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Role of leukocytes in ethanol-induced microvascular injury in the rat brain in situ: potential role in alcohol brain pathology and stroke

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Abstract

Effects of acute and chronic alcohol ethanol administration on in vivo microvascular-leukocyte dynamics was studied in brains of naive and leukocyte-depleted rats by direct, quantitative intravital high-resolution TV microscopy, fluorescence microscopy and myeloperoxidase staining. Administration of alcohol produced dose-dependent venular vasospasm, and rolling and adherence of leukocytes to venular walls; leukocyte velocity concomitantly decreased. Intermediate to high doses of ethanol resulted in infiltration of leukocytes and macrophages across venular walls, and concentration-dependent increases in myeloperoxidase staining in parenchyma, and rupture of postcapillary venules with focal hemorrhages. Use of phosphorus 31-nuclear magnetic resonance spectroscopy on intact animals revealed that the latter were associated with whole brain losses in intracellular levels of ATP and phosphocreatine with concomitant rises in intracellular inorganic phosphate and hydrogen ion concentration. Vinblastine-depletion of circulating leukocytes prevented or ameliorated greatly the alcohol-induced microvascular damage and proinflammatory-like reactions. These new results, when viewed in light of other recent findings, suggest that alcohol-induced cerebral vascular and brain damage is dependent, to a large extent, on recruitment of leukocytes.

Keywords: Leukocyte; Alcohol; Cerebral venule; Cerebral microcirculation; Inflammation; Cell adhesion molecule

1. Introduction

Alcohol is by far the most abused drug in the USA, with a toxic death rate that is second only to carbon monoxide poisoning. More than 100 years ago, the brains of patients suffering from Wernicke–Korsakoff syndrome or alcohol-induced chronic hepatic encephalopathy were found to be infiltrated by white cells and macrophages (Wernicke, 1900; Haller, 1980; Victor et al., 1989). These findings have been ignored for the most part, and their clinical significance remains unknown. Chronic alcohol ingestion is often followed by a toxic leukoencephalopathy of unknown origin (for recent review, see Filey and Kleinschmidt-DeMasters, 2001). There is increasing evidence that alcohol (ethanol) induces, intracranially, hemorrhages, subarachnoid hemorrhage, and cerebral infarction which may lead to sudden death (Altura et al., 1983, 1995; Altura and Altura, 1994; Donahue et al., 1986; Camargo, 1989; U.S. Dept. Health Human Services, 1993).

Studies by our group on the living brain microcirculation of rodents indicate that high doses of alcohol (ethanol) produce an acute proinflammatory-like reaction in the brain before any overt brain tissue damage is apparent (Altura and Gebrewold, 1996, 1998). Such reactions are characterized by venular cerebrovasopasm and leukocyte-endothelial chemoattraction, often followed by leukocyte and macrophage infiltration across the postcapillary microvascular walls, microvessel rupture and some focal hemorrhaging. Whether this alcohol-induced sequelae of pathological events is set into motion by leukocytes, per se, is not known.

Twenty-five years ago, Garcia and Kamijyo (1974) demonstrated, by histopathologic methods, the accumulation of polymorphonuclear cells in ischemically damaged brain. Since these initial experimental findings, a number of work-

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ers, using different animal models, have implicated leukocytes in the pathogenesis of early inflammatory responses in the brain (see e.g., Hallenback et al., 1986; Barone et al., 1992; Carlos et al., 1997; Barone, 1998; Ritter et al., 1998; Stoll et al., 1998).

We tested the hypothesis that either acute or chronic ethanol-induced ischemic brain microvascular injury is set into motion by rolling and recruitment of leukocytes and that leukocyte depletion will either ameliorate or prevent the associated hypoxia, ischemic and brain-related vascular damage.

