Virchows Arch [Cell Pathol] (1986) 50:271–283

Virchows Archiv B

Ammonium chloride-induced alterations in growth kinetics and ultrastructure of murine neuroblastoma cells

A flow cytometric and stereologic study

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Summary. Neuro-2a and L-132 cells have been used as a model in the study of ammonia toxicity. Incubation of neuro-2a cells for 48 h in the presence of 2 mM NH_4Cl caused inhibition of their growth and accumulation of cells in the G_2M phase of the cell cycle as demonstrated by fluorocytometric methods. Mitotic figures were absent in the treated cell preparations. On the other hand, ammonia had no effect on L-132 cells treated in the same way. Electron microscopy of neuro-2a cells incubated with 2 mM NH_4Cl for 5 days showed striking qualitative and quantitative ultrastructural changes compared with control cells. Treated cells doubled in absolute volume and showed marked alterations in the shape and organization of mitochondria. The absolute volume of mitochondria was also increased which, together with a decrease in their total number, suggests that ammonia induces fusion between adjacent mitochondria. Increases in the total number of lysosomes, multivesicular bodies and lipid droplets were also found in treated cells.

Key words: Neuro-2a cells – Ammonium chloride – Cell cycle – Fluorocytometry – Stereology

Introduction

Hyperammonemia is a clinical manifestation in a number of syndromes of unrelated etiology and different severity. In most cases it may arise from gross damage to the liver due to cirrhosis or hepatotoxic substances. Hyperammonemia may also be induced by spontaneous or surgical portacaval anastomoses and the terms hepatic encephalopathy and portal-systemic encephalopathy have been coined to describe these clinical entities (Conn and Lieberthal 1979), which are usually seen in the adult life.

Hyperammonemia in infancy is mainly due by inborn errors of the urea cycle and related pathways, organic acidemias, Reye's syndrome or it may

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be secondary to the administration of salicylates or valproic acid (Walser 1982).

Ammonia induces convulsions and coma. The primary site of its toxic action is still unclear, but it it well established that ammonia interferes with energy metabolism in brain, inducing depletion of high-energy phosphates. Toxic effects of ammonia on the transmission of nerve impulses have also been implied. The adverse effects of ammonia seem to be related to the energy-consuming mechanism of its detoxification, rather than to an effect of ammonia "per se" (for reviews on the mechanism of ammonia toxicity see Duffy and Plum 1982). In this regard, it seems clear that the cerebral compartment concerned in ammonia disposal is the astroglia (Gutierrez and Norenberg 1975).

Pathological alterations in the cellular ultrastructure in association with hyperammonemic conditions are well known. They have been shown in the liver of children suffering from inborn errors of the urea cycle (Walser 1982) and from Reye's syndrome (Conn and Lieberthal 1979) and also in mice subjected to sustained hyperammonemia (O'Connor et al. 1984b). Astrocytic proliferation and the presence of Alzheimer type II astrocytes are common findings in hepatic encephalopathy (Victor et al. 1965) and experimental hyperammonemia (Cavanagh and Kyu 1971; Diemer and Laursen 1977; Gutierrez and Norenberg 1975).

Inborn errors of the urea cycle in infancy are usually accompanied by severe impairment of neural development. Cortical atrophy and spongy degeneration have been shown at necropsy in children dying from these congenital abnormalities (Walser 1982). In spite of this, little is known of the effects of ammonia on the fine structure and behaviour of nerve cells (Martinez 1968; Victor et al. 1975; Horita et al. 1981). In this study we have considered the influence of ammonia on the cell cycle of cultured murine neuroblastoma cells (neuro-2a cells) and its relationship to the morphological and ultrastructural changes encountered.

Material and methods

Cell cultures. The clonal line neuro-2a, C-1300 mouse neuroblastoma, was obtained from the American Type Culture Collection (Rockville, Md, USA). They were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum. Stock cells were grown in Falcon 250 ml plastic tissue flasks in a humidified 37° C incubator in a 5% CO_2 atmosphere, and were subcultured twice weekly.

In a series of experiments, cells were seeded at 200,000 cells/ml in Falcon 25 ml plastic tissue culture flasks in the same medium. In some of the flasks ammonium chloride (Merk) was added so that the final concentrations were 1 and 2 mM (in 5 ml of medium). Control cultures received the same amount of sterile physiological saline. Cultures were divided every two days and fresh medium containing the appropiate amount of ammonium chloride or sterile physiological saline was used for diluting the cell suspension. The osmolarity of the control and experimental media were similar (270 mOsm). For evaluation of the growth curve, a monolayer from each dilution was harvested every 24 h. The medium was decanted and cells were trypsinized and resuspended in their medium; counting was performed in a hemocytometer and viability was checked by the trypan blue exclusion test. Cell growth was observed up to 96 h after seeding and the experiments were repeated three times. To assess the specificity of the ammonia effect on neuro-2a cells, L-132 fibroblasts were used as controls.

