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Original Article

Cytochemical and stereological analysis of rat cortical astrocytes during development in primary culture. Effect of prenatal exposure to ethanol

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ABSTRACT This study has investigated the effect of prenatal alcohol exposure on the qualitative and quantitative ultrastructure of proliferating and differentiated astrocytes in primary culture as well as on the cytochemical activity of several subcellular phosphatase markers, including acid phosphatase, uridine diphosphatase, thiamine pyrophosphatase, 5'-nucleotidase and glucose-6-phosphatase. The astrocytes were obtained from 21-day-fetuses of both control and alcohol-fed rats. Our results show that several cell components, such as mitochondria, rough endoplasmic reticulum and lysosomes, exhibit qualitative and/or quantitative ultrastructural changes during the process of astrocyte maturation. In some cases these morphological changes are accompanied by variations in the cytochemical activity of enzymes located in these and other cell components, suggesting that these enzymes, and therefore the functional state of these organelles, are modulated during astrocyte development. When prenatally exposed to ethanol, both proliferating and differentiated astrocytes showed striking ultrastructural alterations compared with controls, including an increment of lysosomes as well as a decrease in the values of stereological parameters relative to mitochondria, rough endoplasmic reticulum and Golgi apparatus. Cytochemical analysis of these cells indicates that prenatal exposure to ethanol decreased the activities of all the enzymes tested, except for acid phosphatase, which was increased in both groups of treated astrocytes. These results suggest that prenatal exposure to ethanol could affect astrocytes during development in two different but probably complementary ways: a) by causing a delay in astrocyte maturation and, b) by inducing a direct toxic effect on these cells.

KEY WORDS: *astrocytes, development, prenatal alcohol exposure, cytochemistry, stereology*

Introduction

Prenatal exposure to ethanol during development induces in offspring a wide spectrum of adverse effects, the extreme of which is fetal alcohol syndrome or FAS (Jones and Smith, 1973). In humans, alcohol teratogenicity is mainly characterized by dysfunction of the central nervous system (CNS), which is manifested by mental retardation, poor motor coordination, hypotonia, irritability in infancy and hyperactivity in childhood (Abel, 1981, 1982, 1984; Streissguth and Martin, 1983). Many of these symptoms have also been described in laboratory animals (Streissguth and Martin, 1983; Sanchís *et al.*, 1986; Driscoll *et al.*, 1990).

Experimental studies in addition to necropsy observations of FAS-affected children have shown a range of structural and functional alterations in neurons (Clarren *et al.*, 1978; Dow and Riopelle, 1985; Miller, 1986, 1987, 1990). Since many functional aspects of these cells in both adult and developing brain depend on the integrity of neuroglial cells (Bradford, 1986; Kimelberg and Norenberg, 1989; McKay, 1989), it has been postulated that the neuronal alterations found in FAS could be due to some initial damage by ethanol on astrocytes during development (Kennedy and Mukerji, 1986a,b; Miller, 1986, 1987; Guerri *et al.*, 1989; Renau-Piqueras *et al.*, 1989c). This hypothesis has been partially confirmed by us

Abbreviations used in this paper: GA, Golgi apparatus; AcPase, acid phosphatase; GFAP, glial fibrillary acidic protein; IDP, inosine diphosphatase; PEA, prenatally exposed to ethanol; rER, rough endoplasmic reticulum; TPPase, thiamine pyrophosphatase; UDPase, uridine diphosphatase.

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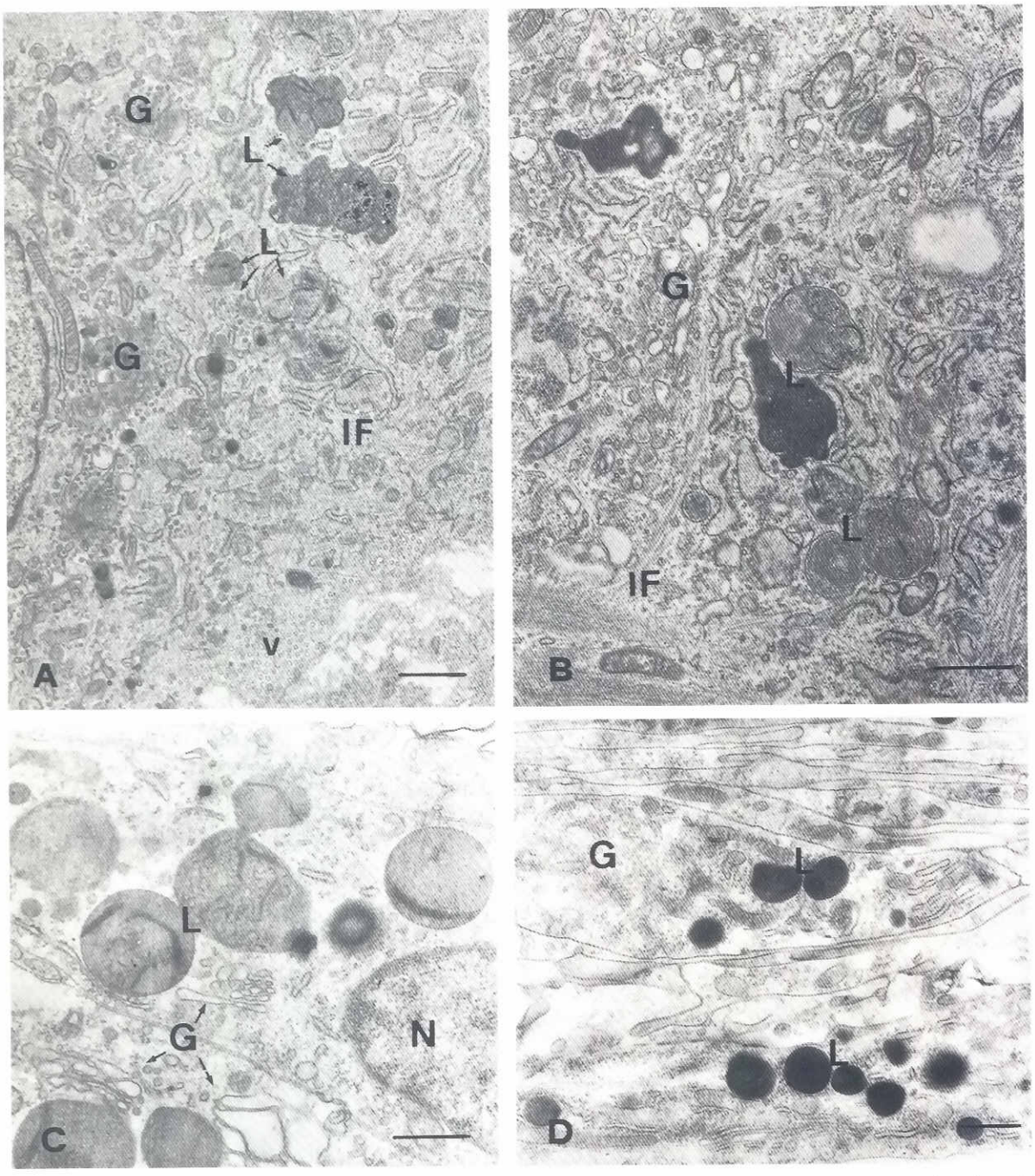


