INTERMITTENT ETHANOL EXPOSURE INCREASES THE NUMBER OF CEREBELLAR MICROGLIA

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(Received 20 April 2001; in revised form 19 October 2001; accepted 30 January 2002)

Abstract — Aims: The number of cerebellar microglia after 5½ months of continuous or intermittent ethanol exposure was studied using the optical disector method. Methods: Male Wistar rats were divided into three groups: an intermittently ethanol-exposed group, a continuously ethanol-exposed group and a control group (n = 6 in each group). The intermittently treated rats had two ethanol-withdrawal periods per week throughout the experiment. The number of microglia was measured in the anterior (folium II) and the posterior (folium X) cerebellar vermis. Tomato (Lycopersicon esculentum) lectin was used to stain the cerebellar microglia. Results: The volumes of folia II and X were similar in all the groups. The number of microglia increased in the molecular layer of folium II in the intermittently ethanol-exposed group compared with the continuously exposed and control groups. In the granular layer, there were no differences between the groups in the number of microglia. Conclusions: The results suggest that the number of cerebellar microglia increases in the anterior vermis before any ethanol-induced cerebellar atrophy is discernible. Repeated ethanol withdrawals seem to be more essential in inducing microgliosis than ethanol intoxication per se.

INTRODUCTION

In human alcoholics, cerebellar atrophy is one of the main neuropathological manifestations. The most pronounced changes typically occur in the anterior superior parts of the vermis (Viktor et al., 1959; Torvik and Top, 1986). In experimental animals, ethanol-induced volumetric changes are found in both the molecular (Rintala et al., 1997) and the granular (Tavares et al., 1987) layer. In humans, the ethanol-induced volume loss is found mostly in the molecular layer (Phillips et al., 1987). In humans, a loss of cerebellar Purkinje cells has been reported when long-term daily ethanol consumption is >40 g/day, and the loss increases until daily intake is >80 g; subsequently the loss remains constant (Karhunen et al., 1994). However, the effects of the pattern of drinking on cerebellar pathology have received little attention so far. In neonatal rats, binge ethanol exposure has been shown to cause more severe cerebellar damage than continuous exposure (Bonthius and West, 1990). In adult animals, Purkinje cell damage is also found after a single, extended withdrawal period (Phillips and Cragg, 1984).

Microglial cells are found throughout the central nervous system (CNS). They are believed to play an active role in CNS inflammatory, immune and degenerative processes, but also in the normal physiology of the brain. Their morphology, immunophenotype and function resemble those of monocytes and macrophages (see Nakajima and Kohsaka, 1993, for a review). Microglia accumulate, proliferate, become activated and phagocyte degenerated neurons in brain ischaemia (Gehrmann et al., 1992; Lees, 1993), Alzheimer’s disease (Perlmutter et al., 1990) and multiple sclerosis (Banati and Graeber, 1994). Microglial cells are sensors of brain pathology, and are able to react to disturbances in neuronal well-being.

Kupffer cells, the resident macrophages in the liver, are activated during heavy ethanol administration (Thurman, 1998), and also during ethanol withdrawal (Bautista and Spitzer, 1992; Lukkari et al., 1999). It is suggested that this Kupffer cell activation causes hepatic hypoxia and increases free radical production, thereby contributing to ethanol-induced liver injury (Thurman, 1998). The impact of cerebellar macrophages, i.e. microglia, on alcohol-induced neuronal damage has not been studied so far. The aim of the present study was to ascertain whether ethanol exposure affects the number of cerebellar microglia, and whether the pattern of ethanol exposure (continuous vs intermittent) plays a decisive role in this regard. In particular, we studied folia II and X in the cerebellar vermis. Folium II was selected as previous studies have suggested that it is probably the most vulnerable area to ethanol-induced degeneration (Viktor et al., 1959; Torvik and Torp, 1986; Rintala et al., 1997). Folium X was used as a reference area.