Cell cycle. For evaluation of the effects of ammonia on the cell cycle, neuro-2a cells were grown as described above in the presence of 2 mM NH_4Cl. Control medium contained similar amounts of physiological saline. The cell cycle was studied by flow cytofluorometry. For fluorescent DNA staining, propidium iodide (PI) was used following the hypotonic method of Fried et al. (1976). Briefly, cells growing in monolayers were rinsed once with 5 ml of serum-free medium, and 5 ml of cold staining solution (PI, 0.05 mg/ml in 0.1% Na-citrate containing 0.1% Triton X-100) were added. Flasks were kept at 4° C overnight. Residual cells were dislodged by hand pipetting and the suspension of stained cells were transferred to test tubes.

Cell fluorescence was measured in a EPICS V cell sorter (Coulter Electronicsm Inc.) using an argon ion laser at 488 nm and 250 mw (filter setting: 515 IF + 515 AB + mirror + 560 SP). Data were plotted as two-parameter histograms of "red" fluorescence versus number of cells, and processed statistically by the Coulter MDADS hardware and EASY-1 software interfaced to the EPICS V. Control of the specificity of DNA staining was assessed by simultaneously plotting two-parameter histograms of light scatter versus red fluorescence.

Electron microscopy. After incubation in 2 mM NH_4Cl for 48 h or 5 days, treated and control neuro-2a cells (four samples per case) were washed in fresh medium, centrifuged and the cell pellets fixed in 1.0% glutaraldehyde – 0.8% formaldehyde in 0.05 M cacodylate buffer (pH 7.4 at 4° C, 2 mM $MgCl_2$) for 15 min at 4° C. The pellets were then cut into 30 small cubes, refixed in the same fixative solution for 45 min at 4° C, washed in 0.1 M cacodylate buffer (pH 7.4, 270 mOsm, 2 mM $MgCl_2$) for 2 h at 4° C, and postfixed in 2% OsO_4 containing 0.8% potassium ferrocyanide for 3 h in the dark at room temperature. The effective osmolarity of the glutaraldehyde-based fixative was adjusted to 270 mOsm as described previously (Renau-Piqueras et al. 1980). After washing in water, the cell blocks were stained with 1% uranyl-acetate for 45 min at room temperature, dehydrated in a graded series of acetones and embedded in Poly/Bed 812 (four blocks randomly chosen per flask and case). Ultrathin sections (one section per block, interference color silver) were stained with uranyl-acetate and lead citrate. Ocassionally, blue sections (about 190 nm) were also cut.

Stereology and morphometry. From each ultrathin section micrographs were made at two levels. At level I ($\times 9576$) the whole cell and the nucleus were assessed. At level II ($\times 20{,}520$) mitochondria, lysosomes, Golgi apparatus and lipid droplets were estimated. In addition, the cell size was also determined using semi-thin sections examined with the light microscope. Stereological analysis of the micrographs was made by planimetry using a Leitz-ASM system for semi-automatic image analysis (O'Connor et al. 1984b; Renau-Piqueras et al. 1985) and using standard stereological formulas (Weibel and Bolender 1973; Williams 1977). The parameters determined are listed in Tables 1 and 2. The minimum sample size (number of micrographs to be measured) for each parameter was determined by the progressive mean (confidence limit, $\pm 5\%$).

As an evaluation of the shape of cells, nuclei and mitochondria the coefficient of form (CF) was determined using the expression $CF = 4\pi \text{ Area}/\text{Perimeter}^2$ (Renau-Piqueras et al. 1985). To assess the area distribution curve of mitochondria a total of 600 mitochondria profiles per case were measured.

Since no statistical differences (analysis of variance, $P \leq 0.05$) were found between cell cultures within each group, the results are expressed as the mean $\pm$ sd of each group. Finally, statistical comparison of the stereologic and morphometric data were made by Student's *t*-test ($P \leq 0.05$).

Results

Cell growth. As can be seen in Fig. 1 A, ammonia strongly inhibited the growth of neuro-2a cells, even at doses in the range of those found in the brains of patients suffering from acute hyperammonemia. This effect was more marked in the first hours of exposure to ammonia and seemed to diminish slightly as the culture time increased. When the effect of ammonia

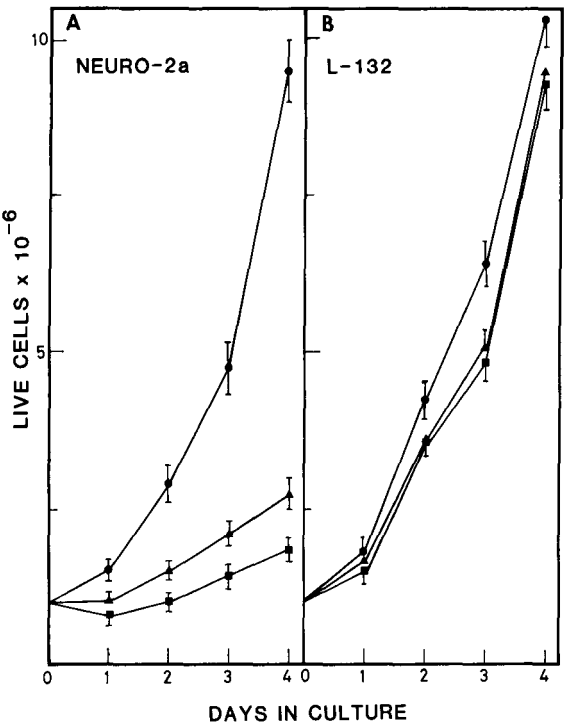


Fig. 1 A, B. Effects of ammonia on the growth of **A** neuro-2a or **B** L-132 cultures. The experimental conditions are described in Material and Methods. Flasks containing saline (●), or ammonium chloride 1 mM (▲) or 2 mM (■). Results are the mean ± sd of three experiments with duplicate flasks