2. Materials and methods

2.1. Animals, leukocyte depletion and microcirculatory studies

Male Wistar rats (125–165 g) were anesthetized with pentobarbital sodium (Nembutal., 40 mg/kg i.m.). Some rats were given vinblastine sulfate (8 mg/kg i.v., Sigma, St. Louis, MO) or comparable volumes of normal physiologic saline i.v., (sham controls). On the fourth day, these rat brains, at which time the circulating levels of white blood cells appeared to reach their nadir, were examined in situ. Circulating levels of white blood cells and platelets were determined just prior to brain-microvascular experimentation with a Spencer hemacytometer. After induction of anesthesia with pentobarbital, in vivo high-resolution, quantitative TV-image intensification microscopy (Altura et al., 1983; Altura and Gebrewold, 1998) was used to examine the effects of ethanol on the adverse brain-damaging actions of this alcohol. Briefly, after using an atraumatic, hemorrhage-free technique to make a small window in a parietal area of the brain (Altura et al., 1983; Ema et al., 1998), the cortical cerebral microcirculation was examined at magnifications up to 3000× (Altura et al., 1983; Altura and Gebrewold, 1998; Ema et al., 1998). Venular lumen sizes were measured, quantitatively, before and after systemically administered ethanol (either into branch of internal carotid artery or intraperitoneally, depending on design). In some in vivo studies, a closed cranial window technique was used for intravital fluorescence microscopy of the brain surface. Before each measurement, leukocytes were stained in vivo by i.v. injection of 0.1 ml of 0.1% Rhodamine CG (Sigma). This was done in order to better visualize the intravenular leukocyte dynamics. The intravital microscopic images were recorded by a SIT video camera. The videotapes were analyzed frame-by-frame with a television monitor at a total magnification of ~ 1000×. To determine if the integrity of the blood–brain barrier was disturbed, we injected (~ 0.5 ml) of a 5% Na^+-fluorescein solution (Sigma) at the end of each experiment. Analysis of the cortical–pial microcirculation, here, as in the above, included measurements of venular diameters, the number of rolling adherent leukocytes in venules (cells per 100 μm/min), the velocity of freely moving leukocytes in venules (μm/s), and the integrity of the blood–brain barrier. Adherent leukocytes were defined as cells attached to the venular walls for >15 s. Vessel segments with a length of 100 μm were studied for 1 min. In a particular vessel segment, the velocities of at least 20 leukocytes were measured at each time point; the results were then used to estimate venular blood flow velocity. At the end of each of the experiments, each animal was sacrificed with an overdose of pentobarbital sodium (i.e., 150 mg/kg).

2.2. Evaluation of alcohol-induced microvascular brain tissue injury

In order to evaluate alcohol-induced microvascular brain tissue injury in our model, (1) tissue damage was examined, quantitatively, by measuring numbers of postcapillary venular vessel ruptures and petechial hemorrhages at postcapillary venular sites; and (2) phosphorus 31-nuclear magnetic resonance (^{31}P-NMR) spectroscopy was performed to assess cellular bioenergetics in intact brains of rats administered various doses of ethanol at 40–60 min after infusion of the alcohol (2, 4 or 6 g/kg; see below). Since a sine qua non of ongoing, progressive brain injury is a fall in brain ATP and phosphocreatine levels concomitant with a rise in intracellular inorganic phosphate ([Pi]) and hydrogen ion levels (for reviews, see Cotran et al., 1999; McIntosh et al., 1998), ^{31}P-NMR spectroscopy was utilized to quantitatively measure whole brain intracellular levels of ATP, phosphocreatine, [Pi], and [pHi] as described previously (Altura et al., 1995).

2.3. Quantitative myeloperoxidase stain

In still other experiments, we examined extravascular leukocyte (i.e., neutrophil) accumulation using a quantitative myeloperoxidase stain. Briefly, after a 60 min infusion (via a Harvard pump) of ethanol (2, 4 or 6 g/kg) via a branch of the internal carotid artery (in anesthetized animals) or after 6 months of daily ethanol feeding in sucrose-water as 36% calories (shams received sucrose-water only) (Altura and Altura, 1994), rats were perfused transcardially with 100 ml of physiological saline (24 °C; at a pressure of ~ 110 mm Hg). Brain samples were then taken from the cerebral cortex, cerebral hemispheres, and the medulla of ethanol-treated and control rats, immediately frozen in liquid nitrogen and stored at −80 °C for later biochemical analysis. The method used to quantify myeloperoxidase activity in rat brains was similar to that described by Barone et al. (1992) with some modifications, as follows: each brain sample was thawed on ice, weighed (wet wt.), and then homogenized (1:20, wt/vol) in 5 mmol/l potassium phosphate buffer (PPB) (pH 6.0, 4 °C) using an ultra-Turrax for three on/off cycles at 5-s intervals and then centrifuged (30,000×*g*, 30 min, 4 °C). The supernatant was then discarded, and the pellet was centrifuged again, as above. After the supernatant was decanted, the pellet was extracted

