Cerebrospinal Fluid Monocyte Chemoattractant Protein-1 in Alcoholics: Support for a Neuroinflammatory Model of Chronic Alcoholism

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Background: Liver inflammation in alcoholism has been hypothesized to influence the development of a neuroinflammatory process in the brain characterized by neurodegeneration and altered cognitive function. Monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2) elevations have been noted in the alcoholic brain at autopsy and may have a role in this process.

Methods: We studied cerebrospinal fluid (CSF) levels of MCP-1 as well as interleukin-1β and tumor necrosis factor-α in 13 healthy volunteers and 28 alcoholics during weeks 1 and 4 following detoxification. Serum liver enzymes were obtained as markers of alcohol-related liver inflammation.

Results: Compared to healthy volunteers, MCP-1 levels were significantly higher in alcoholics both on day 4 and day 25 ($p < 0.0001$). Using multiple regression analysis, we found that MCP-1 concentrations were positively associated with the liver enzymes gamma glutamyltransferase (GGT; $p = 0.03$) and aspartate aminotransferase/glutamic oxaloacetic transaminase (AST/GOT; $p = 0.004$).

Conclusions: These preliminary findings are consistent with the hypothesis that neuroinflammation as indexed by CSF MCP-1 is associated with alcohol-induced liver inflammation, as defined by peripheral concentrations of GGT and AST/GOT.

Key Words: Monocyte Chemoattractant Protein-1, Alcoholism, Inflammation, Cerebrospinal Fluid, Cytokine.

CYTOKINES ARE SOLUBLE polypeptides which function as cellular signaling molecules of the innate immune system. In addition to a classical role as modulators of inflammation-related processes, they can have diverse regulatory actions. In alcoholics, cytokines have been suggested to link inflammatory processes in the periphery with neuroinflammatory changes in the brain (Crews et al., 2006). In the model that has been proposed, one way for this to occur is for excessive alcohol consumption to increase gut permeability, allowing endotoxin to enter the portal circulation and stimulate an inflammatory response in the liver (Bjarnason et al., 1984; Crews et al., 2011; Leclercq et al., 2012; Mandrekar and Szabo, 2009; Wang et al., 2010). Inflammatory cytokines produced in the liver enter the circulation and may influence the brain through peripheral sensory nerves as well as through more direct effects on cells in the brain (Blednov et al., 2011; Mitchell et al., 2009; Quan and Banks, 2007). Alcohol-induced endotoxemia may facilitate the action of circulating cytokines to promote an inflammatory response in the brain which can influence brain function. Neuroinflammation may also be self-perpetuating when accompanied by classically activated microglia which release reactive oxygen species and inflammatory proteases causing cellular damage, effecting further microglia activation (Crews et al., 2006; Mitchell et al., 2009; Yang et al., 2011). It is theorized that central nervous system cytokine concentrations, associated with heavy alcohol consumption, can remain active for an extended period of time, thereby altering neurotransmission, neurogenesis, neurite outgrowth, myelination, and promoting neurodegeneration (Crews et al., 2011; Lull and Block, 2010). Although alcohol-induced activation of microglia may be a marker rather than a cause of alcohol-induced neurodegeneration (Marshall et al., 2013), alcohol-induced immune system changes may be important in the development of the neuropathological and behavioral changes characteristic of alcoholism and could be considered a "neuroinflammatory model" of alcoholism (Crews et al., 2006).

Monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2) is a chemokine which may have special relevance for this model of alcoholism (Blednov et al., 2005, 2012; Crews et al., 2006; Fisher et al., 1999; He

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and Crews, 2008). The administration of alcohol promotes increased MCP-1 in the rodent brain (Ehrlich et al., 2012; Qin et al., 2008) and genetic studies in animals indicate that increased MCP-1 signaling is accompanied by increased alcohol consumption (Blednov et al., 2012). In human alcoholics, MCP-1 expression is increased in multiple areas of the brain at autopsy (He and Crews, 2008).

