these CF-labeled structures lacked AcPase activity (Fig. 5I).

Biochemical determination of AcPase

The activity of AcPase determined in astrocytes after several days of culture is represented in Fig. 6 and expressed as μmol/h/mg protein per plate (3 mm). AcPase activity shows an increase during the first week of culture, reaching a maximum at 15 days, after which the activity diminishes.

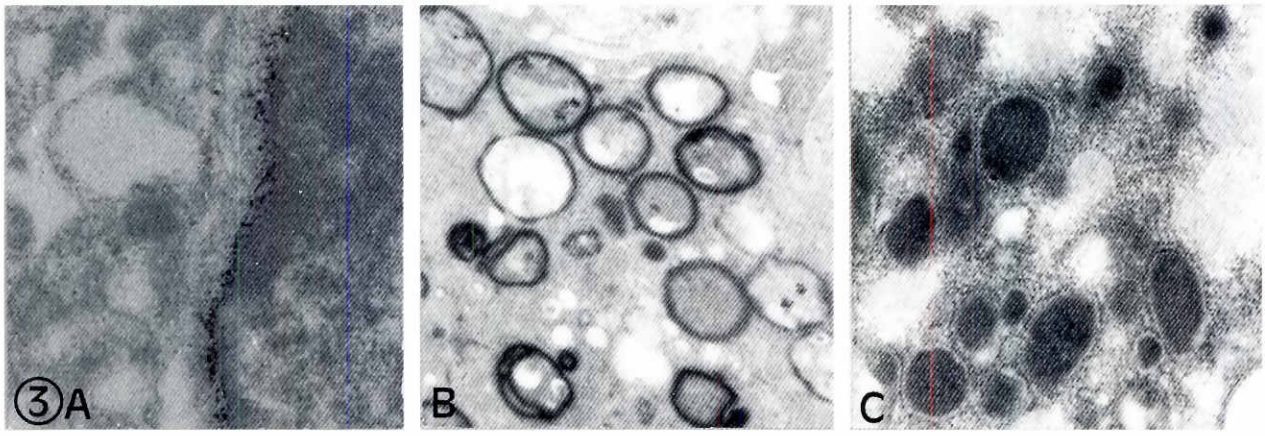

Fig. 3. OsKI and PTA impregnation. Proliferating astrocytes. (A) *An astrocyte after OsKI impregnation is shown. After this technique only the nuclear envelope and some vesicles contained reaction product.* **(B,C)** *Astrocyte after PTA staining. A x20,000; B x12,000; C x26,000.*