MATERIALS AND METHODS

Animals and treatments

Male Wistar rats were used in the study. At the beginning of the experiment, the rats were 2 months old, and the duration of the experiment was 5½ months. Rats were housed in group cages under standard conditions (12 h light/12 h dark cycle; relative humidity 40 ± 5%, temperature 23 ± 1°C). Weight gain, and fluid and food consumption were measured throughout the experiment.

At the beginning of the experiment, rats were divided into three groups: a control group (n = 6), a continuously ethanol-exposed group (n = 6) and an intermittently ethanol-exposed group (n = 6). The only available fluid throughout the experiment was tap water for the control group and 10% (v/v) ethanol for the continuous group. The intermittent group was given 10% (v/v) ethanol on Monday, Tuesday, Thursday and Friday, and water for the rest of the week. This weekly schedule (two withdrawal periods per week) was repeated throughout the 5½-month experiment. Food (Ewos R36; Ewos AB, Sweden) was available ad libitum for all groups.

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Methods

The rats were decapitated under deep pentobarbital anaesthesia. The brains were quickly removed, and the cerebellum carefully dissected. The vermis was separated from the cerebellar hemispheres, and fixed in 4% paraformaldehyde for 24 h. The vermis was cryoprotected with ascending concentrations of sucrose (10, 20 and 30% sucrose in phosphate-buffered saline). The whole vermis was parasagittally sectioned to the thickness of 10 μm at −15°C. Every 36th section was collected from a random starting point for the morphometric analysis (section sampling fraction = 1/36 and the reciprocal sampling fraction s = 36). The next two sections were also collected for histochemical stainings. The presence of folium X was used to determine the beginning and the end of the vermis, because it has only a rudimentary hemispheric part (Heinsen and Heinsen, 1984). Folium X was also used as a reference area in the morphometric analysis, as the volume measurements were most reliable on folium X, due to its negligible hemispheric extent.

The sections used in the volumetric analysis were stained using 0.05% cresyl violet acetate, dehydrated in ascending ethanol (50, 75, 96 and 100%), cleared with xylene and embedded in Aquamount®. Tomato (Lycopersicon esculentum) lectin (Sigma, St Louis, MO, USA) was used for the staining of microglia, as follows. The frozen sections were melted for 20 min at room temperature, and then rinsed twice with 0.05 M Tris-buffered saline (TBS). Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 15 min. After two rinses in TBS and a 15 min pretreatment with 0.3% Triton X-100 in TBS, the sections were incubated in biotin-labelled tomato lectin (3 μg/ml) in TBS with 0.3% of Triton X-100 for 2 h at room temperature, rinsed three times with TBS and then labelled with avidin peroxidase in TBS (1:30 000) for 1 h at room temperature. Diaminobenzidine (Sigma) in TBS was used as a chromogen for 5 min. Finally, the sections were rinsed with TBS and distilled water and embedded in Aquamount. An Olympus Vanox-T (Olympus, Inc., Tokyo, Japan) microscope, an Argus-10 (Hamamatsu Photonics, Hamamatsu City, Japan) image processor and a DTS512N (Sony Precision Technology Inc., Japan) microrouter were used for the morphometric measurements.

The volume of the vermic folia was measured using a point-counting method and the Cavalieri principle (Gundersen and Jensen, 1987). The corresponding area of each grid point \( a(p) \) was 0.055 mm². Cryomicrotome settings were used to determine the section thickness \( t = 10 \mu m \). With a total magnification of \( 43 \), the volumes of folia II and X were measured. The numbers of grid points hitting the molecular \( \Sigma P(mo) \), granular \( \Sigma P(gr) \) and white matter \( \Sigma P(wm) \) layers were counted separately. The volumes were counted with the formula:

\[
V = t \times s \times a(p) \times \Sigma P.
\]

