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Abstract

Fetal exposure to alcohol can lead to extensive pathology in the CNS causing fetal alcohol syndrome or alcohol-related neurodevelopmental disorder. Our previous research has revealed that alcohol has detrimental effects on development of neurons and glial cells, including microglia. However, the effects of alcohol on microglial function as well as interactions between microglia and neurons remain relatively unexplored. Microglia produce immunomodulatory cytokines and chemokines that directly control the survival, development, and function of neurons and glia. In this study, mouse N9 microglial cells were treated with 0.5% (w/v) ethanol for 12-48 hr with or without subsequent challenge with the cellular activator lipopolysaccharide. Microglial expression of the cytokines IL1, IL6, IL10, IL12, TNFα, IFNγ, and MIF, and the chemokines MCP-1, MIP-1α, MIP-1β, MIP-2, IP-10, TCA-3, and RANTES was quantified by ribonuclease protection assay of cellular RNA. The mRNA levels of MIP-1β, MIP-1α, and MCP-1 RNA were increased 94% ($p<0.05$), 23%, and 38% ($p<0.05$), respectively, after 12 hr exposure to ethanol. Increased expression of MIP-1β, MIP-1α, and MCP-1 mRNA was also observed following 24 and 48 hr treatment with ethanol, but the changes were less significant. The mRNA levels of other chemokines and cytokines were not altered significantly under these conditions. These findings suggest that alcohol exposure may alter microglial expression of chemokines within the CNS. MIP-1 and MCP-1 are involved in synaptic development, neuronal maturation and signal transduction, neuronal and glial migration, and angiogenesis in the developing CNS. Increased levels of these chemokines may cause immune cell infiltration and neuroinflammation, with detrimental consequences for neuronal function and survival. Thus, alcohol-induced increase in MIP-1 and MCP-1 may impair neuronal function and survival and contribute to the neuropathology associated with fetal alcohol exposure.

Introduction

Fetal exposure to alcohol can lead to extensive pathology in the central nervous system (CNS), causing mental retardation, behavioral problems, and other developmental neural defects that are associated with fetal alcohol syndrome and alcohol-related neurodevelopmental disorder (Meyer et al., 1990; Mattson et al., 1994; Riley et al., 1995; Stratton et al., 1996). It is well established that alcohol pathogenesis during CNS development involves significant neuropathology and neuronal loss (for example, Bauer-Moffett and Altman, 1977; Pierce et al., 1989; Bonthius and West, 1991; Hamre and West, 1993; Napper and West, 1995; Pierce et al., 1997; Light et al., 2002). However, the mechanisms underlying the neuropathology are not well understood, and the effect of alcohol on interactions between neurons and glia that control neuronal development, survival and function are relatively unexplored. In particular, the effects of alcohol on microglia, the resident immune cells in the brain that exhibit both protective and toxic effects on neurons, merit investigation. Our research has revealed that alcohol has detrimental effects on microglia proliferation, survival, and maturation and that microglia may be more sensitive to alcohol than other neural cells (Kane, 2001). Thus, alcohol may disrupt microglial function or disrupt interactions between neurons and microglia and consequentially contribute to the neuronal pathology associated with fetal alcohol exposure.

The potential relationship between the neuronal pathology associated with alcohol exposure and microglial expression of secreted proteins, including cytokines and chemokines, has not been previously explored. Cytokines and chemokines are regulatory proteins secreted by many cell types and are best known for their ability to act as immune mediators. Within the CNS, cytokines and chemokines can regulate neuronal and glial proliferation, differentiation, function, survival, and death (reviewed in: Ransohoff and Benveniste, 1996; Bajetto et al., 2002). They play essential roles in CNS development and are involved in maintenance of CNS homeostasis. In addition, they mediate neuroinflammatory processes and are involved in neuroinflammatory disorders, such as multiple sclerosis, Alzheimer's disease, and acquired immunodeficiency syndrome-associated dementia (see above reviews).