Fig. 1. Micrographs showing several types of lysosomes (L) in 6- (A,B) and (C,D) 20-day PEA astrocytes. *In (A) many endocytic vesicles (v) accumulate beneath the plasma membrane. (G, Golgi apparatus; IF, intermediate filaments; N, nucleus). A, C, D Bars, 1 μm; B, 0.5 μm. A x10400; B x23350; C x11600; D x9500.*

using astrocytes in primary culture obtained from rat fetuses prenatally exposed to ethanol and controls. We have reported that ethanol reduces the content and synthesis in these cells of proteins, DNA and RNA as well as the activity of several marker and membrane-bound enzymes (Renau-Piqueras *et al.*, 1988; Guerri *et al.*, 1989, 1990c). Moreover, ethanol-exposed astrocytes failed to develop processes and showed striking cytoskeletal alterations in both microtubules and intermediate filaments (Renau-Piqueras *et al.*, 1989c, 1990; Sáez *et al.*, 1990). These studies also demonstrated that astrocytes in primary culture are more susceptible to the toxic effect of ethanol during proliferation than during differentiation (Guerri *et al.*, 1990b).

There are many studies of the ultrastructural changes of mammalian neuroglial cells and their precursors during gliogenesis *in vivo*

TABLE 1

CONTROL ASTROCYTES IN PRIMARY CULTURE. RELATIVE STEREOLOGICAL DATA (MEAN±S.D.)

Component	Parameter	6 days	20 days	Units	p
Mitochond.	Vv	9.23 ± 2.29	7.37 ± 2.28	μm^0	≤0.05
	Sv	1.05 ± 0.13	0.98 ± 0.24	μm^{-1}	n.s
	Nv	1.31 ± 0.43	1.25 ± 0.81	μm^{-3}	n.s
rER	Vv	6.32 ± 1.65	11.44 ± 2.05	μm^0	≤0.05
	Sv	0.45 ± 0.06	1.03 ± 0.09	μm^{-1}	≤0.05
Golgi	Vv	11.27 ± 4.69	11.74 ± 5.94	μm^0	n.s
Lysosomes	Vv	2.48 ± 0.12	4.39 ± 0.68	μm^0	≤0.05
	Sv	0.19 ± 0.04	0.28 ± 0.02	μm^{-1}	≤0.05
	Nv	0.23 ± 0.01	0.24 ± 0.03	μm^{-3}	n.s

Vv, volume density (in percentage); Sv, surface density; Nv, numerical density. Reference volume was cytoplasm.

(Vaughn and Peters, 1967; Caley and Maxwell, 1968; Vaughn, 1969; Phillips, 1973; Skoff *et al.*, 1976; Parnavelas *et al.*, 1983; Sturrock, 1986). However, although primary cultures of astrocytes constitute an important tool for studying the biochemical and functional characteristics of these cells (Hertz *et al.*, 1982; Hansson, 1986) little attention has been paid to the evolution of ultrastructural features of astrocytes during proliferation and differentiation *in vitro* (Trimmer *et al.*, 1982; Fedoroff *et al.*, 1984; Devon and Juurlink, 1988) and none to their cytochemical characteristics.

Knowledge of these characteristics could be of interest in order to better define the functional state of astrocytes during both phases in normal, experimental and pathological conditions.

In the present work we have therefore analyzed, using quantitative electron microscopy and cytochemistry, the possible changes in several subcellular components during the proliferation and differentiation periods of cortical astrocytes in primary culture as well as the effect of prenatal exposure to alcohol (PEA) on these cell components.

Results

Qualitative observations

In both Epon and Lowicryl sections, astrocytes showed a similar ultrastructure. Immunocytochemical observations revealed that most cells (≈90%) were labeled with the anti-GFAP antibody.

Transverse sections of both 6- and 20-day control astrocytes demonstrate that cultures were formed by several layers of overlapping cellular sheets. In these sheets, the adjoining astrocytes showed junctional complexes which were not stained with the anti-GFAP antibody. In both control groups the cells showed a flat profile with a cell body containing the nucleus and long processes filled by intermediate filaments. The nucleus was elongated, with a scant amount of condensed chromatin. In many cells, nuclei contained one or more well-developed nucleoli. The perinuclear cytoplasm was occupied mainly by mitochondria which were more spherical in 6- than in 20-day cells. Cells from both culture periods showed two or more well developed Golgi complexes composed of 4-5 cisternae and many small vesicles which occasionally were coated. In some micrographs these coated vesicles were connected to the cisternae. Several types of lysosomes, including multivesicular bodies, were also found in most of the cells. Rough endoplasmic reticulum (rER) appeared both as tubular profiles and as dilated cisternae. In many cases, tubular rER cisternae were dilated at one extreme. The remainder of the cytoplasm was filled by intermediate filaments, free ribosomes, scattered glycogen granules and, in some cells, lipid droplets. The intermediate filaments, as previously reported (Renau-Piqueras, 1989c), were more abundant in differentiated cells in which they were disposed in parallel arrays, whereas in proliferating astrocytes they formed a reticular network. Finally, nascent or free endocytic vesicles were frequently present in both cell types (Fig 1A).