Although MCP-1 was first recognized as a potent monocyte chemotactic factor (Conductier et al., 2010), it has multiple functions and is produced by many cell types in the periphery as well as the brain. In the brain, MCP-1 has diverse effects (Bridgette et al., 2010; Conductier et al., 2010). MCP-1 can influence neurogenesis and promote the migration of neural stem cells (Mathieu et al., 2010). Inflammatory and neurotoxic effects of MCP-1 are mediated through the activation of microglia and secretion of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Mitchell et al., 2009; Yang et al., 2011) and may contribute to the neuroinflammatory and neurodegenerative changes noted in disorders including major depression (Piletz et al., 2009), Alzheimer disease (Hickman and El Khoury, 2010), multiple sclerosis (Van Der Voorn et al., 1999), ischemic brain damage (Minami and Satoh, 2003), and infections such as HIV (Galimberti et al., 2006). For these reasons, MCP-1 is often considered to be a pro-inflammatory chemokine. MCP-1 also functions as a neuromodulator; it can enhance the excitability of neurons and influence synaptic transmission through presynaptic mechanisms (Zhou et al., 2011). MCP-1 is colocalized in neurons with classical neurotransmitters where it can be released in a calcium-dependent manner through neuronal depolarization (Conductier et al., 2010; Zhou et al., 2011). Actions on the glutamatergic system may prove to be particularly important considering the importance of this system in the pathogenesis of alcoholism (Tsai et al., 1995).

To explore the role of MCP-1 in alcoholism, we measured cerebrospinal fluid (CSF) concentrations of MCP-1 in a sample of alcoholics who were enrolled in a larger study examining the effect of acamprosate on alcohol withdrawal. We reasoned that if the neuroinflammatory model of alcoholism is valid, MCP-1 concentrations would be directly correlated with measures of liver damage and inflammation and with measures of alcoholism severity (Chang et al., 2004). Finally, as the effects of MCP-1 can be mediated through the activation of microglia and secretion of IL-1β and TNF-α (Mitchell et al., 2009; Yang et al., 2011), we also measured CSF concentrations of IL-1β and TNF-α.

MATERIALS AND METHODS

Subjects and Clinical Assessments

The study was conducted using CSF obtained from subjects included in a previously published study that evaluated the effect of acamprosate on measures of glutamate metabolism (Umhau et al., 2010). Descriptive subject characteristics are listed in Table 1. Briefly, otherwise healthy alcohol-dependent subjects were withdrawn from alcohol during a 28-day inpatient protocol at the National Institute of Alcohol Abuse and Alcoholism inpatient unit in the National Institutes of Health (NIH) Clinical Center. These subjects were typically screened over the phone several days before admission. The day after admission, blood was obtained for measurement of liver function tests including gamma glutamyltransferase (GGT), alanine aminotransferase/glutamate pyruvate transaminase (ALT/GPT), and aspartate aminotransferase/glutamic oxaloacetic transaminase (AST/GOT). Withdrawal intensity was evaluated with the Clinical Institute Withdrawal Assessment of Alcohol Scale (CIWA-Ar) rating every 4 hours while awake for the first 5 days after admission (Sullivan et al., 1989). Diazepam was given as clinically necessary for withdrawal. The severity of alcohol dependence was assessed using the Alcohol Dependence Scale (ADS; Skinner and Horn, 1984), and alcohol consumption during the preceding 3 months was quantified using the Time-Line Follow-Back (TLFB; Sobell and Sobell, 1992). After achieving a blood alcohol level of 0 g/dl, subjects were randomized to receive 666 mg acamprosate every 8 hours or matching placebo.

The healthy control participants were recruited through the National Institute of Mental Health Section on Neuroimaging in Mood and Anxiety Disorders using newspaper and radio advertise-

Table 1. Subject Sample Baseline Measures (mean ± SEM)