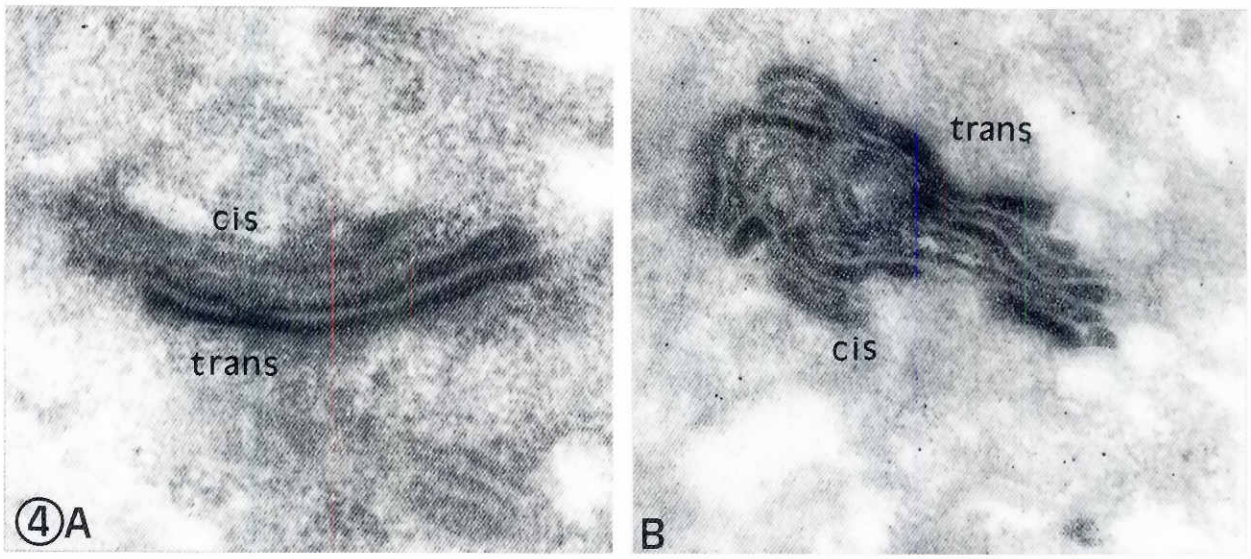

Fig. 4. TPPase activity in astrocytes. *Differentiated astrocytes processed for cytochemical demonstration of TPPase activity. Whereas in* **(A)** *the activity appears stronger in the trans-cisternae of the Golgi complex, in* **(B)** *all cisternae showed a similar amount of reaction product. This micrograph probably corresponds to a tangential or parallel section of the trans Golgi cisternae. Similar images were found in proliferating astrocytes. A x106,000; B x64,000.*

Discussion

We have demonstrated that rat cortical astrocytes in primary culture have a developed endocytic-lysosomal system composed of a heterogeneous collection of structures. This system, moreover, undergoes marked morphological and cytochemical changes related to the maturation stage of the cells. These changes are also heterogeneous in function on the cell component and cytochemical procedure considered.

Osmium tetroxide impregnation has long been considered as a cytochemical procedure of choice for identifying the cis portion of the GC (Farquhar and Palade, 1981). However, we have found that in both proliferating and differentiated astrocytes this procedure results in a strong labeling of all the GC elements, including the trans-cisternae, as had already been reported in previous studies on other types of cells such as fetal hepatocytes or epididymal epithelial cells (Goldfischer, 1982; Yamaoka *et al.*, 1983; Renau-Piqueras *et al.*, 1987). In contrast, striking differences in the osmium tetroxide staining pattern of vesicles and vacuoles of proliferating and differentiated astrocytes were found. Although the chemical nature of this staining is not clear, it appears than when unfixed cells are incubated with osmium tetroxide under the conditions described, at least some part of the osmiophilia is due to a reducing environment, so that the most likely groups to be involved are the -SH groups of cysteine, either in the membrane to create the reducing environment or in extended polypeptides as a result of it (Locke and Huie, 1983). Therefore, variations in the staining pattern of GC cisternae, transition and secretory vesicles, and membranes surrounding vacuoles or lipid droplets could be related to changes in the reducing environment of these cells, including variations in protein synthesis during development (Locke and Huie, 1983). The differences that we have found in the osmium tetroxide staining in proliferating and differentiated astrocytes could be due, therefore, not only to variations in the vesicular composition of the cells but also to the marked changes in specific protein synthesis that take place during astrocyte differentiation (e.g. GFAP, glutamine synthetase, carbonic anhydrase, etc.) (White and Hertz, 1981; Guerri *et al.*, 1990).

In addition to the changes in osmium tetroxide and PTA staining, proliferating and differentiated astrocytes exhibit differences in the cytochemical activity of both TPPase and AcPase. Although both types of astrocyte showed a strong TPPase activity, mainly in the trans portion of the GC, reaction product was more pronounced in differentiated cells. This suggests that astrocytes, principally during differentiation, may be active in those activities that depend on the GC function, including membrane traffic, glycosylation and transport of glycoproteins. In fact, during astrocyte maturation there is an increase in the activity of (Na^+K) ATPase and 5'-nucleotidase, which are glycoproteins in nature, and also in the plasma membrane concanavalin A binding sites (Guerri *et al.*, 1989; Renau-Piqueras *et al.*, 1992). Moreover, differentiated astrocytes also show an increase in the activity of uridine diphosphatase (Mayordomo *et al.*, 1992), an enzyme which plays an important role in glycosylation (Fleischer, 1983).

Both proliferating and differentiated astrocytes show a similar amount of AcPase activity per individual lysosome. However, results in the present work and in previous studies report an augmentation of lysosomes during astrocyte differentiation (Fedoroff *et al.*, 1984; Mayordomo *et al.*, 1992). This agrees with our biochemical data that indicate an increase in the AcPase activity throughout the culture period. These results make it possible to conclude that the increase in the AcPase activity is due to an increase in the number of lysosomes — mainly autophagosomes — rather than to an increase in AcPase synthesis. It has been reported that AcPase activity and proteolysis increases in other cell types during differentiation (Doine *et al.*, 1984; Lee *et al.*, 1992) and that there is also an increase of the degradation of long-lived proteins in confluent cultures as compared with exponentially growing 3T3 cells (Knecht *et al.*, 1984). Moreover, a significant correlation between increases in rates of proteolysis of long-lived proteins and fractional volume