was tested in a cell line of non-nervous origin, no significant inhibition of growth was observed. Fig. 1 B shows the effect of ammonia on L-132 cells, an established cell line from a human lung carcinoma. The inhibitory effect of ammonia on neuro-2a cell growth cannot be explained by a decrease in cell viability since values for the dye exclusion test were similar for control and ammonia-treated cells. Thus, a blocking effect of ammonia on the progression through the cell cycle seems to be a plausible explanation for this inhibition.

Flow cytometry provides an accurate and rapid procedure for determining the distribution of a cell population in the different phases of its cycle. Figure 2 shows the effect of ammonia on the cell cycle of neuro-2a cells. Figure 2A shows the distribution of control cells after 48 h of culture. The histogram shows the G_1 peak and that corresponding to G_2 and M phases, which cannot be distinguished by the fluorocytometric method used and is therefore termed G_2M. Between the two peaks extends the S phase. The histograms shown in Fig. 2B and 2D correspond to cells growing in the presence of 2 mM NH_4Cl. As can be seen, the G_2M phase is clearly increased, especially after 48 h of culture (Fig. 2B). Percentage values show an increase in cells in both G_1 and S phase, accompanied by decreases in cells in both G_1 and S phases. This effect seems partially reversible since after 96 h of culture (Fig. 2D) the pattern of distribution tended to match that of control cells (Fig. 2C). This correlates well with the kinetics of cell growth, as described in Fig. 1, indicating that the increase in the slope of the growth curve seen in the latter stages of cell culture might be asso-