The numbers of microglia were counted with the optical dissector method (Sterio, 1984; Gundersen, 1986) at a magnification of \( x855 \) (objective \( x40 \)). The average of the final section thickness was 5 μm, i.e. the thickness of the sections decreased by 50% during the histochemical processing. This means that when the dissector height used in the measurements was 3 μm, the actual dissector height \( h \) was 6 μm. The microglial nuclei, which were in focus on the reference section but not on the look-up section and were not touching the exclusion line (Gundersen, 1977), were counted from the dissector frame of 10 000 μm². \( \Sigma A \) in the formula below is the sum of the dissector areas. The sums of counted microglia in molecular \( \Sigma Q(mo) \), granular \( \Sigma Q(gr) \) and white matter \( \Sigma Q(wm) \) layers were measured separately starting at a random point and moving throughout the folia in a stepwise manner. The measurements were performed on systematically randomly selected sections and three sections in each animal were analysed. The numerical density of microglia was estimated with the formula:

\[
N_v = \frac{\Sigma Q(\Sigma A \times h)},
\]

and the total number of microglia was calculated from:

\[
N = N_v \times V.
\]

Statistics

The results are expressed as means (± SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) with the Statistical Package for the Social Sciences for Windows Software (7.5 release). Bonferroni-corrected \( t \)-tests were used for further comparing the group means and \( P < 0.0167 \) was considered a statistically significant difference (three parallel comparisons).

RESULTS

Individual ethanol consumption was similar in the intermittent (5.8 ± 0.6 g/kg/day) and the continuous (5.7 ± 1.5 g/kg/day) group on the days when both groups were given ethanol, but the intermittent rats received ethanol only 4 days a week. Therefore, the weekly ethanol consumption was significantly higher \( (P = 0.001) \) in the continuous group (39.6 ± 10.5 g/kg/week) than in the intermittent group (23.0 ± 2.3 g/kg/week).

Individual food consumption was measured in the 23rd week of the experiment, and was equal between the groups (continuous 21 ± 5, intermittent 22 ± 5, control 25 ± 5 g/day).

At the end of the experiments the weights of the rats were 599 ± 35 g in the control group, 600 ± 48 g in the continuous group and 550 ± 32 g in the intermittent group. The difference between the groups was not significant \( [F(2,15) = 3.283, P = 0.066] \). As previously reported, ANOVA for repeated measurements showed a significant difference in weight gain between the groups, the weight gain being smaller in the intermittent group \( (P < 0.001 \) for time \( \times \) group interaction) (Riikonen et al., 1999).

The penetration of staining was complete and microglia were stained uniformly throughout the sections. As previously described, blood vessels and the ependyma were also tomato lectin positive (Acarin et al., 1994), but microglia were easily identified by their typical morphology (Figs 1 and 2). Microglia were scattered throughout the cerebellum, but no microglial accumulations were seen. However, a number of microglia were found in the vicinity of blood vessels. No difference was observed between the groups in the general appearance or distribution of vermic microglia (Fig. 1). Most of the microglia were ramified, and only a few ameboid microglia were found in all groups. Ramified microglia have
small cell bodies and long branched processes (Figs 1 and 2). Amoeboid microglia resemble macrophages with large cell bodies and short processes.

There was no difference between the groups in the volumes of folium II \([F(2,15) = 7.99, P = 0.468]\) or folium X \([F(2,15) = 5.34, P = 0.597]\) (Table 1). Neither was there any difference between the groups when the volume of each layer (molecular, granular and white matter) was analysed separately (Table 1) or when the body weight was used as a covariate (data not shown).

The morphometric results from the number of microglia are expressed in Fig. 3A–D. The number of microglia was calculated from the entire folia II and X (A), and from each layer [molecular (B), granular (C) and white matter (D)] separately. There was a significant difference between the groups in the number of microglial cells in the molecular layer of folium II \([F(2,15) = 6.429, P = 0.0096]\). Post hoc analysis showed that the number of microglia was greater in the intermittent group compared to the continuous \((P < 0.05)\) or the control \((P < 0.05)\) group. The number of folium II microglia in the granular layer was similar in all groups \([F(2,15) = 0.51, P = 0.950]\). There was no significant difference between the groups in the number of microglia in folium X. The results of folium II were similar when the body weight was used as a covariate: there was a significant difference between the groups in the number of microglia in the molecular layer \((F = 6.612, P = 0.005)\), but not in the granular layer \((F = 0.087, P = 0.966)\). \(\Sigma Q_{wm}\) was too small for statistical analysis and therefore the number of microglia in the white matter was not statistically analysed.

