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Characterization of the Morphological Variations of Astrocytes in Culture Following Ethanol Exposure

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Abstract: LUC BARRET, ARIANE SOUBEYRAN, YVES USSON, HÉLÈNE EYSSERID AND RAYMOND SAXOD. Characterization of the Morphological Variations of Astrocytes in Culture Following Ethanol Exposure. *Neurotoxicology* **17**(2): 497-508, 1996. *The nervous system is one of the main targets of ethanol toxicity and it has been suggested that astrocytes might play an important role as their integrity is essential for the normal growth and functioning of neurons. Morphological variations of astrocyte cultures were therefore examined after exposure to various doses of ethanol (0.5, 1 and 2%) for different durations (24, 48, 72 and 96 h). The percentage of cell viability and the cell density were calculated and the changes in astrocyte morphology were assessed by an image analysis system (Samba™ 2005) allowing the characterization of 5 parameters (perimeter, surface, elongation factor, convexity factor and the form factor) of a great number of cells (over 6500). This was necessary because of the high variability in normal cultured astrocyte morphology. A two-way statistical approach (2-factors ANOVA completed by stepwise discriminant analysis) was adopted to emphasize the differences between control and exposed cells. In such conditions, ethanol treated cells became more elongated, less circular and more concave and did not grow like non-exposed cells. The mean pooled values of these parameters tended to be modified as a function of the dose of ethanol. The relationships between parameters clearly separated the groups as a function of the different doses. Finally no significant difference was observed in cell viability and cell density despite lower scores in the groups exposed to the highest dose of ethanol for the longest time. Our results suggest that ethanol might affect astrocytes in two different but probably complementary ways by modifying the cell shape and by altering normal cell development.* © *1996 Intox Press, Inc.*

Key-Words: Ethanol, Astrocytes, Morphometry, Cell Culture

INTRODUCTION

Ethanol is subject to frequent abuse leading to important consequences in terms of individual and social disturbances. The nervous system is one of its main targets. The acute clinical effects due to heavy intake, the withdrawal syndrome characterized by delirium and convulsions, as well as the long term effects of alcohol on memory, mental efficiency, cerebellar structures and the peripheral system are well-known. Pre-natal exposure to ethanol also induces a wide spectrum of adverse effects on the nervous system.

Since astrocyte integrity is essential for normal growth and functioning of neurons (Kimelberg and Norenberg, 1989), experimental studies and necropsy observations have not surprisingly shown a wide range of structural and functional alterations in neurons as well as in astrocytes. In this context, astrocyte cultures could be a useful tool for exploring, for example, *in vitro* ethanol effects on cellular morphology (Rönnbäck *et al.*, 1988). Cell cultures are indeed particularly useful as they avoid the interference of secondary effects such as undernutrition following prolonged and heavy exposure to ethanol which could complicate the data interpretation (Sato *et al.*, 1990). Culture aggregates or monolayer culture systems have also been successfully used to elucidate several cellular neurotoxic actions of CNS xenobiotics (Venadakis *et al.*, 1985).

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Morphometry is an important tool for assessing nerve or cell alterations or for developmental studies of the peripheral nerve (Friede and Samorajski, 1968, Torch *et al.*, 1990, Tranqui *et al.*, 1993). However, apart from a recent study (Davies and Cox, 1991), morphometry has rarely been applied to the study of cell culture alterations following ethanol exposure. It was thus interesting to combine the advantages of both techniques, *i.e.* cell culture and morphometry, in order to assess cellular alterations induced by ethanol.

Our study aimed at describing morphological variations after exposure of secondary monolayer astrocyte cultures to ethanol. These morphological variations were evaluated by 5 parameters (perimeter, surface, elongation, form and convexity factors). The influence of several dosages of ethanol and different durations of exposure were also tested. The cell viability and the cell density were also measured in the same conditions.