in 0.5% hexadecyltrimethyl-ammonium bromide (Sigma) in 50 mmol/l phosphate buffer (pH 6.0, 4 °C) for ~2 min. Samples were immediately frozen on dry ice. The freeze/thaw cycles were then performed with sonications (10 s, 24 °C), between cycles. After the last sonication, the samples were incubated at 4 °C for 20 min and centrifuged (12,500 × *g*, 15 min, 4 °C), and myeloperoxidase activity in the supernatant was assayed as described by Bradley et al., (1982); the changes in absorbance at 460 nm were measured with a spectrophotometer. One unit of myeloperoxidase was defined as the amount that degrades 1 μmol of peroxide per min at 24 °C. Blood alcohol concentrations were measured enzymatically (Altura and Altura, 1994).

2.4. Statistical analyses

Results are expressed as means ± S.E.M. Statistical significance between mean values was determined by means of *t*-tests, paired *t*-tests and analysis of variance (ANOVA). A probability value of 0.05 or less was considered significant.

3. Results

3.1. Effects of ethanol on brain microcirculation, leukocyte dynamics in venules and MPO activity

The number of leukocytes (cells per/100 μm min) rolling along or adhering to the venular endothelium increased after either perivascular or systemic (i.p.) administration of ethanol (to anesthetized rats) from ~4.2 ± 1.6 to as many as 34.6 ± 9.2 cells ($P < 0.001$) and from 1.6 ± 0.4 to as many as 15.7 ± 3.5 cells ($P < 0.01$), depending upon the dose and schedule of ethanol administration (see Table 1, Fig. 1). Leukocyte velocity in cortical venules decreased significantly (e.g., Table 1). These effects of ethanol appeared to be dose-dependent; the greater the dose of ethanol, the lower the velocity and the greater the rolling. Intermediate doses of ethanol (i.e., 4 g/kg; blood alcohol concentration = ~75–150 mg/dl) resulted in infiltration of leukocytes and macrophages. High doses of ethanol (e.g., 6 g/kg; blood alcohol concentration ≅ >250 mg/dl) clearly led to rupture of the blood–brain barrier, causing punctate hemorrhages and massive tissue infiltration of white cells, macrophages and red blood cells. It should be noted that intermediate to high doses of ethanol resulted in infiltration of leukocytes and macrophages usually 30–45 min after blood alcohol levels were in excess of 125 mg/dl; low blood alcohol levels (i.e., <75 mg/dl) caused slight emigration of leukocytes only after 60–70 min. Tissue infiltration of leukocytes was clearly confirmed by the dose-dependent effect of ethanol on brain tissue myeloperoxidase activity 60 min post-ethanol infusion; the greater the dose of ethanol, the greater the amount of tissue myeloperoxidase activity (Table 2). Rats fed ethanol–sucrose solutions (36% calories) for 6 months also demonstrated increased parenchymal brain tissue myeloperoxidase activity (Table 2). Although

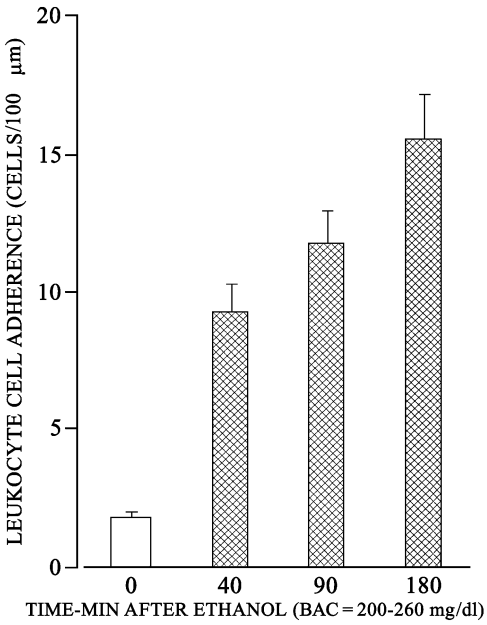

Fig. 1. Ethanol administration (6 g/kg i.p.) results in venular leukocyte adherence in rat brain.