Variable	Alcoholics	Controls
Total *N*	28	13
Gender	9 females, 19 males	7 females, 6 males
Race	9 Black, 19 White	4 Black, 9 White
Age (years)	34.8 ± 1.3	35.5 ± 3.4
Body mass index	24.8 ± 0.7	26.5 ± 1.6
Acamprosate treatment	11 acamprosate, 17 placebo	
Smoking status	25 smokers, 3 nonsmokers	13 nonsmokers
Alcohol Dependence Scale (ADS)	26.4 ± 1.3	N/A
Cumulative diazepam dose (mg)	30 ± 7.8	
Time-Line Follow-Back (TLFB; 90 days)		
Heavy drinking days	70.1 ± 4.2	
Peak Clinical Institute Withdrawal Assessment of Alcohol Scale (CIWA-Ar)	13.5 ± 1.0	
Serum chemistry values		
Gamma glutamyltransferase (GGT)[a,b]	121.0 ± 26.2	
Alanine aminotransferase/ glutamate pyruvate transaminase (ALT/GPT)	72.6 ± 11.5	22.0 ± 2.5
Aspartate aminotransferase/ glutamic oxaloacetic transaminase (AST/GOT)	89.5 ± 16.0	23.3 ± 1.9
Other laboratory values		
Mean corpuscular volume (MCV)	94.7 ± 1.3	87.0 ± 2.0
Platelets	244.0 ± 16.1	279.5 ± 23.7
Homocysteine[a,c]	10.9 ± 1.0	
Immunoglobulin IgG[a,d]	1,016.0 ± 45.5	
Red blood cell folate[a,e]	715.0 ± 34.8	
CSF MCP-1		
Day 4	468.0 ± 23.8	287.5 ± 8.3[f]
Day 25	419.0 ± 16.2	

CSF, cerebrospinal fluid; MCP-1, monocyte chemoattractant protein-1.
[a]Laboratory values not available for controls.
[b]Normal laboratory range = 7 to 38 μ/l.
[c]Normal laboratory range = 0 to 13 μmol/l.
[d]Normal laboratory range = 642 to 1,730 mg/dl.
[e]Normal laboratory range = 496 to 1,477 ng/ml.
[f]Only 1 measurement was taken from controls, and CSF was not taken systematically on day 4 or day 25 as it was for alcoholics.

ments in the Washington, DC greater metropolitan area. Volunteers between 18 and 55 years of age were evaluated using the Structured Clinical Interview for DSM-IV-TR (First and Pincus, 2002) and an unstructured interview with a psychiatrist. Screening also included physical examination, urine drug testing, and laboratory assays that included blood count, hepatic function, viral titers, and pregnancy status. Volunteers were excluded if they met DSM-IV-TR criteria for substance abuse within 1 year or substance dependence within the lifetime, had major medical or neurological disorders (including laboratory evidence of hepatic dysfunction), pregnancy, anemia, positive drug or viral (HIV, hepatitis) screen, exposure to psychotropic medications within the previous 3 weeks, or peripheral stigmata of alcoholism on physical examination. Informed consent was obtained in accordance with the Declaration of Helsinki and the NIH Institutional Review Board.

CSF Sampling and Cytokine Analysis

CSF samples were obtained by lumbar puncture the morning after an overnight bed rest. Alcoholics were sampled on day 4 and day 25 of abstinence from alcohol (Umhau et al., 2003). After collecting 3 to 5 ml of CSF for clinical analysis, CSF for analysis of MCP-1, IL-1β, and TNF-α was placed on ice and subsequently frozen at -80°C until assayed. MCP-1, IL-1β, and TNF-α levels were analyzed using Meso Scale Discovery (MSD, Gaithersburg, MD) electrochemiluminescence Ultra Sensitive Human Cytokine Assay. The MSD method was followed except for using 1% bovine serum albumin in phosphate-buffered saline to prepare the standards and block the plates to mimic the protein concentration of CSF. The plate was analyzed using the MSD SI2400 plate reader. MCP-1, IL-1β, and TNF-α concentrations are reported in pg/ml.

Statistical Analysis

The effects of acamprosate treatment and day of measurement on CSF MCP-1 as well as IL-1β and TNF-α levels were analyzed using 2-way analysis of variance with treatment (acamprosate vs. placebo) as the between subjects factor and day (day 4 vs. day 25) as the within subjects factor. CSF MCP-1, IL-1β, and TNF-α levels in alcoholics on each day were also compared to levels in healthy controls (measured only once) using 1 way ANOVA.