MATERIALS AND METHODS

Cell Culture

Primary cultures of astrocytes were prepared aseptically from cerebral hemispheres of 1- and 2-day-old Sprague-Dawley rats (Iffa-Credo, France) according to previously described methods (Booher and Sensenbrenner, 1972, Klein and Fricker, 1992) with a few modifications. After mechanical dissociation, the cells were centrifuged (8 min, 160 g) twice in PBS and the pellet resuspended in 50% V/V HAM's F-12 (Gibco) / Minimal Essentiel Medium (Gibco) with 10% fetal bovine serum (FCS, Boehringer Mannheim, Meylan, France), 0.03% L-glutamine (Gibco), 0.6% D-glucose (Sigma), 0.11% sodium bicarbonate (Sigma), 0.12% Hepes (Sigma), 0.1 mg/ml-100U/ml streptomycin-penicillin (Boehringer Mannheim). Tissue culture dishes (Falcon, 87 mm diameter, Beckton Dickinson, Polylabo, Strasbourg, France) were filled with 5 ml of 0.001% poly-Lysine (Sigma) for 30 min, emptied and air dried. Dissociated cells were plated at a density of 5×10^4 viable cells per cm^2 and cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed 1 h after seeding and then every 3 days. After 7 days *in vitro* (DIV), confluent primary cultures were washed with PBS. Purified astrocytes were harvested by manual shaking and flushing in trypsin (0.08%) / EDTA (0.037%) (Sigma). The suspension was centrifuged (5 min, 160g) and the cell pellet resuspended in the culture medium and filtered through a 20 µm nylon mesh. For morphometric studies of secondary cultures, cells were seeded at a low density (6000 cells per cm^2) in 24-well multidishes (Nunc, Gibco) on 14 mm diameter glass coverslips (Polylabo) coated with 400 µl of 0.001% poly-L-Lysine for 30 min, rinsed with sterile distilled water and PBS, and dried before use.

Ethanol Treatment

In order to study the morphological effects of ethanol on cells, secondary cultures at 4 DIV were treated with medium containing ethanol at 0.5%, 1.0% or 2.0% w/v (*i.e.* 108.5 mM, 217 mM or 434 mM). Control cultures received the same medium without ethanol. To reduce ethanol evaporation which may represent 70% of the ethanol dose in an open system of culture at 37° (Borgs *et al.*, 1993), culture plates were wrapped in parafilm and media were replaced every 24h. In such conditions, ethanol evaporation is less than 30% at the end of each 24 h period (data not shown) in agreement with previous works (Lokhorst and Druse, 1993). Coverslips were removed after 24, 48, 72 or 96 h, and then treated for immunocytochemistry.

Immunocytochemistry

Astrocytes were identified by the immunocytochemical localization of glial fibrillary acidic proteins (GFAP). Following intoxication experiments, cultures were fixed with 4% paraformaldehyde in phosphate buffer (0.1M pH7.4) for 20 min at room temperature. Cells were rinsed twice with PBS and incubated with 0.3% hydrogen peroxide and 0.1% sodium azide in PBS in order to inhibit endogenous peroxidase (Li *et al.*, 1987). After 2 washes with PBS, non-specific sites were blocked by incubating 1 h with 3% normal goat serum (NGS, Jackson Immunoresearch, USA) in PBS containing 0.3% Triton X-100 (PBS-T). The primary antibody, rabbit polyclonal anti- GFAP (1:1000 in PBS-T with 1% NGS, DAKO, France) was applied overnight at 4°C. Cultures were then rinsed twice 10 min in PBS.

The secondary antibody, peroxidase-conjugated affinipure goat anti-rabbit IgG (H+L) (Jackson Immunoresearch) was applied overnight at 4°C at a dilution of 1:100 in PBS-T containing 1% NGS then washed successively in PBS and Tris buffered saline (TBS, 0.05M, pH 7.6). The peroxidase activity was then revealed by incubation a few minutes with 0.05% 3,3'-

diaminobenzidine tetrahydrochloride (Sigma) and 0.01% hydrogen peroxide in TBS. After further rinsing, cultures were cover-slipped. As controls, some cultures were treated with the same protocol except for omission of the primary or the secondary antibody.

Cell Viability and Density Determinations

The total number of viable cells was estimated using the trypan blue exclusion test.

The cell density (number of cells/ mm^2) was calculated with a Samba™ 2005 cell image analysis system (Alcatel-TITN Answare, France) equipped with a CCD video camera (CP 3003 Tokina, Japan) placed on the camera output of an Axioskop Zeiss microscope (objective x 1.25, Oberkochen, FRG).

The digitized surface measuring 4.07 x 4.02 mm was divided into four fields. Each field was analysed and corrected for heterogeneous illumination by dividing its image by the image of an empty field. A binary mask of the cells was obtained using an intensity thresholding technique. All the pixels (image elementary points) with grey levels smaller than the intensity threshold were considered as cell pixels, all the others were considered as background pixels. After the segmentation step a connected component labelling algorithm was applied in order to give a unique number to each cell. During this process an area criterion (minimum and maximum allowed size) was used in order to eliminate small artifacts and cell aggregates. The number of isolated cells was finally counted and the density measured.

Morphometric Analysis

The main cell parameters were calculated on the same system as above i.e. the Samba™ 2005 cell image analysis system but equipped in this case with an objective x10. Cell separation was made according to the procedure of adaptative grey level thresholding which gave the most reliable results. Adaptative grey level thresholding consisted in calculating the grey level histogram for each new video field. Then an optimal intensity threshold for separating cells from the background was calculated on the basis of the statistical analysis of the grey level histogram (Fisher's discriminant criterion). Cell clusters were automatically separated using a labelling algorithm and in case of failure, the mask of the cells was shown to the operator who could then delimit the contour of the cell to be analysed by means of interactive graphic tools. For further details, see Usson *et al.*, 1991.