**DISCUSSION**

In the present study, we found that the number of microglia increased in the anterior superior part of the cerebellar vermis subsequent to intermittent ethanol exposure. This increase was found only in the molecular layer of folium II. No cerebellar atrophy was observed after 5½ months of continuous or intermittent ethanol exposure in any of the cerebellar layers, even when body weight was used as a covariate.

Microglia are brain macrophages of monocytic origin (Ling et al., 1980). From a morphological point of view, microglia are divided into amoeboid and ramified. Ramified (resting) microglia represent the majority of microglia found in normal adult CNS. During brain degeneration, microglia become...
hypertrrophic and phagocytic, and change their phenotype and morphology towards macrophages, to become amoeboid or reactive microglia (Streit et al., 1988). The reactive microglia change back to the ramified form when the CNS damage is repaired (Giulian and Baker, 1986; Suzumura et al., 1990, 1991). Only a few amoeboid microglia were found in the present study, and no statistical analysis on the number of reactive/amoeboid microglia could therefore be performed. It seems that the nature of ethanol-induced CNS lesion is slow, and only a few activated microglia are needed to phagocytose the damaged tissue at a time.

Microglia are activated and/or their number increases in several CNS diseases. In Alzheimer’s disease, microglia are associated with senile plaques (Perlmutter et al., 1990). In brain ischaemia, activated microglia phagocytose degenerated or dead neurons (Gehrmann et al., 1992). In multiple sclerosis, microglia may present CNS antigens to circulating T-cells (Banati and Graeber, 1994). The main targets of HIV-1 infection in the CNS are microglia and macrophages (Price et al., 1988). When the cells are infected, they release cytokotxins to induce neuronal degeneration (Guilian et al., 1990). As shown in the present study, intermittent ethanol exposure also increases the number of cerebellar microglia; this finding was observed prior to the appearance of any signs of cerebellar atrophy. Microgliosis was seen in the anterior superior part of the vermis; in the same area where ethanol-induced cerebellar atrophy is most prominent in human alcoholics (Viktor et al., 1959; Torvik and Torp, 1986; Phillips et al., 1987) and in experimental animals (Rintala et al., 1997). It is therefore possible that microglia contribute to brain atrophy by phagocytosing degenerated CNS tissue. In line with this view, cerebral atrophy has been found to correlate strongly with cortical microgliosis in AIDS patients (Gelman, 1993). At the time of undertaking the present study, our suggestion was that the changes are most likely to be found in the anterior superior (folium II) part of the vermis. In line with previous studies and according to our hypothesis, the ethanol-induced microglial changes were found in folium II, but not in the folium X.

Silver staining, immunohistochemistry and lectin histochemistry have been used to visualize microglia. Silver staining is rather an unspecific method labelling not only microglia, but also oligodendrocytes. Immunohistochemistry usually gives stronger labelling to amoeboid, than ramified, microglia (OX-42, OX-18 and RMG1) or does not stain ramified microglia at all (OX-41, OX-6, ED3 and anti-vimentin). Tomato lectin was used for microglial staining in the present study as it labels reliably both reactive and resting microglia. Lectins are proteins or glycoproteins of non-immune origin (Goldstein and Hayes, 1978; Alroy et al., 1988). They have been isolated from many natural sources, including seeds, roots, bark, fungi, bacteria, seaweed, sponges, molluscs, fish eggs, body fluids of invertebrates and lower vertebrates and from mammalian cell membranes. Although the physiological functions of the lectins are unknown, they have a wide variety of applications in vitro. Their unique ability to recognize specific sugar residues of complex glycoproteins makes them highly valuable in cytological and histological studies for the identification of different cell types (Goldstein and Hayes, 1978). The tomato

Fig. 3. The total number of microglia in the whole folium II and X of the cerebellar vermis (A), in the molecular layer (B), in the granular layer (C), and in the white matter (D) of intermittently and continuously ethanol-exposed and control rats. The results are expressed as means ± SD. *#Significantly different from the control group and the continuously ethanol-exposed group respectively (P < 0.05, Bonferroni-corrected t-tests).
lectin has been reported to specifically label both amoeboid and ramified microglia, as well as endothelial cells and ependyma, without any binding to neurons and other glial cells (Acarin et al., 1994). Similar results were obtained in the present study. The specific morphology of microglia made their distinction from blood vessels and ependyma reliable.