Six- and 20-day prenatally exposed to alcohol (PEA) astrocytes showed a gross ultrastructural appearance similar to that of controls. However, some striking alterations were found in several cell components. Thus, compared with controls, proliferating astrocytes showed an increased number of lysosomes (Fig. 1), which were recognized by the morphological criteria (Fawcett, 1981) and by the presence of AcPase activity. These included dense lysosomes, multivesicular bodies, primary lysosomes, autophagic vacuoles, lysosomes containing myelinic figures and lysosomes with laminated inclusions. Moreover, these cells had a decreased number of mitochondria, which were more elongated than in 6-day control astrocytes. GA and rER appeared less developed than in control cells. In many astrocytes of both treated groups, intermediate filaments appeared disorganized. 20-day PEA astrocytes mostly resembled 6-day control astrocytes, although they showed an abundance of lysosomes, some swollen mitochondria and a less developed rER and GA. Also seen in some cells were alterations in the cytoskeletal organization. In these latter cells intermediate filaments appeared organized in a fashion similar to that found in proliferating control cells.

Stereology

Results obtained after stereological analysis of the four astrocyte groups are summarized in Tables 1-5 and Fig. 2. Comparison of 6- and 20-day control astrocytes showed significant differences ($P<0.05$) between stereological parameters concerning mitochondria, rER and lysosomes. Thus, the Vv of mitochondria of 20-day astrocytes was smaller than that of 6-day cells. However, the

TABLE 2

CONTROL ASTROCYTES IN PRIMARY CULTURE. ABSOLUTE DATA (MEAN±S.D.)

Component	Parameter	6 days	20 days	Units	p
Mitochond.	A	0.08 ± 0.04	0.09 ± 0.05	μm^2	≤0.05
	V	0.03 ± 0.01	0.05 ± 0.01	μm^3	≤0.05
	CF	0.79 ± 0.01	0.70 ± 0.02	–	≤0.05
Lysosomes	A	0.10 ± 0.07	0.13 ± 0.06	μm^2	≤0.05
	V	0.04 ± 0.01	0.06 ± 0.01	μm^3	≤0.05
	CF	0.98 ± 0.04	0.97 ± 0.10	–	n.s

A, area; V, volume; CF, coefficient of form.

TABLE 3

PEA ASTROCYTES IN PRIMARY CULTURE. RELATIVE STEREOLOGICAL DATA (MEAN±S.D.)

Component	Parameter	6 days	20 days	Units	p
Mitochond.	Vv	4.33 ± 0.66	5.01 ± 060	μm^0	n.s
	Sv	0.93 ± 0.12	0.78 ± 0.10	μm^{-1}	n.s
	Nv	0.89 ± 0.14	1.00 ± 0.14	μm^{-3}	n.s
rER	Vv	4.31 ± 0.99	8.01 ± 1.34	μm^0	≤0.05
	Sv	0.29 ± 0.03	0.68 ± 0.03	μm^{-1}	≤0.05
Golgi	Vv	6.03 ± 0.76	9.03 ± 1.01	μm^0	≤0.05
Lysosomes	Vv	7.74 ± 0.87	8.45 ± 0.89	μm^0	n.s
	Sv	0.35 ± 0.04	0.42 ± 0.05	μm^{-1}	n.s
	Nv	0.57 ± 0.09	0.52 ± 0.08	μm^{-3}	n.s

Vv, volume density (in percentage); Sv, surface density; Nv, numerical density. Reference volume was cytoplasm.

Vv of lysosomes was greater in differentiated than in proliferating astrocytes. The former cells also showed a more developed rER. Analysis of absolute stereological values showed that both mitochondria and lysosomes were greater in differentiated cells and that the mitochondria of these astrocytes were more elliptical than those of 6-day cells.

Important too were the high values found in both cell groups for the Vv of the GA, confirming the qualitative observations which indicated that this cell component is well-developed in astrocytes.

As summarized in Tables 3 and 4, stereological analysis of 6- and 20-day PEA astrocytes confirmed most of our qualitative observations concerning these cells. Both cell groups showed, as compared with controls (Table 5), decreased values of the relative stereological parameters concerning mitochondria, GA and rER. In contrast, both PEA cell groups showed a significant increase in the relative values concerning lysosomes (Table 5).

Cytochemistry

When astrocytes were incubated as monolayers in the cytochemical medium without previous permeabilization or without cryostat sectioning, little if any reaction product was observed. Addition of detergent to the cytochemical medium results in an enhancement of the intracellular staining, which was qualitatively similar to that found in cryostat sections. However, the ultrastructural preservation was poorer in Triton X-100 treated cells than in astrocytes processed for cryostat.

Proliferating control astrocytes consistently showed AcPase activity in lysosomes, whereas the trans-cisternae of GA lacked reaction product (Fig. 3A,B). In 20-day control astrocytes this activity was also restricted to lysosomes although the intensity of deposits was always greater than in 6-day cells (Fig. 3C). When 6-day PEA astrocytes were examined, heavy deposits of cerium phosphate were found in lysosomal elements. The distribution of this activity and its intensity were similar in both 6- and 20-day PEA astrocytes and greater than that found in the corresponding controls (Fig. 3).

Analysis of 6-day control astrocytes incubated for the demonstration of UDPase showed a weak activity located in the dilated portions of the rER (Fig. 4A). This activity was also found in 20-day control astrocytes, where the deposits were located in one or two cisternae of the GA in addition to the activity found in the dilated rER (Fig. 4B). However, the reaction was clearly stronger in the GA cisternae than in the rER, where it was reduced to a few spots of reaction product. This activity was absent in 6-day PEA cells and only a few 20-day PEA astrocytes showed a little activity located in both trans-GA and rER. The same cytochemical pattern was found in all cases when inosin diphosphatase (IDPase) was assayed.