For this analysis, the cell parameters studied were the perimeter, the surface, the elongation factor which represents the ratio between the maximum and the minimum diameter of the cytoplasm, the convexity factor which represents the ratio between the area of the cell and the area of the convex hull of the cell and finally the form factor (equal to the square of the perimeter divided by the projection area times 4 π) which reflects the deviation of the cell shape from a perfect circle.

These measurements are not correlated because of the way they are calculated and because they express different features of the cell shape. For example, there is no expected correlation between the elongation and the convexity as illustrated by Fig. 1. It can be seen in this figure that the ratio max. diameter/min. diameter (elongation) in the pairs A and B is identical but the convexity factor is dramatically different, the same result is also observed for the pairs C and D. In the cases of the pairs A and C and of the pairs B and D, it is the opposite as the convexity factor is similar but the elongation factor is significantly different.

Study Design and Statistical Methods

Three series of culture each including four groups (control, 0.5%, 1% and 2% ethanol) and four durations of incubation (24h, 48h, 72h and 96h), were performed. For each time, about 150 cells were measured in each group. The values of the parameters measured in each series of culture were then pooled according to the duration of the exposure and to the alcoholic concentration and compared to matched controls. The statistical significance of the results was tested by a 2-factor analysis of variance (ANOVA) completed by post-HOC test.

However, because of the large heterogeneity of the cell morphology even in the control group, we completed this study by the use of the moving means clustering method to classify the cells homogeneously according to the five cells parameters which were measured. This was applied to each group for each duration of exposure and for each series of cultures (3 series were performed, each of them including the 4 groups and the 4 different durations of incubation). The stability of these classifications was checked by the repetition of the cluster analysis.

A stepwise discriminant analysis (SDA), was then applied to confirm and emphasize the differences between experimental and control groups by measuring

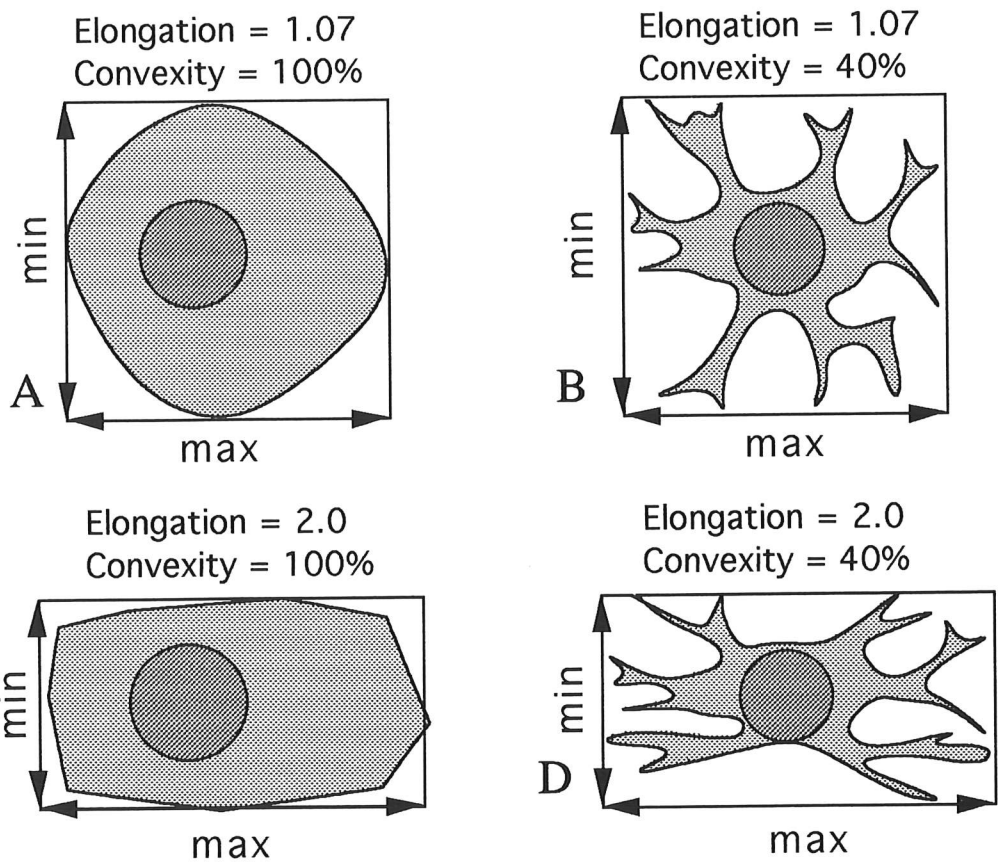

FIG. 1. Examples of cell measurements showing the absence of correlation between the cell parameters *e.g.* the elongation factor (ratio between the maximum and the minimum diameter of the cytoplasm), and the convexity factor (ratio between the area of the cell and the area of the convex hull of the cell.

the capacity of the morphological cell parameters to separate these different experimental groups. The variables were added one by one as a function of their intrinsic discriminant power (for more details see Torch *et al.*, 1990, Usson *et al.*, 1987).