The relationships between subject characteristics (e.g., age, sex, race, smoking status, body mass index, ADS, peak CIWA-Ar Score, cumulative diazepam dose, number of heavy drinking days, serum chemistry values, and indicators of nutritional status such as IgG, red blood cell folate, homocysteine, mean corpuscular volume (MCV), and the platelet count) and CSF MCP-1 levels in alcoholics were analyzed using multiple regression analyses, where the final model was determined using the best subsets selection procedure and Mallows' Cp as the selection criteria. Separate multiple regressions were run for values measured on day 4 and on day 25. We did not perform multiple regression analyses for IL-1β or TNF-α because there were no significant differences between alcoholics and controls on day 4.

RESULTS

There was a trend for CSF MCP-1 levels to decrease from day 4 (mean = 455.9, SE = 22.0) to day 25 (mean = 425.7, SE = 16.7), $F(1, 21) = 3.51$, $p = 0.07$ (see Fig. 1). There was no effect of acamprosate treatment on CSF MCP-1 levels, neither overall, $F(1, 21) = 2.25$, $p = 0.15$, nor across the 2 time points, $F(1, 21) = 0.20$, $p = 0.66$. Consequently, we combined the 2 treatment groups for subsequent analyses.

Compared to healthy volunteers, MCP-1 levels were significantly higher in alcoholics on day 4, $F(1, 35) = 12.32$, $p = 0.001$ and also on day 25, $F(1, 30) = 31.45$, $p < 0.0001$ (see Fig. 1).

On day 4, there were no significant differences between alcoholics and controls for IL-1β, $F(1, 33) = 1.59$, $p = 0.22$, or TNF-α, $F(1, 33) = 0.004$, $p = 0.95$. On day 25, there was no significant difference between alcoholics and controls for IL-1β, $F(1, 28) = 0.00002$, $p = 1.0$; however, concentrations for TNF-α were lower in alcoholics compared to controls, $F(1, 28) = 10.49$, $p = 0.003$.

The multiple regression analysis for CSF MCP-1 measured on day 4 resulted in a model that included sex, cumulative benzodiazepine dose, GGT, and AST/GOT ($R^2 = 0.60$; see Table 2 for parameter estimates). Both GGT (partial $R = 0.48$) and AST/GOT (partial $R = 0.59$) were positively associated with CSF MCP-1, as was benzodiazepine dose (partial $R = 0.53$). Males had higher MCP-1 values than females (partial $R = 0.32$).

The multiple regression analysis for CSF MCP-1 measured on day 25 resulted in a model that included only age and ALT/GPT ($R^2 = 0.32$; see Table 3 for parameter estimates). Age (partial $R = 0.45$) was positively associated with CSF MCP-1 measured on day 25. Although ALT/GPT was included in the final model, it was not significantly associated with MCP-1 measured on day 25.

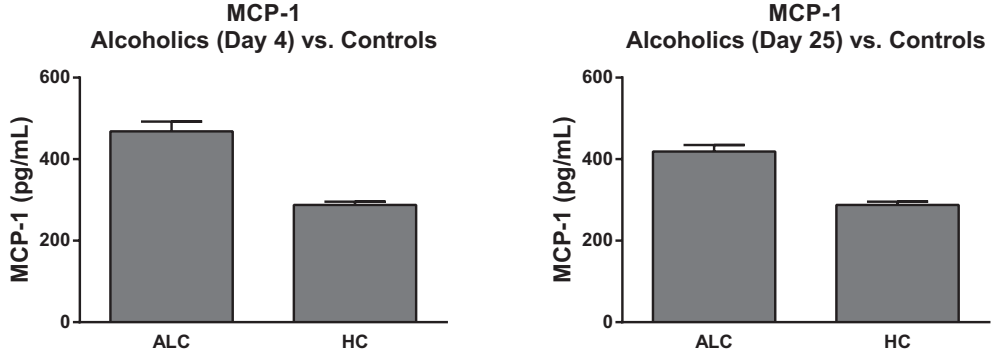

Fig. 1. Cerebrospinal fluid MCP-1 levels measured in alcoholics (ALC) on day 4 and on day 25 compared to healthy controls (HC), $F(1, 35) = 12.32$, $p = 0.001$, and $F(1, 30) = 31.45$, $p < 0.0001$, respectively. MCP-1, monocyte chemoattractant protein-1.