In control cells, TPPase activity was mainly located in the trans-GA (Fig. 4C,D). Whereas in 20-day control astrocytes two or three cisternae contained cerium phosphate deposits, only one was labeled when proliferating cells were examined. This distribution was similar in treated astrocytes, although differences existed among control and PEA cells. Thus, the intensity and amount of reaction product were both lower in PEA cells than in controls, and

TABLE 4

PEA ASTROCYTES IN PRIMARY CULTURE. ABSOLUTE DATA (MEAN±S.D.)

Component	Parameter	6 days	20 days	Units	p
Mitochond.	A	0.14 ± 0.01	0.11 ± 0.06	μm^2	n.s
	V	0.08 ± 0.01	0.06 ± 0.01	μm^3	n.s
	CF	0.65 ± 0.08	0.81 ± 0.13	–	≤0.05
Lysosomes	A	0.25 ± 0.03	0.12 ± 0.06	μm^2	≤0.05
	V	0.18 ± 0.02	0.06 ± 0.01	μm^3	≤0.05
	CF	0.91 ± 0.10	0.96 ± 0.09	–	n.s

A, area; V, volume; CF, coefficient of form.

TABLE 5

STATISTICAL COMPARISON (+= P≤0.05; -= N.S) OF THE EFFECTS OF PRENATAL EXPOSURE TO ETHANOL ON PROLIFERATING AND DIFFERENTIATED ASTROCYTES. DATA ARE PRESENTED IN TABLES 1-4

Component	Parameter	6d C vs. 6d PEA	21d C vs. 21d PEA
Mitochond.	Vv	+	+
	Sv	-	-
	Nv	+	-
	A	+	-
	V	+	-
	CF	-	-
rER	Vv	+	+
	Sv	+	+
Golgi	Vv	+	+
Lysosomes	Vv	+	+
	Sv	+	+
	Nv	+	+
	A	+	-
	V	+	-
	CF	-	-

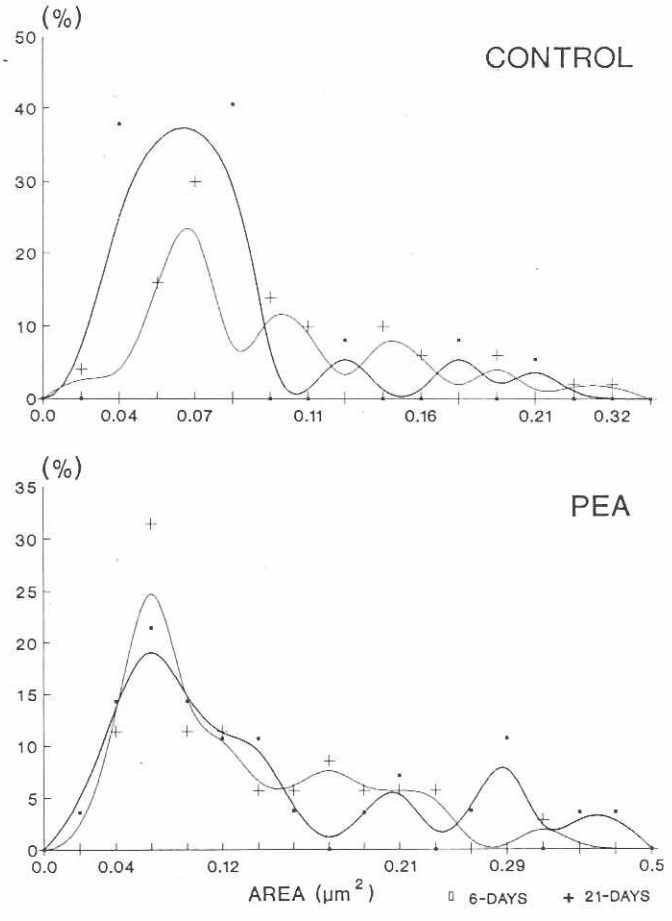


Fig. 2. Graph showing the distribution of mitochondrial area in control and PEA astrocytes. *A total of 200 mitochondrial profiles per group were measured using point counting procedures (Lindberg and Vorwerk, 1970, 1972).*

there was some difficulty to observe cells containing reaction product. However, the TPPase activity was lower in proliferating than in differentiated PEA cells. The cytochemical activity of 5'-nucleotidase, as previously reported in detail (Renau-Piqueras *et al.*, 1990, 1992), was restricted in all cases to the plasma membrane and endocytic vesicles. This activity was more intense in 20- than in 6-day control cells. While in proliferating astrocytes the reaction product appeared as small spots, in differentiated astrocytes the cytochemical activity formed a continuous layer on the cell surface. The 5'-nucleotidase activity was clearly lower in PEA cells than in controls. In 6-day PEA cells the activity was difficult to see and in 21-day PEA astrocytes it was similar to that found in 6-day control cells.

Finally, we have been unable to demonstrate G-6-Pase activity in the different groups of astrocytes analyzed.