Table 1
Influence of perivascularly administered ethanol on venular leukocyte rolling and velocity in rat brain in vivo[a]

Ethanol (mg/dl)	Venular rolling (cells/100 μm min)	Rolling velocity (μm/s)
Early responses (< 40 min after ethanol administration)		
0	4.2 ± 1.6	34 ± 2.2
50–70	14.2 ± 3.4[b]	24 ± 0.8[b]
100–150	21.3 ± 6.7[b]	17 ± 0.6[b]
≥250	34.6 ± 9.2[b]	6.8 ± 0.4[b]
Late responses (>75 min after ethanol administration)		
50–70	19.5 ± 5.8[b]	21.4 ± 1.4[b]
100–150	25.7 ± 6.3[b]	12 ± 0.8[b]
≥250	42.4 ± 8.5[b]	4.7 ± 0.3[b]

[a] Values are means ± S.E.M. ($N = 6–7$ animals each; 13–75 vessels per measurement).
[b] $P < 0.01$ compared to 0 mg/dl ethanol.

Table 2
Influence of different doses of intra-arterial ethanol on myeloperoxidase (MPO) activity in rat brain cerebral cortex 60 min post-infusion and 6 months of chronic ethanol[a]

Ethanol (blood alcohol concentration, mg/dl)	Myeloperoxidase (U/g wet tissue)
60 min post-infusion	
Sham—0	0.03 ± 0.013
37.5 ± 4.7	0.12 ± 0.042[b]
78.6 ± 12.5	0.18 ± 0.053[b]
265 ± 27	0.29 ± 0.12[b]
6-month ethanol feeding	0.16 ± 0.044[b]

[a] Values are means ± S.E.M. ($N = 6–12$ animals each).
[b] $P < 0.01$ by comparison with sham controls.

not shown, this was also associated with an increase in leukocyte rolling and a decrease in leukocyte velocities.

3.2. Effects of neutrophil depletion on microvascular effects of ethanol

Since some monocytes, in addition to neutrophils could be seen infiltrating the brain parenchyma 3–4 h post-ethanol administration, we sought to appraise the roles of neutrophils and monocytes in this process by depleting circulating leukocytes in rats, using vinblastine. White blood cell levels (4 days post-vinblastine treatment) were ~ 1400 ± 246/mm^3 or 10–15% of control (8500 ± 2960/mm^3, $P<0.05$, $n=10$). Small nonsignificant ($P>0.05$) decreases were noted in the platelet counts (226,200 ± 125, vs. 326,400 ± 76,000) and hematocrit levels (43.5 ± 6 vs. 46.8 ± 2%). These leukopenic rats, after high doses of ethanol (4 and 6 g/kg), clearly demonstrated a marked reduction in (~ 80–90%), but not elimination of venular cerebrovasospasm and intensity of postcapillary damage (as assessed by reduced numbers of petechial hemorrhages and decreased rupture of the blood–brain barrier measured with Na^+-fluorescein). For example, control-sham rats exhibited ~ 3.2 ± 0.08 venular ruptures and microhemorrhages per postcapillary venule vs. ~ 0.2 ± 0.08 ($P<0.01$) in leukopenic rats.

3.3. Effects of ethanol on microvascular brain tissue injury

The data presented in Table 3 indicate that administration of high doses of ethanol (4 and 6 g/kg) produce concentration-dependent increased ruptures of venular vessels and petechial homorrhages at postcapillary venular sites. Using ^{31}P-NMR spectroscopy, the results in Table 4 demonstrate that 40–60 min after ethanol administration, there is clear concentration-dependent biochemical compromise of cellular bioenergetics; ethanol results in a concentration-dependent fall in whole brain intracellular [PCr]/[ATP] ratios and phosphocreatine coupled with intracellular rises in inorganic phosphate and hydrogen ion concentration. Taken together, the ongoing in situ vessel pathology and compromise of brain intracellular bioenergetics clearly point to cardinal signs of brain tissue pathology (Cotran et al., 1999; McIntosh et al., 1998). Although not shown, vinblastine-induced leukopenia in rats ameliorates the alcohol-induced alterations in brain cellular bioenergetics ~ 45–80%, depending upon the animal ($n=4$).