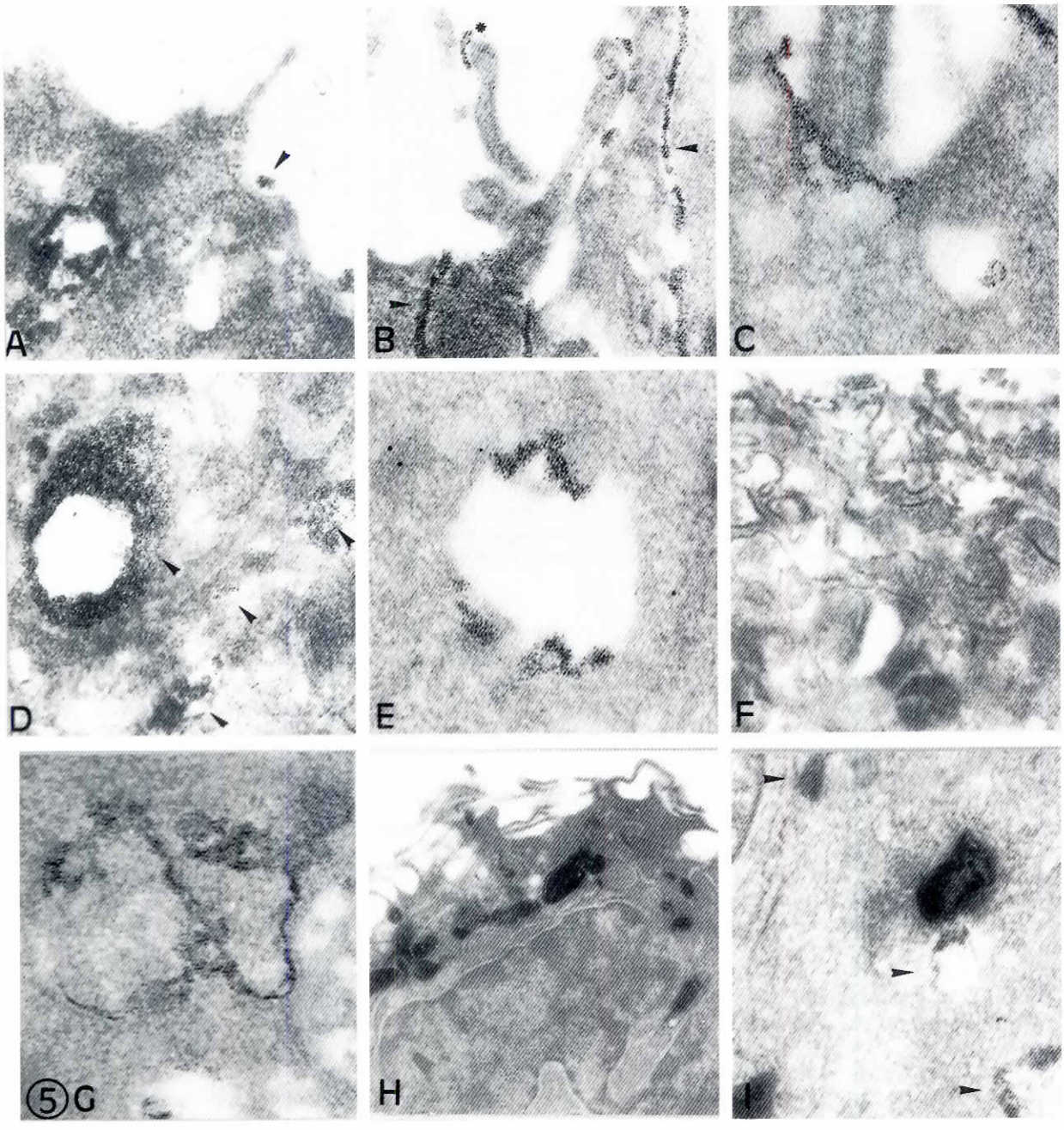

Fig. 5. Detail of astrocytes after incubation in presence of CF and AcPase activity. **(A)** *A pit decorated with CF particles (arrowhead) and several endosomes containing CF are shown.* **(B)** *CF particles appear in extracellular spaces (arrowhead) and between microvilli (*).* **(C)** *Labeling is located at the basis of a cilium.* **(D)** *Several types of endosome containing CF are shown (arrowhead).* **(E)** *Closeup showing a large vacuole with a lucent matrix displaying CF labeling in the periphery. Gold particles correspond to anti-clathrin binding sites.* **(F)** *Several tubular profiles with CF are seen.* **(G)** *A large tubular endosome heavily loaded with CF is shown.* **(H)** *Detail of an astrocyte incubated for the demonstration of AcPase showing several lysosomes containing reaction product.* **(I)** *In most cells CF-labeled structures lacked AcPase activity (arrowhead). A x15,000; B x50,600; C x75,000; D x10,000; E x70,500; F x27,000; G x95,000; H x18,000; I x42,500.*