Discussion

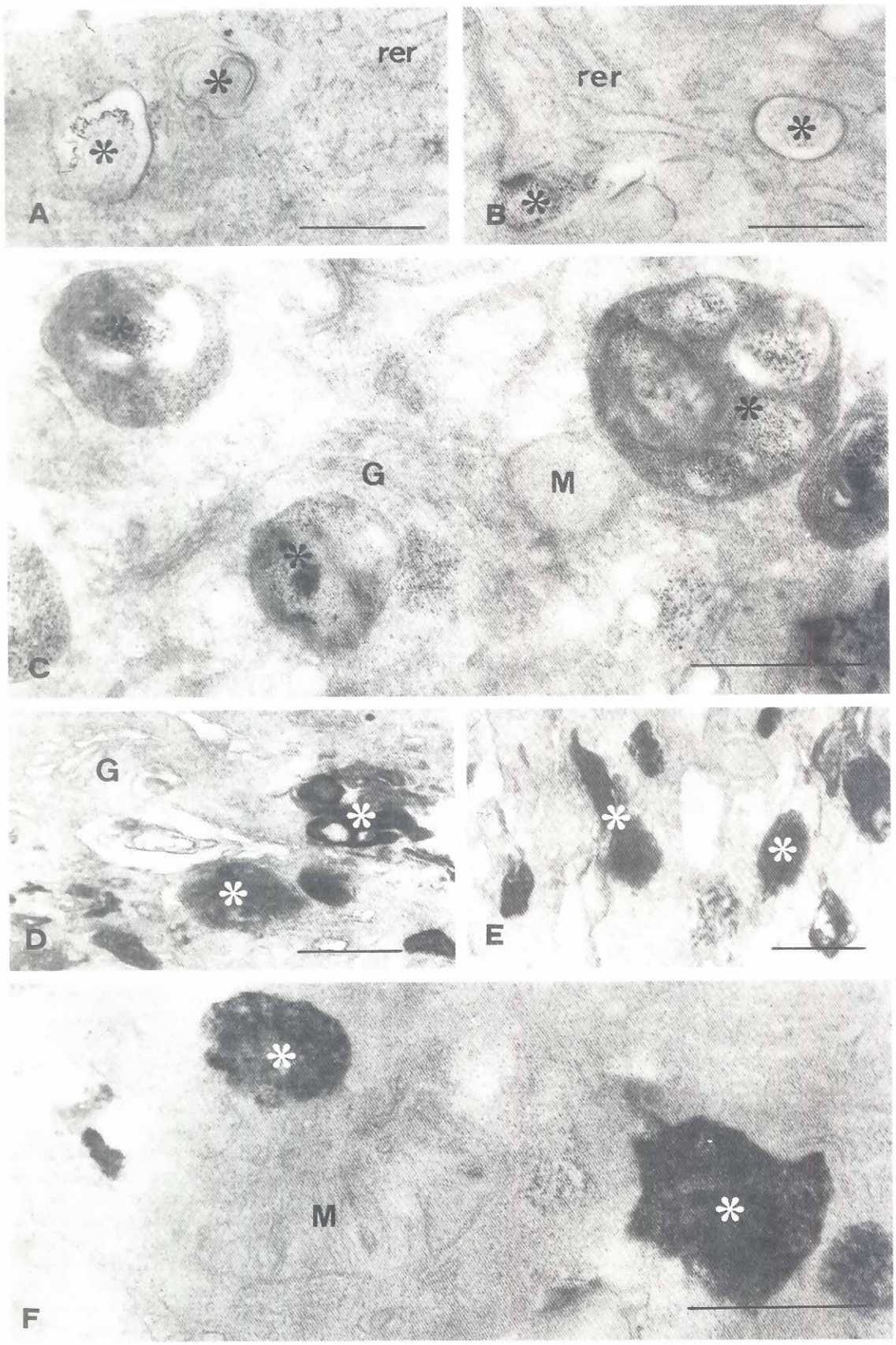
Cortical astrocytes obtained from 21-day-old fetuses, when cultured as primary cultures, grow rapidly during the first 7-10 days (proliferative phase), after which their number increases slowly (differentiation phase) (Renau-Piqueras *et al.*, 1989c). Astrocytes in these two phases differ and can be characterized by several parameters, such as the synthesis of protein, DNA and RNA, the activity of several enzymes, the amount and distribution pattern of cytoskeletal proteins as well as the number of plasma membrane concanavalin A binding sites (Renau-Piqueras et *al.*, 1988, 1989c, 1990, 1992; Guerri *et al.*, 1989, 1990b,c; Sáez *et al.*, 1990).

Our results show that proliferating and differentiated control astrocytes also differ in their ultrastructural organization, which correlated to that for 1- and 4-week-old cultures, respectively, in the type-C colonies described by Fedoroff *et al.* (1984).

In addition to modifications in cytoskeletal organization, which have been described in detail (Trimmer *et al.*, 1982; Fedoroff *et al.*, 1984; Renau-Piqueras *et al.*, 1989c; Sáez *et al.*, 1990), it appears that several cell components, such as mitochondria, rER and lysosomes, exhibit qualitative and/or quantitative ultrastructural changes during the process of astrocyte maturation in primary culture. In some cases, these morphological changes are accompanied by variations in the cytochemical activity of enzymes located in these cell components. The increase in the volume density of rER occurs parallel to that of the UDPase activity, suggesting a functional maturation of this cell compartment during astrocyte differentiation. In contrast, other organelles, such as the GA, do not change morphologically during this process, but display an important increase in the cytochemical activity of the enzymes analyzed during differentiation. Finally, other cell components, such as lysosomes, show no changes during the entire culture period in the amount of cytochemical reaction product per individual organelle. In this latter case, however, there was an augmentation in number during culture, thereby resulting in an increment in the total activity of AcPase per cell.

Thus, the increase which occurs in the activity of various enzymes during astrocyte maturation in primary culture, appears to be related to the functional state of different cell components. This agrees with previous studies showing that the activity of certain astrocyte marker enzymes, such as glutamine synthetase and butylcholinesterase, increase mainly during the first weeks of culture (Guerri *et al.*, 1989). On the other hand, the activity of several membrane-bound enzymes, including (Na+K)ATPase and 5'-nucleotidase (the latter also studied in the present work), showed a striking increment during the differentiation period (Guerri *et al.*, 1989).

An interesting finding is the increment in the volume density of rER throughout the culture period and which, as mentioned, is accompanied by an increase in UDPase activity. This, together with a prominent GA and an increase in the TPPase and UDPase activities in this cell component during astrocyte differentiation, strongly suggests that these cells, mainly during this latter period, may be active in biosynthesis, glycosylation and transport of glycoproteins. In fact, during astrocyte maturation in primary culture there is an increment of both the activity of (Na+K)ATPase and 5'-nucleotidase, which are glycoproteins in nature, as well as of the plasma membrane concanavalin A binding sites (Renau-Piqueras *et al.*, 1990, 1992). On the other hand, the presence of abundant clathrin coated vesicles, nascent endocytic vesicles and multivesicular bodies suggests that astrocytes in primary culture can also be active in endocytosis, which could be closely related with the functional activity of these cells in regulating the extracellular medium in the brain (Bradford, 1986; Kimelberg and Norenberg,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1989). Recent studies showed that astrocytes in primary culture can act as vehicles for the translocation of macromolecules such as transferrin and serum albumin (Juurlink and Devon, 1990) or cationized ferritin (unpublished results).