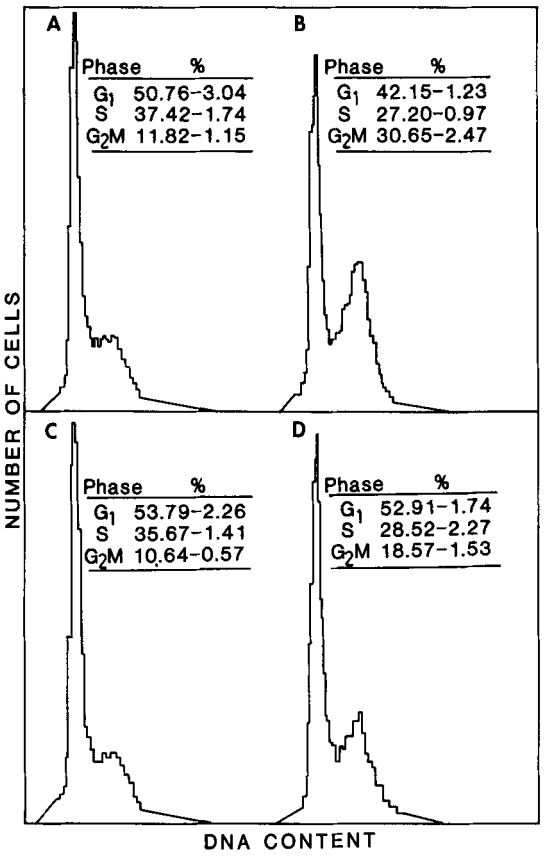
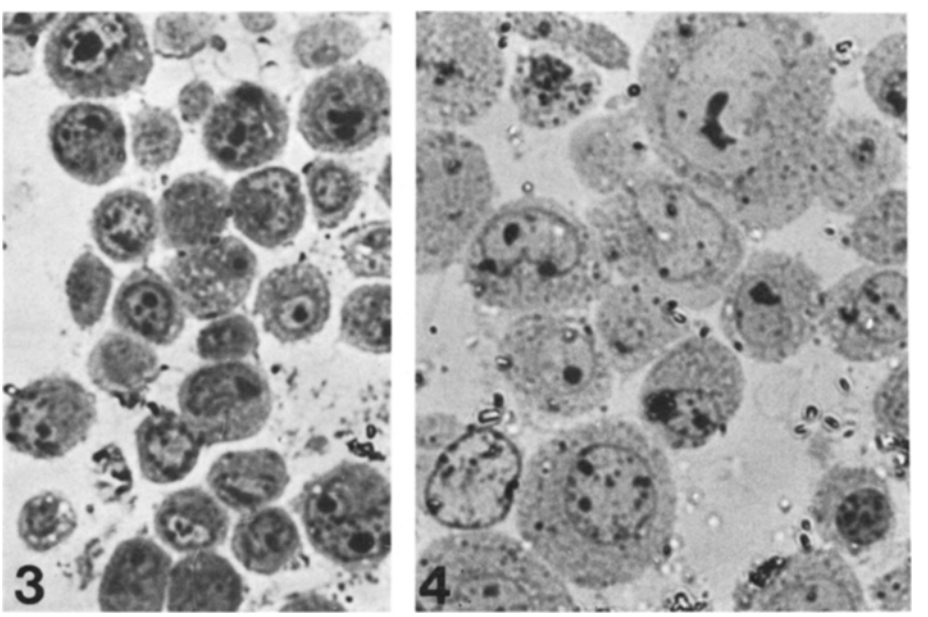
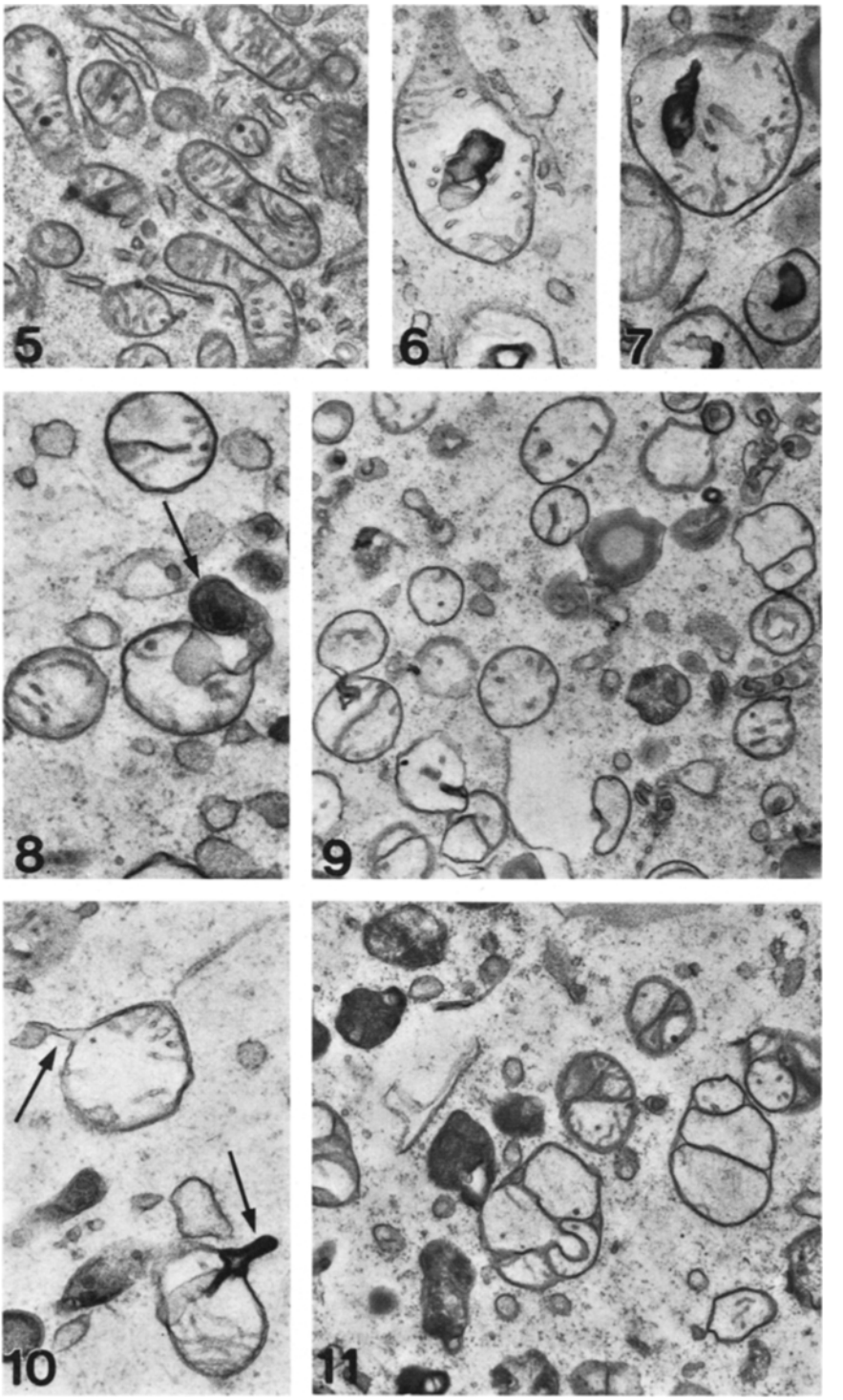