RESULTS

Cytopathological Examination

No significant structural modifications were visible by light microscopy in the cultures examined after exposure to ethanol although certain cells tended to be vacuolated, particularly after exposure to the highest doses of ethanol and for the longest times. As already noted by other authors, cultures were composed mainly of type 1 astrocytes with a flat polygonal morphology in our experimental conditions, but some stellate type 2 astrocytes were also present. One of the main features of the astrocytic cultures was the great heterogeneity in the shape of the cells whatever was duration of the incubation or the degree of alcoholic exposure (see Fig. 2).

Cell Viability

The cell viability ranged from 89 to 93% in controls, from 79 to 87% in 0.5% alcohol, from 85 to 90% in 1% alcohol and from 75 to 88% in 2% alcohol. The least viability was thus obtained in the cells exposed to the highest concentrations but the values were not stastistically different between control and exposed cells (ANOVA).

Cell Density

In the exposed groups, the cell density significantly decreased with the duration of the incubation (mean cell density ranging from 27.8 cells/mm^4 after 24 h of

Bright-Field Micrographs of GFAP-Immunoreactive Astrocytes in Secondary Cultures Exposed to Different Concentrations of Ethanol for 96 h.

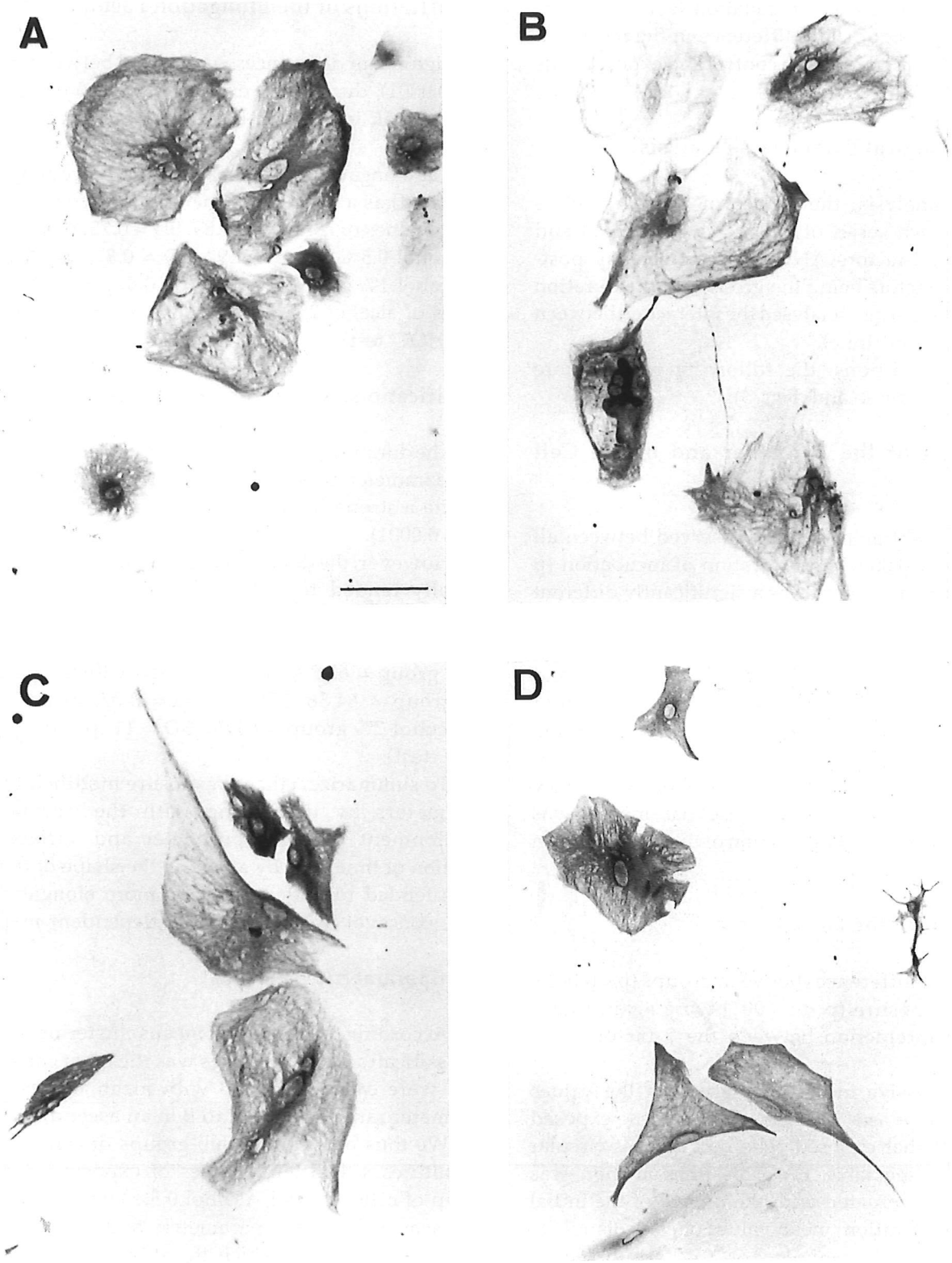

FIG. 2. A : control culture consists of flattened polygonal (type 1) astrocytes. B-D : cultures treated with 0.5% (B), 1% (C) and 2% (D) ethanol. Labeled cells present elongated and flat polygonal shaped cytoplasmic extensions. Bar represents 100 mm.

incubation to 11.8 after 96 h, p = 0.02, ANOVA). The same phenomenon was observed in the control cells but to a lesser extent (mean cell density ranging from 29 cells/mm^2 after 24 h of incubation to 19.2 after 96 h). However, no significant difference in the mean cell density was observed between control and exposed cells (2-factors ANOVA).

Cell Morphological Parameters Analysis

For this analysis, the values of the parameters measured in each series of cultures were pooled and analysed by a 2-factors ANOVA completed by post-HOC test (the factors being the group and the duration of exposure, the test also analysed the interaction between the group and the time).

In such conditions, the following results were obtained (see table 1 and Fig. 3).

Modifications of the Perimeter and of the Cell Surface

Significant differences were observed between all the groups ($p < 0.0001$) and duration of incubation ($p < 0.0001$). Moreover, there was a significantly different evolution of the different groups in function of the time whatever the parameter considered ($p < 0.0001$).