The results concerning the activities of UDPase, TPPase and 5'-nucleotidase suggest that these enzymes are modulated in the rER and GA during astrocyte development as occurs in other cell types (Doine *et al.*, 1984).

It is also of interest to point out that whereas the TPPase activity in astrocytes shows a labeling pattern similar to that reported in other tissues, including liver (Morré and Ovtracht, 1977; Farquhar and Palade, 1981), the cytochemical activity of UDPase differs from that described in hepatocytes. Thus, whereas UDPase is a good marker for ER and GA in both adult and fetal hepatocytes (Farquhar and Palade, 1981; Robinson and Karnovsky, 1983b; Renau-Piqueras *et al.*, 1985b, 1987a), this activity was scant in the rER of developing astrocytes. On the other hand the qualitative and quantitative changes in mitochondria throughout the proliferative and differentiation periods could be related to changes in the functional activity of these organelles in astrocytes. It has been reported that the activity of several mitochondrial enzymes related to energy transduction increases from the proliferative to differentiation period (Tholey *et al.*, 1982; Avola *et al.*, 1986; Renau-Piqueras *et al.*, 1988). As previously suggested (Avola *et al.*, 1986), these results indicate a relationship between the increase in brain energy metabolism and the differentiation of astrocytes in primary culture.

Both 6- and 20-day PEA astrocytes showed striking ultrastructural and cytochemical alterations as compared with controls. Qualitatively, the main alteration observed in both treated cell types is an increase in lysosomes, mainly autophagosomes, together with an increase in the size of mitochondria, which is more apparent in proliferating cells. Stereological analysis reveals that prenatal exposure to ethanol decreases the volume density of mitochondria and GA as well as the numerical and surface density of mitochondria and rER, respectively. In contrast, stereological parameters concerning lysosomes are clearly increased. Although these alterations were found in both 6- and 20-day PEA astrocytes, proliferating cells appeared more affected than differentiated ones, which could be related to previous findings (Guerri *et al.*, 1990b).

Functional analysis of cell components by means of the study of the cytochemical activity of enzyme markers shows that prenatal exposure to alcohol decreases all the activities tested, except for that of AcPase, which appears increased per individual lysosome in both 6- and 20-day cultures. This effect could be interpreted as damage induced by ethanol or ethanol metabolites on astrocytes during *in utero* exposure.

Since the activity of the enzymes tested is related to the functional activity of the cell components containing these enzymes, the effect of ethanol on the remaining activities studied could be interpreted as the result of a delay in the astrocyte maturation induced by this toxic agent. In fact, it has been shown in previous studies that prenatal exposure to ethanol alters this process both *in vivo* and in primary culture (Renau-Piqueras *et al.*, 1989c; Guerri *et al.*, 1990c; Miller and Potempa, 1990; Davies and Cox, 1991).

It has also been demonstrated that growth kinetics of astrocytic cultures are vulnerable to ethanol *in vitro* and that these changes are, in part, qualitatively manifested in the ultrastructural configurations of cultured astrocytes (Davies and Cox, 1991). On the other hand, studies performed on brain tissue revealed alterations in glial cells. Thus, after administration of an ethanol diet begun on gestational day 12 and continued until postnatal day 7, the glia were greatly distended and had effectively isolated the dendrites from the surrounding neuropil (Smith and Davies, 1990). Also observed were enlarged glial processes wrapped around the parallel fibres in the molecular layer of the cerebellum (Popova and Shchekalina, 1980; Tavares and Paula-Barbosa, 1984) as well as an increase in the amount of perineuronal and total glia (Popova and Shchekalina, 1980). Studies carried out at different days of pre- and postnatal development showed that prenatal exposure to ethanol induces optic nerve hypoplasia in rat, alters the ultrastructure of both astroglial and oligodendroglial cells and decreases the number of these cells (Pinazo-Durán, 1991 in preparation).

Prenatal exposure to ethanol also causes ultrastructural changes in neurons concerning nucleus, mitochondria, ER and GA, which appeared distended and vesiculated (Clarren *et al.*, 1990; Smith and Davies, 1990). Some of these alterations are similar to those observed in fetal liver (Renau-Piqueras *et al.*, 1985b, 1987a, 1989a). It has been reported that many of these changes observed in neurons refer to their maturation process. Thus, a significant delay in differentiation of the neurons occurs in several brain regions (Volk, 1984; Maglóczky *et al.*, 1990; Smith and Davies, 1990). Moreover, recent studies have reported that the extent of ultrastructural alterations in neurons increased as the mean peak plasma ethanol concentrations increased among the full gestational exposed animals (Clarren *et al.*, 1990). However, it remains to be clarified whether these ethanol-induced neuronal alterations are due to a direct effect of the toxin on these cells or are a consequence of the changes observed in astrocytes.

In addition to the effects of prenatal exposure to ethanol on the growth kinetics of astrocytes, a direct cytotoxic effect of ethanol on these cells should also be considered as a complementary mechanism. For example, the increase in the number of lysosomes and, thereby, of the overall activity of AcPase, could be the result of this direct toxic effect. Moreover, the decrease in the 5'-nucleotidase, a membrane-bound enzyme which is synthesized in the rER, glycosylated in the GA and then transported to the plasma membrane, could be due to inhibitory effects of ethanol on protein synthesis, glycosylation or glycoprotein transport. In fact, the results presented here, together with others from previous studies on liver, indicate that prenatal exposure to ethanol alters the function of such cell components as rER, GA, and mitochondria, which are directly involved in these functions (Renau-Piqueras *et al.*, 1985a,b, 1987a,b, 1989a,c; Sancho-Tello *et al.*, 1987; Guerri *et al.*, 1990a). We have also demonstrated, as mentioned above, that astrocyte cytoskeleton, a cell component involved in glycoprotein transport, is also altered by prenatal exposure to ethanol (Renau-Piqueras *et al.*, 1989b; Sáez *et al.*, 1990).