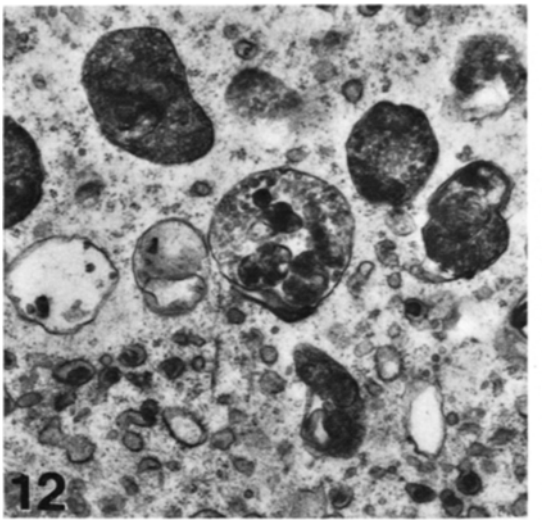
Fig. 2 A–D. Effects of ammonia on the cell cycle distribution in neuro-2a populations. Cells were cultured in the presence of 2 mM ammonium chloride, as described in Methods. After 48 h (panel **B**) and 96 h (panel **D**), cells were prepared for DNA analysis by flow cytometry, as described. Controls were cell populations growing for 48 h (**A**) or 96 h (**C**). Figures are derived from computer generated histograms and are representative of each experimental condition. Numerical data are the mean ± sd of three experiments with duplicate flasks. Values are calculated from individual DNA content through sequential analysis by the Multiparametric Data Adquisition and Extended Analysis Systems of the flow cytometer. The scale varies depending on the number of particles analyzed

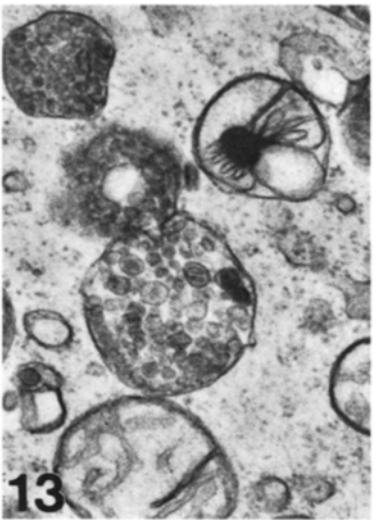


Figs. 3, 4. Micrographs showing semi-thin sections of control neuro-2a cells (Fig. 3) and 2 mM NH_4Cl treated neuro-2a cells (Fig. 4). Micrographs are at the same magnification and illustrate the variation in the cell size after five-days treatment



Figs. 5–11. Effects of ammonia on mitochondria of neuro-2a cells. Five days of treatment. Figure 5 shows mitochondria in control cells. Figures 6–11 refer to ammonia treated cells. Figures 6, 7 and 9 illustrate the changes of mitochondria in size and shape as well as in the matrix organization. Figures 8 and 10 show the formation of blebs (*arrows*). Figure 11 show early stages of autophagic vacuoles. (Fig. 5, ×16,573; Fig. 6, ×19,730; Fig. 7, ×18,838; Fig. 8, ×24,428; Fig. 9, ×16,522; Fig. 10, ×26,439; Fig. 11, ×17,381)





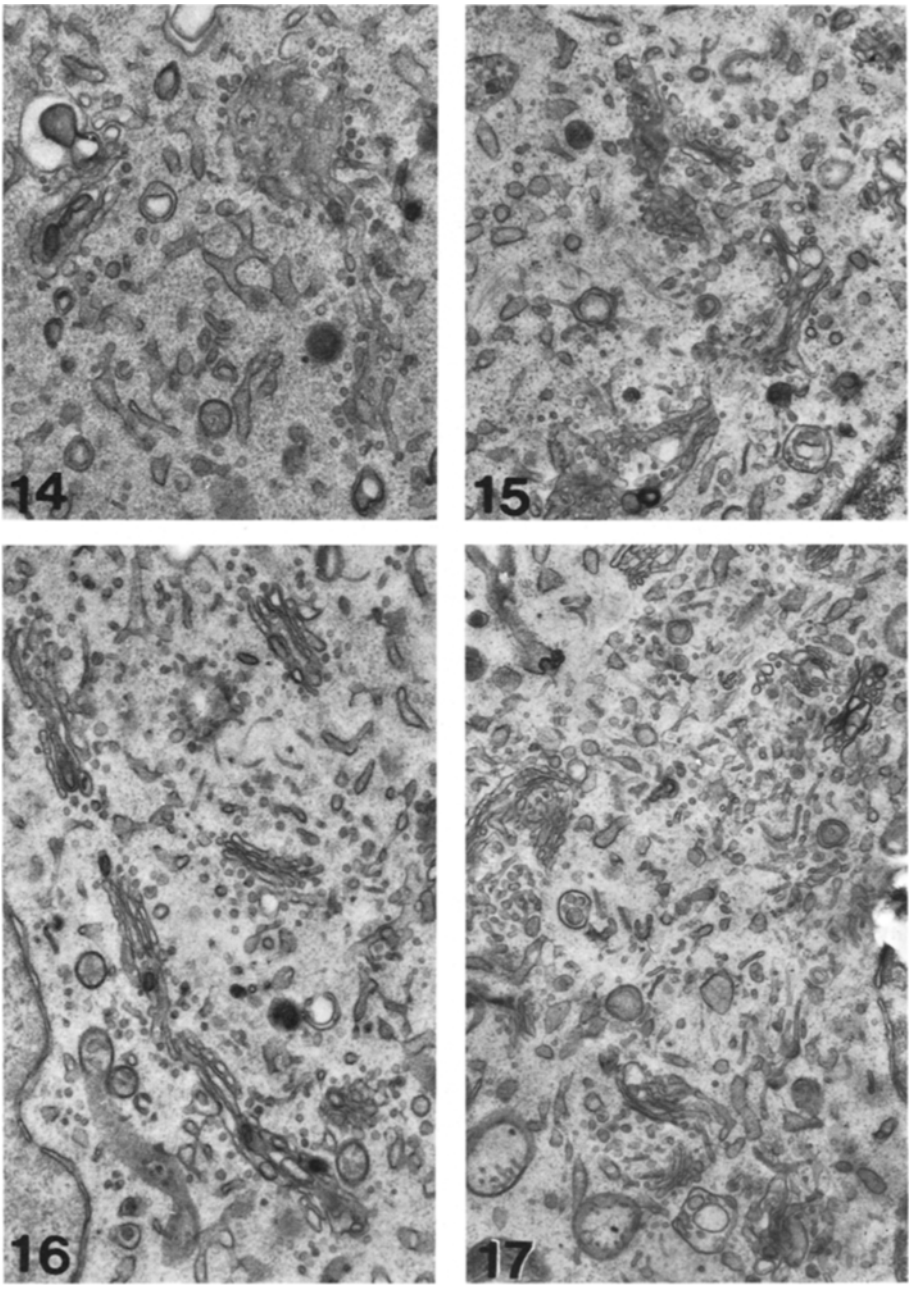
Figs. 12, 13. Micrographs showing areas of cytoplasm of treated neuro-2a cells containing increased number of MVB. (Fig. 12, ×15,811; Fig. 13, ×29,990)