In a previous study, performed on the same material as the present one, we found that a 5½-month intermittent ethanol exposure decreased the number of peripheral sympathetic neurons by 28%, compared to the continuously ethanol-exposed group (Riihonen et al., 1999). Also in the cerebellum, binge ethanol exposure has been shown to induce more severe damage in neonatal rats, than continuous consumption (Bonthius and West, 1990), and a loss of Purkinje cells has been reported after a single ethanol withdrawal in adult rats (Phillips and Cragg, 1984). It has previously been suggested that ethanol-induced CNS degeneration is too slow to cause any noticeable microglial reaction (Streit, 1994). This may be the case when ethanol exposure is continuous and chronic. But as we show in the present study, microgliosis is found in the cerebellum after intermittent ethanol exposure. Microgliosis probably reflects a more severe neuronal degeneration caused by binge ethanol consumption, compared to the degeneration caused by continuous ethanol exposure.

To date, only a few studies have been published on ethanol-induced alterations in microglia. Chronic ethanol treatment appears to accelerate the degeneration of microglial processes, and therefore accentuate the effects of normal ageing on microglia (Kalehua et al., 1992). However, no age-related changes were found in the number of microglia in the dentate gyrus, CA I or hilus of mouse hippocampus (Long et al., 1998). Ethanol exposure has been found to increase superoxide anion production and to depress nitric oxide levels in cultured (resting) microglia (Colton et al., 1998). This may result in an increased oxidative stress in the CNS and further degeneration of neurons, since the latter are particularly vulnerable to oxidative stress (Cohen and Warner, 1993). Neuronal degeneration, in turn, may lead to an increased number of microglia and microglial activation. A putative pathogenic vicious circle with ethanol/withdrawal-induced neuronal degeneration and microglial alterations inducing each other is presented in Fig. 4. Microglial functions have been found to deteriorate during chronic ethanol treatment in vitro, which may reflect an increased risk of CNS infections in vivo (Aroor and Baker, 1998). The functions of microglia were beyond the scope of the present study, but CNS infections were not found post mortem in either the ethanol-exposed or the control rats.

The resident liver macrophages, the Kupffer cells, are believed to play an important role in ethanol-induced liver injury. Ethanol consumption increases the release of gut-derived endotoxins. Elevated endotoxin levels stimulate Kupffer cells to release eicosanoids, TNF-α and oxygen radicals (Bautista and Spitzer, 1992, 1999). Chronic ethanol exposure also increases the number of Kupffer cells (Shiratori et al., 1989) and increases their activity by up-regulating the CD14 receptor (Järveläinen et al., 1997). Increased levels of oxygen radicals in the liver are found during acute and chronic alcohol intoxication and during alcohol withdrawal (Bautista and Spitzer, 1992). Analogously with the role of Kupffer cells in alcoholic liver disease (Nordmann et al., 1992), we suggest that microglia may have a role in ethanol- and withdrawal-induced neuronal degeneration.

In conclusion, intermittent ethanol exposure increases the number of microglia in the anterior superior part of rat vermis, which has been shown to be the most vulnerable part of the cerebellum to ethanol-induced degeneration. The microgliosis found in the present experiment occurred selectively in the molecular layer of folium II, in the same area where cerebellar atrophy was previously found after lifelong ethanol exposure (Rintala et al., 1997). The number of microglia increased before any volumetric changes of the cerebellar layers were found. Therefore, microglia may be used as early markers of ethanol-induced nervous system damage, before any macroscopic changes are discernible.

Acknowledgements — We thank Ms Olli Kemppainen for technical assistance, and Esko Väyrynen, MA for checking the English text. The study was supported by the Finnish Foundation for Alcohol Studies and The Medical Research Fund of Tampere University Hospital.

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