It could be of interest to point out that in other experimental or

Fig. 3. AcPase activity (*) in control and PEA astrocytes. *This activity is scarce in both* **(A, B)** *6- and 20-day control cells, whereas in PEA cells lysosomes show an intense labeling* **(D and E**, *6-days;* **F**, *20-days). No activity was found in the Golgi apparatus (G)* **(C and D).** *(rer, rough endoplasmic reticulum; M, mitochondria). Bars: 0.5 μm. A x44550; B x40250; C x62200; D x37500; E x34150; F x65800.*

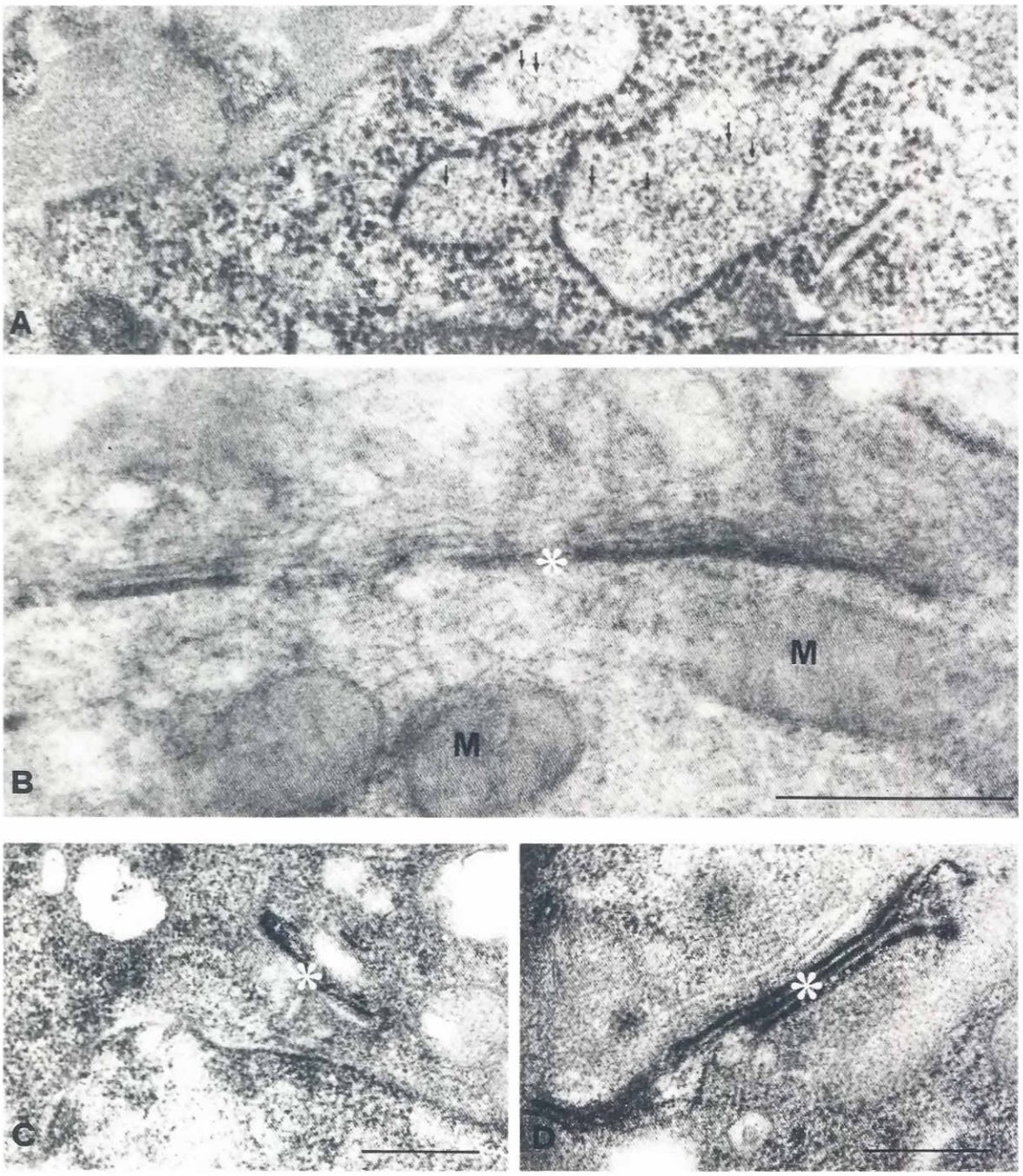

Fig. 4. UDPase activity in astrocytes. (A) *6-day control astrocytes.* **(B)** *20-day control astrocytes. In both cases the UDPase activity was found in the dilated portions of the rER where it was very weak (*)* **(A),** *and in the trans-cisternae of the Golgi apparatus (*)* **(B).** *No reaction product was found in PEA astrocytes.* **(C-D) TPPase activity in control (A,B) astrocytes.** *This activity was located in one-three trans-cisternae (*) of the Golgi apparatus of most control cells* (**C**, *6-days;* **D**, *20-days). (M, mitochondria; N, nucleus). Bars: 0.5 μm. A x73500; B x76800; C x38000; D x41300.*

pathological situations astrocytes show ultrastructural and/or enzymatic changes different to those observed after prenatal alcohol exposure. Thus, TPPase and AcPase showed little changes in cytochemical activity in reactive astrocytes (Al-Ali and Robinson, 1982). In contrast, these cells showed an increase in the activity of G-6-Pase, which was accompanied by an increment of rER (Al-Ali and Robinson, 1982).

Finally, since it has been demonstrated that astrocytes play an important role in neuronal function in both adult and developing brain, the results we report here could be related to some of the

neuronal alterations observed in children and animals prenatally exposed to ethanol.

Materials and Methods

Animal treatment

Adult virgin female Wistar rats with an initial body weight of 200-250 g were used. Animals were fed before and during gestation as previously reported (Sanchís *et al.*, 1986). Briefly, rats were divided into two diet-based groups: (a) the alcoholic animals received an ethanol-liquid diet (5% w/v) in which ethanol provided 36% of total calories (Lieber and DeCarli, 1976); (b) a control group was given a similar diet, except that maltose-dextrin replaced ethanol isocalorically. Both groups were maintained on their diets for 4-5 weeks before mating. After this time, females in proestrus or initial estrus were placed overnight in cages with males of the same strain. The appearance of sperm in the vaginal washing the following morning defined day 0 of gestation.