Table 3
Effects of different doses of intra-arterial ethanol on venular ruptures and microhemorrhages per postcapillary venule 40–60 min post-alcohol infusion

Ethanol (blood alcohol concentration, mg/dl)	Number of venular ruptures–microhemorrhages per postcapillary venule
Sham—0	0
39.5 ± 4.5	0.6 ± 0.04[b]
80.3 ± 11.6	3.6 ± 0.07[c]
258 ± 29	5.8 ± 0.09[c]

[a] Values are means ± S.E.M. ($N=6–12$ animals each).
[b] $P<0.01$ by comparison with sham controls.
[c] $P<0.01$ by comparison with all other values.

Table 4
Effects of different doses of intra-arterial ethanol on cellular bioenergetics in intact rat brain after 40–60 min[a]

Ethanol (g/kg)	pHi	[PCr] (mM)	[Pi] (mM)	[PCr]/[ATP] ratio
Sham—0	7.26 ± 0.05	4.74 ± 0.28	1.26 ± 0.08	2.38 ± 0.13
2	7.23 ± 0.06	4.56 ± 0.26	1.42 ± 0.06	2.16 ± 0.12
4	7.15 ± 0.04[b]	4.22 ± 0.24[b]	2.08 ± 0.22[b]	2.02 ± 0.09[b]
6	6.98 ± 0.07[c]	3.74 ± 0.38[c]	3.24 ± 0.56[b]	1.64 ± 0.08[b]

[a] Values are means ± S.E.M. ($N=6–12$ animals each).
[b] $P<0.01$ by comparison with sham controls (paired t-test).
[c] $P<0.01$ by comparison with all other values (paired t-test).

4. Discussion

Damage to the cerebral microvasculature could result from a variety of insults, including blunt trauma, ischemia, viral or bacterial infection, immunologic challenge and alcohol abuse. Alcohol abuse is the fourth leading cause of death in the USA, killing more than 100,000 people each year (U.S. Dept. Health Human Services, 1993). This total is probably underestimated, as death certificates generally do not include alcohol abuse as a cause of death. Approximately 10% of all alcohol-dependent persons have some type of clinically diagnosable brain syndrome (Victor et al., 1989; U.S. Dept. Health Human Services, 1993), and approximately 75% of all detoxified long-term alcoholics have some degree of cognitive brain impairment.

Efforts to understand the pathogenesis of alcohol-induced cerebrovascular injury, and hence to develop effective countermeasures, have been seriously curtailed by: (1) failure to note the significance of white blood cell and histiocyte infiltration of brain disorders (for reviews, see Wernicke, 1900; Haller, 1980; Victor et al., 1989); (2) the lack of reliable in vivo models of the human brain; (3) failure to exploit in situ, high-resolution TV microscope recording systems when using extant brain models; (4) failure to scrutinize brain sections for myeloperoxidase activity, a marker of neutrophil (and monocyte/macrophage) invasion of brain tissue; and (5) failure to simultaneously monitor brain intracellular bioenergetics.

Although the results described herein should be thought of as still preliminary, these new data are dramatic and, nevertheless, exciting in our opinion. To our knowledge, this is the first demonstration that circulating leukocytes are required for alcohol-induced microvascular brain tissue injury, at least in the in situ rat brain. Our data suggest that not only are leukocytes required, in part, for the alcohol-induced venular cerebrovasospasm, and subsequent alterations in brain bioenergetics, but that these white blood cells

are also needed for most of the subsequent rupture of, and transudation of, blood elements across postcapillary venules.