ciated with a partial restoration of the normal cell cycle distribution. This suggests an adaptive mechanism by neuro-2a cells against ammonia injury.

Electron microscopy

Qualitative results. After 48 h treatment, the ultrastructure of neuro-2a cells was similar to that of control cells. However, when cells were incubated for 5 days in the presence of ammonia, striking ultrastructural alterations were observed when compared with control cells. Treated cells were larger than control cells (Figs. 3, 4) and showed several changes in the mitochondria including an electron-lucent matrix, disorganization of cristae and, in some cases, small blebs on the outer membrane. Occasionally, probable fusion between adjacent mitochondria was observed. Some examples of these alterations are illustrated in Figs. 5–11. In addition, mitochondria in treated cells were larger and more spherical than those of control cells (Figs. 5–7). Another finding in ammonia-treated cells was a notable increment in the lysosomal compartment, particularly in multivesicular bodies (MVB) (Figs. 11–13) which increased from 12% of the total lysosome population in control cells, to 56% in ammonia-treated cells. Finally, the Golgi apparatus in treated cells appeared slightly disorganized and more developed than in control cells (Figs. 14–17). Mitotic figures were absent in preparations of treated cells either by light or by electron microscopy. Binucleated cells were not found in these preparations.

Quantitative results. Stereological analysis showed no differences between 48-h and 5-day control cultures of neuro-2a cells. Moreover, the stereological parameters of 48-h treated cells were similar to those of 48-h controls.



Figs. 14–17. Figures 14 and 16 correspond to areas of Golgi apparatus in control cells, and Figures 15 and 17 illustrate the Golgi apparatus in treated cells. (Fig. 14, ×22,572; Fig. 15, ×14,500; Fig. 16, ×17,784; Fig. 17, ×14,030)

Table 1. Relative stereological data (mean ± sd) of control and five days ammonium treated neuro-2a cells

Component	Parameter[a]	Control	Ammonia	Units
Nucleus	$Vv_{n,c}$[b]	30.88 – 3.45	31.26 – 5.72	μm^0
	$Sv_{n,c}$	0.26 – 0.04	0.16 – 0.02*	μm^{-1}
Mitochondria	$Vv_{m,cy}$	6.27 – 1.75	9.19 – 2.27*	μm^0
	$Sv_{m,cy}$	0.73 – 0.28	0.66 – 0.28	μm^{-1}
	$Nv_{m,cy}$	0.93 – 0.22	0.30 – 0.01*	μm^{-3}
Lysosomes	$Vv_{ly,cy}$	0.84 – 0.31	1.23 – 0.39*	μm^0
	$Sv_{ly,cy}$	0.15 – 0.02	0.17 – 0.01*	μm^{-1}
	$Nv_{ly,cy}$	0.47 – 0.05	0.31 – 0.05*	μm^{-3}
Golgi	$Vv_{g,cy}$	7.92 – 1.92	10.19 – 2.01*	μm^0
	$Sv_{g,cy}$	0.29 – 0.01	0.39 – 0.01*	μm^{-1}
Lipids	$Vv_{l,cy}$	1.92 – 0.15	0.69 – 0.13*	μm^0
	$Sv_{l,cy}$	0.16 – 0.09	0.07 – 0.01*	μm^{-1}
	$Nv_{l,cy}$	0.13 – 0.02	0.08 – 0.01*	μm^{-3}