Astrocyte cultures

Primary cultures of astrocytes from 21-day-old fetuses were prepared from brain hemispheres as described (Renau-Piqueras *et al.*, 1989c). Fetuses were obtained under sterile conditions from both control and alcohol-fed rats and astrocytes were cultured without ethanol in the medium. The purity of astrocyte cultures was assessed using a mouse anti-GFAP monoclonal antibody and fluorescence microscopy (Renau-Piqueras *et al.*, 1989c). Since these cultures grow rapidly for 7-10 days (proliferative period) after which the cell number increases slowly (differentiation period) (Renau-Piqueras *et al.*, 1989c; Guerri *et al.*, 1990c), all the experiments described below were done in triplicate on 6- and 20-day cultures.

Electron microscopy and stereology

Monolayers growing in 35-mm Nunc tissue culture plastic dishes were randomly selected (3 dishes per group and culture period, 3 different cultures), washed three times in cold phosphate buffered saline and fixed in 1.5% glutaraldehyde + 1.0% formaldehyde in 0.05 M cacodylate buffer, pH 7.4, for 60 min at 4°C. After washing in this buffer (0.1 M), the monolayers were postfixed in 2% OsO_4 containing 0.8% potassium ferrocyanide for 60 min at 4°C. The cells were then treated with 0.1% tannic acid in buffer for 1 min at room temperature, washed in buffer and stained in block with 2.0% aqueous uranyl acetate for 120 min at room temperature, dehydrated in cold ethanol, detached from the plastic with propylene oxide and embedded as monolayers in Epon 812 (10 capsules per dish).

For stereological analysis, the sampling method was carried out according to Cruz-Orive and Weibel (1981) and Weibel (1979). Stereological analysis of micrographs was performed using point counting and standard procedures, as described (Weibel, 1979; Renau-Piqueras *et al.*, 1985a). The following relative stereological parameters were evaluated: a) the volume density, Vvi (mitochondria, rough endoplasmic reticulum (rER), Golgi apparatus(GA) and lysosomes), b) the surface density, Svi (mitochondria, rER and lysosomes) and, c) the numerical density, Nvi (mitochondria and lysosomes). As absolute stereological parameters, the mean transsectioned area, Ai, the mean volume, Vi, and the coefficient of form, CF, of mitochondria and lysosomes were determined (Lindberg and Vorwerk, 1970, 1972; Renau-Piqueras *et al.*, 1985a; DePaz *et al.*, 1986a,b).

The minimum sample size (MSS) in each case was determined by the progressive mean technique (confidence limit, ±10%) (Williams, 1977). The MSS for all the stereological parameters analyzed in both control and treated cells was always less than 17. However, 25 micrographs were analyzed for each. All the stereological data were statistically analyzed by the ANOVA test using the Oxstat programme (Oxstat ver. 3.1 (I), IBM).

Cytochemistry

The cytochemical activity of several phosphatases widely used as subcellular markers in various cell types was analyzed (Morré and Ovtracht, 1977): a) acid phosphatase (AcPase) for the lysosomal system; b) uridine diphosphatase (UDPase) for ER and the transportion of the GA ;c) thiamine pyrophosphatase (TPPase) as a specific marker for the trans-GA, d) glucose-6-phosphatase (G-6-Pase), for ER ,and e) 5'-nucleotidase for the plasma membrane.

Since information on the cytochemical activity of these enzymes in astrocytes in primary culture is scarce, several procedures were used for the demonstration of those enzymes located inside the astrocytes. a) The monolayers were incubated, after specific fixation, in the cytochemical medium, postfixed and embedded in Epon, as described above. b) Fixed cells were incubated in the cytochemical medium containing Triton X-100 at 0.0001% to permeabilize cell membranes (Robinson, 1985; Báguena-Cervellera *et al.*, 1987). c) Monolayers were detached from the plastic, centrifuged, and cryostat sections (20 µm) were incubated in the corresponding cytochemical medium (Renau-Piqueras *et al.*, 1987a). The substrates were as follows: 1.0 mM ß-glycerophosphate for AcPase, 1.0 mM UDP for UDPase, 1.0 mM thiamine pyrophosphate for TPPase, 1.0 mM glucose-6-phosphate for G-6-Pase, and 1.0 mM AMP for 5'-nucleotidase. In all cases cerium ions were used as capture agent (Robinson and Karnovsky, 1983a,b; Renau-Piqueras *et al.*, 1987a). Controls were sections incubated without substrate. After incubation, the cells were processed for electron microscopy as described (Renau-Piqueras *et al.*, 1987a). Ultrathin sections (80 nm) were examined at 60 kV without counterstaining.

Immunocytochemistry

For immunocytochemistry the cells were processed as previously described (Renau-Piqueras *et al.*, 1989c). Briefly, the cell monolayers were fixed with 0.5% glutaraldehyde and 4.0% formaldehyde in 0.1 M PIPES buffer (pH 7.3) for 60 min at 4°C, detached from the plastic using a rubber policeman, and embedded in Lowicryl K4M. For localization of GFAP with the immunogold procedure, ultrathin sections mounted on nickel grids were placed on a droplet of 0.1% BSA-tris buffer (20 mM Tris-HCl, 0.9% NaCl, pH 8.2, containing 0.1% BSA, Type V) supplemented with 5% heat-inactivated fetal calf serum (FCS) for 30 min at room temperature and then transferred to droplets of 0.1% BSA-Tris containing 1% FCS and a mouse anti-GFAP monoclonal antibody (10 µg/ml, Boehringer Mannheim GmbH) for 12 h at 4°C in a moist chamber. After three rinses with 0.1% BSA-Tris for 10 min each, the grids were placed on droplets of 0.1% BSA-Tris containing 0.05% Tween 20, 5% FCS, and goat anti-mouse IgG-gold complex (10 nm, Sigma, 1:100 dilution). The incubation was for 12 h at 4°C as above. After two rinses with 0.1% BSA-Tris and a rinse in distilled water, the sections were air dried and counterstained with uranyl acetate. Immunocytochemical controls were incubated in absence of the first antibody.

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