The migration of leukocytes from the microvascular system, and their infiltration into parenchymal tissues, and across the blood–brain barrier, is a key early event in brain tissue inflammation and injury. The entry of leukocytes into tissue-impaired injured sites requires precise molecular mechanisms to enable the white blood cells and macrophages to recognize the injured sites from within the microvasculature and to allow contact with the endothelium so that the cells can exit and infiltrate blood vessel walls. These processes of recognition and contact are mediated by several adhesion molecules which include selectins, integrins and members of the Ig superfamily, which act in a sequential manner with other regulatory molecules. Dysregulation of these adhesion molecules and precise signal transduction pathways can contribute to continued recruitment and leukocyte activation with unrelentless inflammation (Carlos et al., 1997; Barone, 1998; Ritter et al., 1998; Stoll et al., 1998).

Recently, we have reported that antioxidants, inhibitors of activation of transcription factor nuclear factor-kappa β (NF-κβ) (which inhibit synthesis of selectins), Ca^{2+} antagonists, and inhibition of certain signal transduction pathways (e.g., protein kinase C, mitogen-activated protein kinases, tyrosine kinases, and phosphatidyinositol 3-kinases) could all, to varying degrees, either prevent or attenuate markedly ethanol-induced cerebrovasospasm and cerebral microvascular injury (Altura et al., 1983, 1999; Altura and Altura, 1994; Altura and Gebrewold, 1996, 1998; Morrill et al., 1997; Ema et al., 1998; Li et al., 1999, 2001; Zheng et al., 1998; Yang et al., 2000, 2001). Moreover, we have recently shown, elsewhere, that ethanol administration results in rapid lipid peroxidation of cerebral vascular smooth muscle cells and subsequent activation of NF-κB in these cerebral vascular cells (Altura et al., in press). Antioxidants and inhibitors of NF-κB activation either prevent or ameliorate the ethanol-induced membrane peroxidation and NF-κB activation (Altura et al., in press; unpublished findings). In addition, alcohol-induced brain injury is dependent upon an early brain tissue loss of intracellular free Mg ($Mg^{2+}]_i$) (Altura and Altura, 1994). We thus hypothesize that after the initial cellular loss of $[Mg^{2+}]_i$, an entry and intracellular release of $[Ca^{2+}]_i$ occurs (Altura and Altura, 1994; Altura et al., 1995, 1999; Yang et al., 2000, 2001). These divalent cation changes then result in membrane lipid peroxidation (Altura and Gebrewold, 1996, 1998; Altura et al., 1999, in press; Morrill et al., 1997; Li et al., 1999, 2001), triggering the release and generation of reactive oxygen species, which in combination with elevated $[Ca^{2+}]_i$ result in early venular vasospasm and reperfusion injury, triggering early release and activation of selectins followed by integrin activation, thus resulting in rolling, adhesion and infiltration of leukocytes and macrophages into brain parenchymal tissue; the greater the brain concentration of ethanol, the greater the cell infiltration. The observed increasing levels of myeloperoxidase reported herein are consistent with this hypothesis. Moreover, in recent in vivo studies on rats, we have found that chronic treatment of rats with either vitamin E or pyrrolidine dithiocarbamate (an inhibitor of NF-κB in rat brain tissues) (Altura and Gebrewold, 1996, 1998; Altura et al., in press), ameliorates greatly the ethanol-induced rises in tissue MPO and leukocyte infiltration (Altura et al., unpublished findings). Increasing brain concentrations of alcohol would eventuate in increasing deficits in cerebral blood flow, postcapillary venular rupture, stroke and/or brain neuronal damage. In vivo [^{31}P]-nuclear magnetic resonance (NMR), spectroscopy shown herein (and elsewhere) and optical spectroscopic data on the intact brain (Altura and Altura, 1994; Altura et al., 1995; Barbour et al., 1993), demonstrating continuing loss of brain phosphagens (phosphocreatine, ATP), concomitant rises in intracellular inorganic phosphate, reduced cytochrome oxidase aa_3, and deoxyhemoglobin, and brain intracellular acidosis, are certainly consistent with the latter hypothesis.

Lastly, it is important to point out here that the brain pathology attributed to chronic ingestion of alcohol, observed in patients with Wernicke–Korsakoff syndrome, hepatic encephalopathy, central pontine myelinolysis, cortical atrophy, toxic leukoencephalopathy, and alcohol-induced stroke, particularly after binge-drinking, may owe a great deal of their brain lesions to microvascular proinflammatory-like events reported herein.

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