In normal cells, the values of the perimeter and of the surface tended to increase with the duration of exposure (see table 1). On the other hand, the evolution of these parameters in exposed cells was irregular, particularly for the highest doses of alcohol. A tendency to a progressive decrease of these parameters was observed at 72 and 96 h as a function of the concentration of ethanol.

Modifications of the Form Factor

Significant differences between groups ($p < 0.001$), duration of exposure ($p < 0.0001$) were again found with a deep interaction between the 2 factors ($p < 0.001$).

The progressive increase observed in the values of the form factor as a function of time in the exposed groups meant that exposed cells became less circular than controls (see table 1). This phenomenon was significantly accentuated with the degree of the initial alcoholic concentration (mean values of controls = 2.58, SD = 1.2, mean values of alcohol 0.5% group = 2.66, SD = 1.8, mean values of alcohol 1% group = 2.7, SD = 1.6, $p < 0.01$, mean values of alcohol 2% group = 2.83, SD = 2.2, $p < 0.0001$, post-HOC test).

Modifications of the Elongation Factor

Significant differences were found between groups ($p < 0.0001$), duration of exposure ($p < 0.001$) but with only a weak interaction between these 2 factors ($p = 0.053$).

The elongation factor increased (cell becoming more elongated) as a function of the alcoholic concentration (mean values of controls = 1.89, SD = 0.75, mean values of alcohol 0.5% group = 1.92, SD = 0.81, mean values of alcohol 1% group = 1.98, SD = 0.8, p = 0.001, mean values of alcohol 2% group = 2.06, SD = 1, $p < 0.0001$, post-HOC test).

Modifications of the Convexity Factor

The duration of the incubation significantly modified this parameter ($p < 0;0001$) but not the group factor despite a strong interaction between groups and time ($p < 0.0001$).

However, the degree of the alcoholic concentration globally tended to reduce this parameter *i.e.* cells tended to become more concave (mean values of controls = 65.68, SD = 9.3, mean values of alcohol 0.5% group = 65.39, SD = 10, mean values of alcohol 1% group = 64.86, SD = 10, p = 0.02, mean values of alcohol 2% group = 64.96, SD = 11, p = 0.04, post-HOC test).

To summarize, ethanol exposure modified the cell parameters by interfering with the normal cell development (reduced perimeter and surface as a function of time) and by affecting the shape of the cells (cells tended to be less circular, more elongated and more concave) in time and dose-dependent manners.

Multiparametric Analysis

According to the moving means clustering method, a classification in two classes was the most consistent; they were called A and B with mean values of the perimeter ranging from A to B in an ascending order.