* Significant differences between control and treated neuro-2a cells ($P \leq 0.05$)

[a] The first subindex corresponds to the cell component and the second to the reference volume

[b] Volume density values in %. Vv, Volume density; Sv, Surface density; Nv, Numerical density; c, Cell; n, Nucleus; cy, Cytoplasm; m, Mitochondria; ly, Lysosomes; g, Golgi apparatus; l, Lipid droplets

Table 2. Absolute stereological data (mean ± sd) of control and five days ammonium treated neuro-2a cells

Component	Parameter	Control	Ammonia	Units
Cell	Vc[a]	1,615.01 – 275.92	3,542.74 – 341.53*	μm^3
Nucleus	Vn	561.52 – 192.60	1,176.83 – 232.54*	μm^3
Cytoplasm	Vcy	1,053.33 – 211.10	2,365.91 – 265.31*	μm^3
Mitochondria	Nt_m	989.20 – 26.68	693.36 – 29.91*	μm^0
	Vm.s[b]	0.09 – 0.02	0.31 – 0.07*	μm^3
Lysosomes	Nt_{ly}	498.77 – 18.67	790.44 – 72.70*	μm^0
Lipids	Nt_l	142.77 – 9.88	165.09 – 9.78	μm^0
	Vl.s[c]	0.18 – 0.03	0.12 – 0.08	μm^3

* Significant differences between control and treated cells ($P \leq 0.05$)

[a] Absolute cell volume, in semi-thin sections: Control cells, 1,671.85 ± 84.59 μm^3; treated cells, 3,032.91 ± 407.96 μm^3

[b] Mean value for an individual mitochondrion

[c] Mean values for an individual lipid droplet; V, Absolute volume; Nt, Total number of components per cell; c, Cell; n, Nucleus; m, Mitochondria; ly, Lysosomes; l, Lipid droplet

Data summarized in Tables 1 and 2 indicate that after 5 days treatment, control and treated neuro-2a cells are significantly different in several variables including the absolute nuclear and cellular volume, the absolute volume of a single mitochondria, the volume and surface density of lysosomes and Golgi apparatus and the total number of lysosomes and lipid droplets,

Table 3. Coefficient of form (CF) of cells, nuclei and mitochondria. Control and five-days ammonium treated neuro-2a cells (values as mean ± sd)

Component	Parameter	Control	Ammonia	Units
Cell	CF_c	0.90 – 0.04	0.82 – 0.12	μm^0
Nucleus	CF_n	0.74 – 0.12	0.76 – 0.13	μm^0
Mitochondria	CF_m	0.67 – 0.16[a]	0.85 – 0.08[a, *]	μm^0

* Significant differences between control and treated cells ($P \leq 0.05$)

[a] These values correspond to theoretical ellipses with, a (long axis) = 1.0 μm and b (short axis) = 0.393 μm, in control cells, and a = 1.0 μm and b = 0.557 μm, in treated cells

all of which appeared increased in treated cells. On the other hand, the numerical density of mitochondria and lysosomes, the volume, the surface and numerical density of lipid droplets, the total number of mitochondria and the absolute volume of a single lipid droplet were greater in control cells.

Finally, the cells also differed in the mitochondrial CF (Table 3) as well as in their respective mitochondrial area distribution curves (not shown).

Discussion

In the present work we have shown that ammonia in concentrations similar to those occurring in pathological states (Benjamin 1982) induced marked alterations in neuro-2a cells "in vitro". Thus, growth was strongly inhibited when ammonia was added to the culture medium. This compound, however, has little effect on the growth of lung fibroblasts, indicating a preferential action of ammonia on cells of neural origin, which agrees with previous results (Tsiang and Superti 1984). Our results concerning the effect of ammonia on the neuro-2a cell cycle clearly show an accumulation of cells in the G_2M phase which could be related to the retarded proliferation ability of these cells. DNA-staining by PI does not separate the G_2 phase from mitosis. Nevertheless, the absence of mitotic figures, as shown by microscopy, indicates that the ammonia-induced blocking effect takes place prior to mitosis. The absence of astrocytic mitoses has been reported in experimental hepatic encephalopathy (Norenberg and Lapham 1974), after methionine sulfoximine treatment (Gutierrez and Norenberg 1975) and in both human and experimental Alzheimer II astrocytosis (Cavanagh and Kyu 1981; Norenberg and Lapham 1974). This effect of ammonia on the cell cycle of neuro-2a cells could be related to decreases in both protein synthesis and in energy-generating processes. Inhibition of protein synthesis has been demonstrated in the developing brain in the presence of hyperammonemia due to methionine sulfoximine treatment and in other situations (reviewed by Duffy and Plum 1982). Moreover, several studies have reported that ammonia may interfere with energy-generating processes in brain mitochondria (reviewed by Benjamin 1982). In addition, specific effects on microtubular assembly

could be involved in the ammonia-induced inhibition of mitosis. Similar effects on astrocytes have been ascribed to an increased membrane permeability (Cavanagh and Kyu 1971; Diemer and Laursen 1977). In this regard, it has been shown that some compounds affecting membrane properties, such as local anaesthetics or ethanol, also influence microtubules (Marx 1976). We have recently reported that ammonia increases membrane fluidity (O'Connor et al. 1984a). Thus, a double effect of ammonia on cell membranes and microtubules appears possible.