of lysosomes has also been found in these cells, suggesting a causal relationship between the two (Knecht *et al.*, 1984). The turnover of cytoskeletal and Triton X-100 soluble proteins in cultured astrocytes displays biphasic decay kinetics, with an initial phase corresponding to a fast-decaying pool with a half-life of 12-18 h. A larger pool, about 60%, decayed more slowly, exhibiting a half-life of 8 days (Chiu and Goldman, 1984). It is possible, therefore, that lysosomes in astrocytes play an important role in the proteolysis of long-lived proteins, including cytoskeletal proteins. On the other hand, it is important to point out that most of structures showing AcPase activity lacked CF, which suggests that, as occurs in other cell types, the endocytic and the lysosomal systems

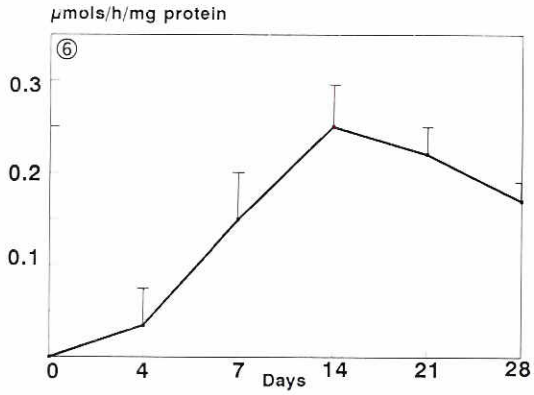

Fig. 6. Enzymatic activity of AcPase in astrocytes as a function of culture time. *Each point represents the average ± S.D. of three to four different cultures. Results are expressed as µmol/h/mg protein.*

constitute two different compartments (Helenius *et al.*, 1983). However, experiments done to clarify this point and to establish the kinetics of endocytosis in astrocytes are still in progress.

Astrocytes in primary culture internalize large amounts of CF by adsorptive (non specific) endocytosis following a route similar to that described for other cell types, including nervous cells (Farquhar, 1983; Báguena-Cervellera *et al.*, 1987). This confirms previous studies showing that transferrin and BSA are internalized and translocated by astrocytes in primary culture (Juurlink and Devon, 1990).

Moreover, other macromolecules such as insulin-like factors, IGF-I and IGF-II are also rapidly internalized after binding to their respective receptors which are then trafficked and processed by different intracellular pathways in neonatal rat astrocytes (Auletta *et al.*, 1992).

All the results presented here indicate that astrocytes have developed machinery to internalize macromolecules from the surrounding medium. This material could be translocated, as reported (Juurlink and Devon, 1990), or degraded by the lysosomal system (Auletta *et al.*, 1992). The main conclusion of this work is that astrocytes can play an important role in the movement and regulation of macromolecules in the extracellular space of the CNS and that this function appears to be more developed in differentiated astrocytes, i.e., when astrocytes perform their specific function. Moreover, since astrocytes synthesize several factors (Jacobson, 1991), the possibility that the vacuolar system in astrocytes is involved in intracellular transport and secretion of proteins should also be considered.

Materials and Methods

Astrocyte cultures

Primary culture of astrocytes from 21-day-old rat fetuses were prepared from brain hemispheres as described (Renau-Piqueras *et al.*, 1989, 1992; Gómez-Lechón *et al.*, 1992). The purity of astrocyte cultures was assessed using a mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody and fluorescence microscopy (Renau-Piqueras *et al.*, 1989; Sáez *et al.*, 1991). Since these cultures grow rapidly for 7-10 days (proliferative period), after which the cell number increases slowly (differentiation period) (Renau-Piqueras *et al.*, 1989; Guerri *et al.*, 1990), all the experiments described below were done in triplicate on 7- and 20-day cultures.