Table 2. Multiple Regression Results for CSF MCP-1 Measured on Day 4

Variable	Parameter	Std. Err	*t*	*p*
Intercept	202.4019	58.58	3.45	0.002
Sex (male = 1)	123.68	43.69	2.83	0.01
Benzodiazepine dose	1.34	0.66	2.04	0.05
Gamma glutamyltransferase	0.33	0.13	2.47	0.03
AST/GOT	0.78	0.24	3.26	0.004

AST/GOT, aspartate aminotransferase/glutamic oxaloacetic transaminase; CSF, cerebrospinal fluid; MCP-1, monocyte chemoattractant protein-1.

Table 3. Multiple Regression Results for CSF MCP-1 Measured on Day 25

Variable	Parameter	Std. Err	*t*	*p*
Intercept	202.86	85.29	2.38	0.03
Age	5.27	2.39	2.20	0.04
ALT/GPT	0.47	0.25	1.84	0.08

ALT/GPT, alanine aminotransferase/glutamate pyruvate transaminase; CSF, cerebrospinal fluid; MCP-1, monocyte chemoattractant protein-1.

DISCUSSION

This is the first human study to demonstrate that CSF levels of MCP-1 are increased in alcoholics and are associated with liver inflammation as measured by peripheral concentrations of GGT and AST/GOT. Previously, we found that magnetic resonance imaging measures of brain shrinkage in alcoholism were correlated with liver inflammation as quantified by serum GGT (Chen et al., 2012). The present study expands on this finding by demonstrating that the severity of liver inflammation is correlated with neuroinflammation as indexed by the concentration of MCP-1 in CSF. Although MCP-1 is a normal constituent of CSF, MCP-1 is increased with neuroinflammation and neurodegeneration (Conductier et al., 2010; Franciotta et al., 2001). MCP-1 can be derived from neurons, astrocytes, endothelial cells, and microglia as well as from the choroid plexus (Mitchell et al., 2009; Yang et al., 2011). Cytokine production by the choroid plexus is stimulated by circulating inflammatory mediators, including lipopolysaccharide or cytokines derived from an inflamed liver (Crews et al., 2006; Singh et al., 2007). Inflammatory cytokines can also promote inflammatory changes in the brain by way of the circumventricular organs, through the secretion of immune mediators by cells of the blood–brain barrier, by active transport across the blood brain barrier and through the influence of vagal afferents (Blednov et al., 2011; Mitchell et al., 2009; Quan and Banks, 2007). Circulating cytokines may also pass into the brain, allowing detection in the CSF (Banks, 2005; Fisher et al., 1999; Singh et al., 2007; Yadav et al., 2010). Irrespective of the manner in which peripheral inflammatory signals access the brain, their effect could be to promote the development of central cytokine changes and neuroinflammation.

Our results suggest the possibility that a progressive normalization of neuroinflammation occurs with abstinence, as there was a trend for MCP-1 levels to decrease over 3 weeks of early abstinence. Any normalization during this time is incomplete, however, as demonstrated by the observation that MCP-1 levels in alcoholics remain higher than those in healthy control subjects. Our data do not allow us to determine whether this normalization trend is real; re-measurement of CSF MCP-1 following a longer, more significant period of abstinence may be necessary to determine whether CSF MCP-1 levels decrease or remain elevated.

In contrast with the results at day 4, we found that the association of CSF MCP-1 with hepatic function was minimal at day 25, a finding which suggests that with progressive abstinence, changes in the inflammatory state occur at different rates in the liver and the brain. It is important to emphasize that these relationships cannot determine cause and effect. It is possible that neuroinflammation can, at least to some extent, influence the liver, and it is also possible that alcohol consumption is influencing both liver inflammation and central nervous system cytokines in parallel. GGT might be simply an index of previous alcohol consumption; alcohol may be directly influencing the synthesis of MCP-1. Future studies that accurately characterize alcohol consumption patterns may be needed to clarify what may be a very complex interaction between alcohol consumption and inflammation.