Another explanation for the accumulation of cells in the stages of higher DNA content could be the conversion of some cells to tetraploidy. This effect has been invoked to explain the absence of mitoses in Alzheimer II astrocytosis (Lapham 1961). Since in our experiments this accumulation is associated with inhibited proliferation, which seems to be partially reversible with time, the possibility of amitotic cell division is unlike. Thus, it appears that a block in the G_2 phase is responsible for the ammonia-induced arrest in growth.

The relevance of these results to the pathological alterations seen in hepatic or portal-systemic encephalopathies are not evident, given the fact that neurons do not divide in adults. Nevertheless, there are a number of hyperammonemic syndromes in early infancy which result in impaired development of the brain and cortical atrophy. In these cases, an ammonia-induced arrest of neuronal proliferation could be of importance in determining the pathological features found. The use of a neuroblastoma line provides a model of actively dividing neural cells. The growth rate of the neuroblastoma cells is superior to that of primary cultures of cells from fetal or newborn brains and this is of advantage in amplifying their sensitivity to the adverse effects of substances such as ammonia. Nevertheless, we believe that it would be of interest to extend these investigations to primary cultures, as a model more pertinent to the infant brain.

On the other hand, ammonia induces a notable increment in the volume of neuro-2a cells as demonstrated by both light and electron microscopy. Increase in cell volume has been noted in many hyperammonemic states (reviewed by Duffy and Plum 1982) although the mechanism leading this effect remains largely unknown. It has been reported that ammonia enhances water uptake and induces a slight swelling of neurons (Benjamin 1982), but this alone would not be sufficient to explain how neuro-2a cells double in volume after ammonia treatment. Since many cells double in volume prior to mitosis (Knutton et al. 1975; Pasternak 1976; Steen and Lindmo 1978), the inhibition of mitosis and the arrest and accumulation of neuro-2a cells in G_2 phase could be an additional contribution to the swelling of these cells.

Another finding present in most treated cells is an increase in the mitochondrial volume accompanied by changes in their shape and in the ultrastructure of the cristae and outer membranes. Such effect of ammonia or ammonia derivates on mitochondria have been reported "in vivo" (Wakabayasi et al. 1984; O'Connor et al. 1984b) and the increase in the mitochondrial volume has been ascribed to fusion of adjacent mitochondria (Waka-

bayasi et al. 1984). Our quantitative results are consistent with this since, in addition to the increase in the absolute mitochondrial volume, we have observed that after ammonia treatment the mitochondrial volume density increased only 1.47 times whereas both the mitochondrial numerical density and the total number of mitochondria per cell diminished 3.10 and 1.43 times, respectively. However, this cannot exclude the possibility that as a response to ammonia, a new population of mitochondria capable of division may have been generated, since during five days of treatment a considerable turnover may have occurred. Moreover, the alterations in the mitochondrial membranes imply alterations in the function of these organelles, as has been previously suggested by Benjamin 1982, and Duffy and Plum 1982.

Exposure of neuro-2a cells to 2 mM NH_4Cl for 5 days causes an increase in the volume and surface density of the Golgi apparatus as well as in the stereologic variables concerning lysosomes. Although an effect of ammonia on the lysosomal compartment has been described (Seglen 1976; Maxfield 1982), we have found that this effect is particularly notable on the MVB, which could be related to the effect on the Golgi apparatus. Both systems, the MVB as endosomes and the Golgi apparatus, play important roles in the processes of endocytosis and membrane recycling (Helenius et al. 1983). Our results suggest that ammonia could affect these phenomena and, in this regard, it has been shown that 20 mM ammonium chloride inhibits endocytic processes in murine neuroblastoma cells faster than in fibroblasts (Tsiang and Superti 1984). Finally, we have observed that ammonia increases the lipid content in neuro-2a cells, which agrees with previous results in the liver of urease-treated mice (O'Connor et al. 1984b).

In summary, the observations reported here concerning the cell cycle, cell size and ultrastructure of neuro-2a cells resemble, in some aspects, those described in central nervous system cells in several pathological states which have in common increased levels of ammonia. It should be of interest, therefore, to extend these observations with further studies using neuro-2a cells and primary cultures of neuronal and glial cells.

Acknowledgements. We are indebted to Dr. F. Thompson for critically reading this manuscript. This work was supported partially by Laboratorios Glaxo (Spain), the KUMC-IIC International Molecular Cytology Program and FISS. The EPICS V cell sorter is property of Tabacalera S.A.

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Received July 9 / Accepted October 9, 1985