We thus obtained 12 sub-groups of cells (3 series of cultures x 4 different times of exposure) for each group of cells (control, alcohol 0.5...) characterized by the mean values of 5 parameters and divided into 2 classes (A and B) to which the stepwise discriminant analysis was applied.

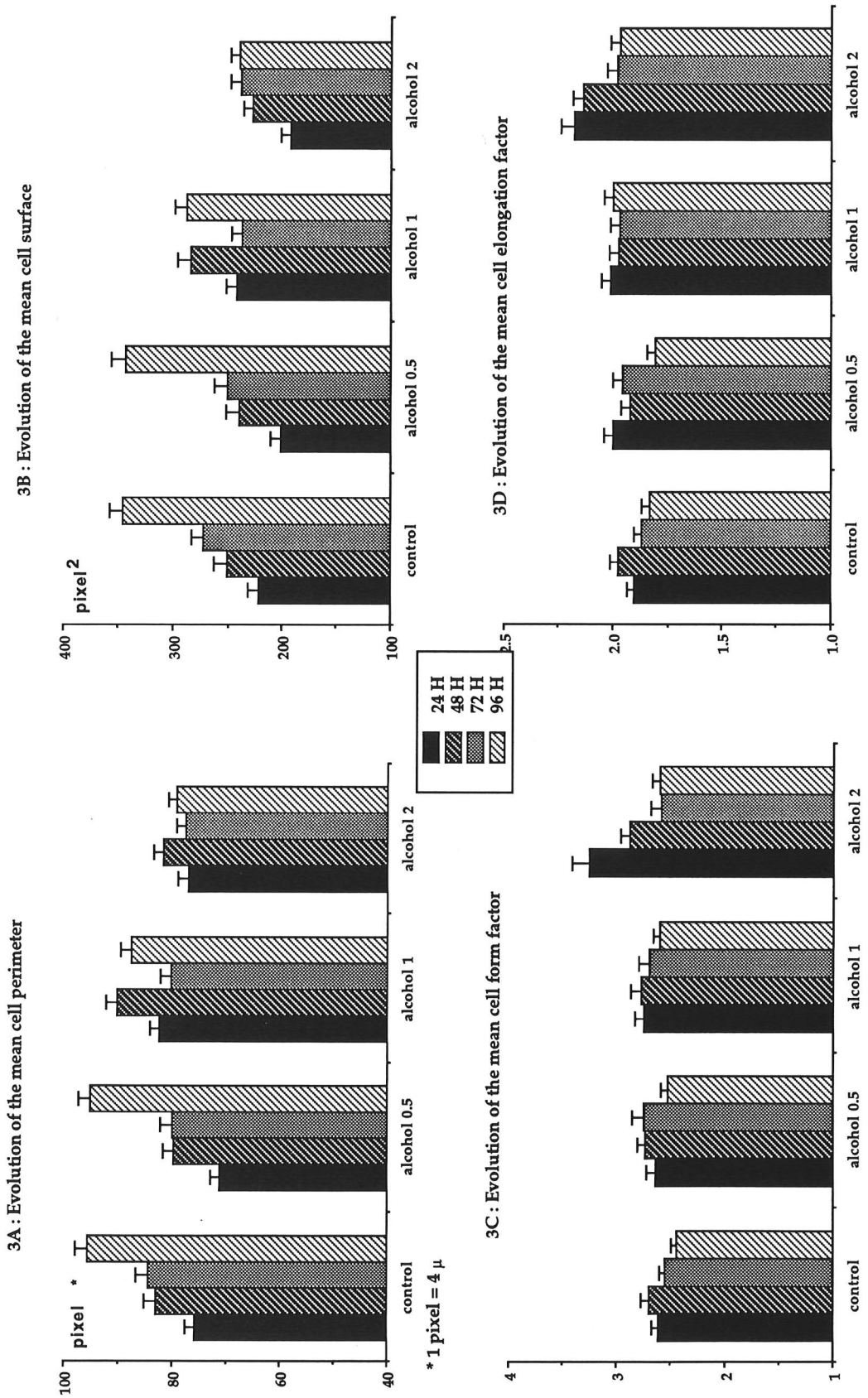

FIG. 3. Evolution of the main cell parameters in function of the time of exposure and of the alcoholic concentration (mean ± S.E.M.)

TABLE 1. Values of the Cell Parameters in the Different Experimental Groups.