With our inpatient sample of recovering alcoholics, we were able to control for a number of factors potentially influencing MCP-1, including smoking status, race, gender, age, and body mass index as well as indicators of nutritional status, for example, IgG, red blood cell folate, homocysteine, MCV, and the platelet count (Conductier et al., 2010; Hammons et al., 2009; Li et al., 2007). In addition, subjects did not have viral hepatitis or HIV. It should be noted that levels of AST/GOT, ALT/GPT, and GGT can be influenced by a number of factors, but in our population, the predominant influence is likely to be alcoholic liver disease (Pratt and Kaplan, 2000). The fact that there was no significant difference on day 4 between alcoholics and controls for concentrations of IL-1β or TNF-α is contrary to the findings of Mitchell and colleagues (2009) and Yang and colleagues (2011) and suggests additional studies are indicated to understand how alcohol may influence the relationship between MCP-1 and other CSF cytokines. Although the primary objective of our study design was to examine the effect of acamprosate on the glutamatergic system in alcohol withdrawal, the design of the study lends itself to an initial investigation of the neuroinflammatory model of alcoholism. A confound from acamprosate is made unlikely by 2 observations. First, drug treatment did not have a significant effect in the analysis of MCP-1 levels. Second, when our analysis was restricted to those alcoholic subjects who had not received acamprosate, the findings were very similar. Our observation that acamprosate did not regulate central MCP-1 levels in early alcohol abstinence is of some interest in its

own right, because acamprosate is structurally closely related to the amino acid taurine, reported to reduce the levels of MCP-1 (Elvevoll et al., 2008; Liu and Quinn, 2002). Our data make a similar effect of acamprosate unlikely.

Previous research in animals indicates that MCP-1 activity can influence alcohol consumption (Blednov et al., 2005). Of interest, our measurements of CSF MCP-1 did not correlate with the severity of alcohol dependence as measured by the ADS or alcohol consumption as recorded by the TLFB. However, CSF MCP-1 did correlate with the cumulative diazepam dose. Because diazepam was administered based on the clinical features of alcohol withdrawal, this may indicate that CSF MCP-1 is greater in those with more severe alcoholism. Our finding of increased MCP-1 concentrations in CSF is also consistent with autopsy results showing that in alcoholics MCP-1 expression is increased in multiple areas of the brain (He and Crews, 2008). The relative greater level of MCP-1 in males has been noted previously (Jilma-Stohlawetz et al., 2001).

Future research is indicated to explore the relationship between the expression of cytokines and the drive to consume alcohol. It is possible that other cytokines which we did not measure could exert an effect on alcohol consumption. Also, other factors, such as genetics, dietary status, gut permeability, and the intestinal biome could have a role on liver inflammation and the development of neuroinflammation and neurodegeneration (Kirpich et al., 2008; Leclercq et al., 2012).

In conclusion, we found that CSF concentrations of MCP-1 are elevated in treatment seeking alcoholics in early abstinence and are associated with peripheral concentrations of GGT and AST/GOT. The correlation of CSF MCP-1 levels with potential markers of liver inflammation is an expected, yet, up to this point, untested proposition of the neuroinflammatory model of alcoholism. Although these findings should be considered preliminary, as our sample size is small, they add to the growing body of data supporting a role for cytokine-induced neuroinflammation in the pathophysiology of alcoholism. These findings also demonstrate the potential for using spinal fluid cytokine concentrations to provide a window into neuroinflammatory processes which may alter neurotransmitter function and promote the neuropathology of alcoholism (Tsai et al., 1995).

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FINANCIAL DISCLOSURES

Dr. Zarate is listed as a co-inventor on a patent for the use of ketamine in major depression and has assigned his patent rights on ketamine to the U.S. government. Dr. Drevets is currently an employee of Johnson & Johnson Pharmaceuticals. Dr. Drevets has consulted for Pfizer Pharmaceuticals, Johnson and Johnson Pharmaceuticals, Eisai, Inc., and Myriad/Rules Based Medicine, Inc. All other authors report no biomedical financial interests or potential conflict of interests.

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