Osmium impregnation

Osmium impregnation was carried out following two procedures: a) In the first procedure, osmium tetroxide treatment was performed according to Friend (1969) and Renau-Piqueras *et al.* (1987). The cells were incubated in unbuffered 2% osmium tetroxide for 24 h at 40°C. After this period, the monolayers were immersed in fresh 2% osmium tetroxide for another 24 h at 40°C. b) Monolayers of cells were fixed 60 min at 22°C in a 2% aqueous solution of osmium tetroxide. The cells were then transferred in the same solution to an oven maintained at 42°C for a total period of 34 h. During the first 10 h, the osmium tetroxide solution was changed every 2 h. Subsequent to another change of solution after 20 h, the monolayers were allowed to stay in osmium for an additional period of 14 h (Rambourg *et al.*, 1987).

After washing in water, the cells were treated in both cases for 90 min in 0.5% uranyl acetate, dehydrated in graded ethanols, and embedded in Poly/Bed-812. Ultrathin sections (90 nm) were counterstained with uranyl acetate and examined at 80 or 100 kV in a Philips EM 301.

Osmium tetroxide-potassium iodide impregnation

Astrocytes were washed in 1% potassium iodide and postfixed overnight at 22°C in freshly prepared 1% osmium tetroxide with 1% potassium iodide with the pH adjusted to 5.9. Cells were washed in water, dehydrated in ethanol and embedded in Poly/Bed-812 (Locke and Huie, 1983).

Phosphotungstic acid staining

Phosphotungstic acid (PTA) staining of astrocytes was carried out according to De Bruyn *et al.* (1977). Lowicryl K4M ultrathin sections (see below) were picked up with a platinum wire loop and transferred to the surface of bidistilled water. Sections were then floated 5 min on 0.5 N hydrochloric acid solution before being floated for 20 min on a solution of 4% PTA in 1 N hydrochloric acid (pH 0.2-0.3). The sections were again floated on a 0.5 N hydrochloric acid solution for 5 min, washed in distilled water and mounted on formvar-coated nickel grids.

Endocytosis of cationized ferritin

Monolayers of astrocytes were rinsed with culture medium (DMEM) without serum and then incubated with culture medium containing 800 µg/ml of cationized ferritin (CF) (Sigma) (Renau-Piqueras *et al.*, 1985a; Báguena-Cervellera *et al.*, 1987) in a shaking bath at 37°C for 15 min. This time was selected after several experiments. The reaction was stopped with cold medium and then the cells were fixed with 4% formaldehyde-0.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 for 120 min at 22°C, detached from the plastic using a rubber policeman, incubated for 60 min at 22°C in 50 mM NH_4Cl, dehydrated in methanol and embedded in Lowicryl K4M (Bendayan *et al.*, 1987; Hobot, 1989; Renau-Piqueras *et al.*, 1989).

Cytochemistry

For cytochemical demonstration of AcPase and TPPase, monolayers of cells were fixed in ice-cold 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 5% sucrose. The cells were detached from the plastic and centrifuged and maintained in the fixative solution for 45 additional min. Pellets were then washed three times in the appropriate reaction buffer and immersed in the same buffer containing 10% DMSO. After 60 min, they were frozen in liquid nitrogen-cooled isopentane and 20 µm frozen sections were obtained in a cryostat. The sections were pre-incubated at 37°C for 60 min in medium without substrate (Renau-Piqueras *et al.*, 1987). The different enzymatic activities were then assayed by immersing sections in the appropriate solutions, which also contained cerium ions as capturing agent (Robinson and Karnovsky, 1983; Renau-Piqueras *et al.*, 1985b, 1987). Incubation was at 37°C for 90 min with shaking (the medium was changed after 45 min). For AcPase the incubation medium was composed of 0.1 M acetate buffer (pH 5.0), 1 mM ß-glycerophosphate (disodium salt) and 2 mM $CeCl_3$. For TPPase the incubation medium was: 0.1 M Tris-maleate (pH 7.2), 1 mM thiamine pyrophosphate chloride, 2 mM $MnCl_2$ and 2 mM $CeCl_3$. The reaction was stopped by washing sections in cacodylate buffer followed by refixing in cold glutaraldehyde. The samples were embedded in Lowicryl K4M. Control sections were incubated without substrate. In some cases, monolayers incubated with CF were also processed for cytochemical demonstration of AcPase and TPPase.

Biochemical determination of AcPase

Astrocytes were resuspended in 10 mM Tris-HCl buffer (pH 7.0) and stored at -70°C. Cells were later thawed, homogenized in the same buffer and used for enzymatic analysis. AcPase activity was assayed according to Bergmeyer and Bernt (1974). Protein concentration was determined by the method of Lowry *et al.* (1951) using BSA as a standard. Results were expressed as μmol/h/mg protein.

Acknowledgments

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