	Exposure Time	Number of Cells	Perimeter* (M ± SD)	Surface** (M ± SD)	Form Factor (M ± SD)	Elongation Factor (M ± SD)	Convexity Factor (M ± SD)
Control		461	75.85 ± 36.42	221.75 ± 196	2.61 ± 1.5	1.90 ± 0.76	65.25 ± 9.40
0.5% Alcohol	24 h	451	71.20 ± 36.78	201.07 ± 217	2.64 ± 1.7	2.00 ± 0.85	64.97 ± 9.94
1% Alcohol		447	82.40 ± 36.68	241.70 ± 205	2.75 ± 1.7	2.01 ± 0.77	64.50 ± 10.2
2% Alcohol		386	77.17 ± 37.30	192.47 ± 165	3.26 ± 3.2	2.18 ± 1.16	61.80 ± 12.4
Control		334	82.92 ± 39.13	250.54 ± 218	2.70 ± 1.3	1.97 ± 0.77	64.56 ± 9.39
0.5% Alcohol	48 h	370	79.64 ± 38.52	239.47 ± 233	2.73 ± 1.5	1.92 ± 0.79	64.86 ± 10.3
1% Alcohol		422	90.14 ± 40.24	284.40 ± 243	2.77 ± 1.4	1.97 ± 0.77	64.08 ± 10.6
2% Alcohol		412	81.76 ± 34.32	277.70 ± 171	2.88 ± 1.6	2.14 ± 0.98	64.03 ± 10.6
Control		433	84.34 ± 44.31	271.84 ± 238	2.55 ± 1.1	1.87 ± 0.75	65.79 ± 9.25
0.5% Alcohol	72 h	447	80.07 ± 43.10	250.70 ± 262	2.75 ± 2.4	1.95 ± 0.89	65.38 ± 10.8
1% Alcohol		422	80.32 ± 37.91	237.87 ± 198	2.70 ± 1.9	1.96 ± 0.90	64.89 ± 11.3
2% Alcohol		411	77.56 ± 36.61	238.91 ± 199	2.58 ± 2.1	1.98 ± 0.93	67.36 ± 11.1
Control		451	95.67 ± 39.61	345.37 ± 225	2.44 ± 1.0	1.83 ± 0.72	67.13 ± 8.95
0.5% Alcohol	96 h	411	95.18 ± 42.69	342.78 ± 286	2.53 ± 1.1	1.80 ± 0.68	66.31 ± 9.19
1% Alcohol		414	87.64 ± 35.83	288.10 ± 209	2.59 ± 1.2	1.99 ± 0.86	66.02 ± 9.63
2% Alcohol		394	79.31 ± 28.13	239.67 ± 165	2.60 ± 1.6	1.96 ± 0.91	66.54 ± 11.0

*Expressed in pixel (1 pixelL = 4 µm). ** Expressed in pixel^2

According to this analysis, the exposed groups of cells were clearly separated from the controls and also clearly separated between themselves. Indeed, 11 out of 12 sub-groups for the control, alcohol 0.5% and alcohol 2% groups and all the sub-groups of the alcohol 1% group were correctly reclassified (see table 2).

In table 3, the parameters were classified as a function of their decreasing discriminatory power. It is noticeable that the most discriminant parameters were those describing the cell shape (form, elongation and convexity factors) of class B then of class A, the perimeter and the surface being apparently less discriminant. It must also be noted that none of them had enough discriminatory power to support a correct classification in itself. The final percentage of misclassification, resulting from the influence of all the parameters, was of 6.25%.

It was thus possible to correctly re-classify the different sub-groups in their original group, a fact favouring the existence of particular characteristics inherent in each group.

Both statistics used, demonstrated the ability of ethanol to mainly alter the cell shape parameters in a dose dependent fashion.

TABLE 2. Confusion Matrix (stepwise discriminant analysis).

	Reclassified Subgroups			
	Control	0.5% Alcohol	1% Alcohol	2% Alcohol
Control	11	1	0	0
0.5% Alcohol	0	11	1	0
1% Alcohol	0	0	12	0
2% Alcohol	0	0	1	11

TABLE 3. Discriminatory Power of the Cell Parameters in Class A and B of Cells Obtained in Each Experimental Group After Automatic Classification (moving means clustering method).

Cell Parameters	Discriminatory Power	Percentage of Misclassification
Form factor Class B	4.076	62.50
Convexity factor Class B	1.257	66.67
Elongation factor Class B	1.830	52.08
Convexity factor Class A	1.016	43.75
Elongation factor Class A	5.292	37.50
Perimeter Class A	1.606	27.08
Surface Class A	4.759	18.75
Perimeter Class B	0.350	12.50
Surface Class B	1.010	6.25

DISCUSSION

The normal functioning of the central nervous system could be severely affected by ethanol ingestion and it has been suggested that ethanol toxicity might be mediated to some extent by glial cells (Zou *et al.*, 1993). In the present study, astrocytes in culture were exposed to different concentrations of ethanol (0.5, 1 and 2%) for different durations of exposure (24, 48, 72 and 96 h). These concentrations, apart from the 2% concentration, are usually considered to be within the range of clinically observed values (Davies and Cox, 1991). The variations in their morphology were investigated by the means of an analysis image system. Over 6500 cells were measured because of the large variations of the normal astrocyte morphology in culture which were susceptible to be accentuated by numerous others factors such as the cell density, the cell to cell contact (Goldman and Chiu, 1984). In our experimental conditions, the cell viability and the cell density were not significantly modified between control and exposed cells, such factors could not have accounted so for the observed morphological variations. These morphological variations consisted mainly in modifications in the shape of the cells which became more elongated, less circular and more concave with a possible dose-dependent effect as the mean pooled values of these parameters tended to be modified as a function of the dose of ethanol.

However, because of the already mentioned large morphological variations of the cells even in the controls, results were analysed by different statistical approaches to better emphasize the variations between control and exposed groups. A classical two-factor ANOVA was thus completed by a stepwise discriminant analysis which stressed the differences between experimental and control groups by measuring the capacity of the morphological cell parameters to separate the experimental groups from the control. The efficiency of this procedure has been previously demonstrated on morphological studies of nerve transverse sections (Barret *et al.*, 1991, Barret *et al.*, 1992). According to the stepwise discriminant analysis, the modifications of the measured parameters were able to separate the different experimental groups. The most discriminant parameters were those describing the shape of the larger cells, the perimeter and the surface being apparently less discriminant. It must nevertheless be said that none of them had enough discriminatory power to support a correct classification in itself which implied that the modifications of the parameters should be considered as a whole.

Alterations of the cell shape and of the cell growth kinetics have also been the prominent features of several experimental studies (Davies and Venadakis, 1984, Davies and Cox, 1991, Mayordomo *et al.*, 1992). Unfortunately direct comparison between studies is difficult because of different methodological approaches. Nevertheless, some similarities exist such as dose-dependent altered growth kinetics and variations in the cell profile (Davies and Cox, 1991). In other astrocyte cultures, vacuolated cells were also observed after direct exposure (Davies and Venadakis, 1984) and stereological and qualitative modifications of mitochondria, rough endoplasmic reticulum and lysosomes were obtained after pre-natal exposure (Mayordomo *et al.*, 1992). However, no information on the cell morphology was available in this latter study.

The comparison of our results with histopathological examinations is hazardous because of the large differences in the reactions of a cell in its normal cellular environment and those of an isolated cell to toxic exposure. The morphology of in vivo astrocytes is however generally affected by ethanol intoxication. For example, hypertrophied astrocytes were characterized by morphometry in the area 3 of the primary somatosensory cortex (Miller and Potempa, 1990) and by confocal microscopy in areas surrounding blood vessels in layers I to IV of the cortex and in layer V (Goodlett *et al.*, 1993), a result we did not observe in cell culture.

Finally, an essential question remains : could these modifications be related to ethanol toxicity and by what mechanisms?

Numerous factors can affect directly or indirectly cell morphology such as cell density, substrate interactions, and modifications of cytoskeletal stucture and of cell metabolism.

Ethanol is suspected to act on most of these factors. For example, ethanol can be oxidised at the brain level into reactive species which can affect the cell functioning. A production of ethanol-derived free-radicals was also observed and may have the same effects (Gonthier *et al.*, 1991).

The role of other factors such as the osmolality is also to be discussed. Indeed, it has been shown that exposure to iso-osmotic ethanol causes cell swelling whereas hyperosmotic did not (Kimelberg *et al.*, 1993). It is interesting to note that the latter condition is achevied, for example, when ethanol is added in terms of volume

per cent to the medium of the cell culture like in our experiments. However, the osmolality seems unlikely by itself to explain the observed astrocyte morphological variations which were more time dependent than dose-dependent.

Finally ethanol rapidly enters the cell and may thus alter the membranes inducing cellular reactions towards this aggression as well as modifying normal cellular functioning.

On the basis of this study alone, is so impossible to identify the precise factor responsible for the observed astrocytes morphological variations.

It is nevertheless possible to exclude the role of a factor affecting the cell density. Indeed, the lower density observed in exposed and control cells as a function of the duration of the incubation, is generally considered to favour cell proliferation (Venadakis *et al.*, 1985). The constant increase in the cell perimeter and surface observed in control cultures could be explained by such a factor. However, this was not seen in exposed cells despite a similar evolution of the density as a function of time. This could stress so an effect of ethanol not related to a factor affecting the cell density.

In conclusion, our results suggest that ethanol could affect astrocytes in two different but probably complementary ways by modifying the cell shape and by altering the normal cell development. The mechanism involved may be directly toxic as a consequence of its metabolism and or of its ability to alter cellular and sub-cellular membranes because of its lipophilic properties.

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