The thrust of this investigation included analysis of

alcohol effects on the levels of mRNA encoding cytokines and chemokines that can be secreted by microglia, including the cytokines interleukin (IL)-1, IL6, IL10, IL12, tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), and migration inhibitory factor (MIF), and the chemokines monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, IFNγ inducible protein (IP-10), thymus-derived chemotactic agent-3 (TCA-3), and regulated on activation normal T cell-expressed and secreted (RANTES). IL1β, IL6, and IL10 can enhance survival (Plata-Salaman, 1991; Patterson, 1992; Mehler and Kessler, 1994; Mehler and Kessler, 1995), and IL6, IL12, and IFNγ can promote maturation (Barish et al., 1991; Mehler and Kessler, 1994; Mehler and Kessler, 1995), of neurons or neuroblasts. IL1 and TNFα can upregulate other cytokines, stimulate glial proliferation (Ransohoff and Benveniste, 1996; Oppenhheim and Feldman, 2001), and induce neuroinflammatory processes (Korner et al., 1997; Probert et al., 1997). The chemokine RANTES can promote migration, survival and differentiation of neurons (Bolin et al., 1998; Meucci et al., 1998) and stimulate proliferation and survival of astrocytes (Bakhiet et al., 2001). RANTES, MIP-1α, MIP-2 and MCP-1 can regulate synaptic development and neuronal signal transduction (Giovanelli et al., 1998; Meng et al., 1999; Meucci et al., 1998). RANTES is chemotactic to monocytes and lymphocytes (Bukara and Bautista, 1999). MIP-1α and MCP-1 are chemotactic to astrocytes (Heesen et al., 1996; Tanabe et al., 1997). TCA-3 is chemotactic to microglia and macrophages (Hayashi et al., 1995).

Alcohol can either suppress or amplify the production of cytokines and chemokines in peripheral monocytes and macrophages (Szabo et al., 1995, 1998; Arbabi et al., 1999; Bukara and Bautista, 1999; Zhang et al., 2001). Because microglia are derived from monocytes that migrate into the developing CNS (Hickey et al., 1992; Ling and Wong, 1993), alcohol may also alter microglial production of cytokines or chemokines. There are few reports in the literature regarding the effects of alcohol on the expression or secretion of chemokines or cytokines within the CNS. Alcohol was shown to reduce TNFα expression induced by hormones (DeVito et al., 1996) or injury (Liao et al., 2003) in mixed glial cultures. Decreased levels of IP-10 (Ren et al., 1999; Davis and Syapin, 2004) and MCP-1 (Thibault et al., 2000) were reported in cultured astrocytes and neuroblastoma cells, respectively. Increased levels of IP-10 chemokine mRNA have been suggested in a rodent model of fetal alcohol exposure (Yang et al., 2002). To our knowledge, there are no reports of the effects of ethanol on chemokine or cytokine mRNA or protein expression by microglia. Because changes in expression of these proteins may contribute to the neural pathology associated with prenatal exposure to alcohol, the specific effects of alcohol on cytokine and chemokine mRNA expressed by microglia were explored in the present study.

Materials and Methods

Mouse N9 Microglia Cell Cultures.--Cultures of the mouse N9 microglia cell line, provided by Dr. Paola Ricciardi-Castagnoli, Cellular Pharmacology Center, Milanon, Italy, (Righi et al., 1989; Corradin et al., 1993) were plated in 25 cm^2 culture flasks (Corning) containing Minimum Essential Medium with Earle's salts and L-Glutamine (Cellgro, Cat. No. 10-010-CV) with 10% fetal bovine serum (Gibco), penicillin (100 units/ml, Gibco), and streptomycin (100 μg/ml, Gibco) and incubated at 37°C in a 5% CO_2:95% air atmosphere. Cells were sub-cultured routinely to maintain 25-80% confluence.

Treatment of N9 Cells with LPS and Ethanol.--On the day of the experiment, the medium in each flask was replaced with fresh medium as described above except containing 1% fetal bovine serum and, if indicated, 0.5% (w/v; 110 mM) ethanol. This concentration of ethanol has been shown to produce significant microglial cell death in culture (Yang et al., 1999). Flasks with or without ethanol were equilibrated with 5% CO_2:95% air, sealed to prevent ethanol evaporation, and incubated for 12, 24, or 48 hr at 37°C. After 12, 24, or 48 hr, 1 μg/ml of bacterial lipopolysaccharide (LPS, *Escherichia coli* serotype 026:B6; Sigma, St. Louis, MO) was added to the indicated flasks. The flasks were resealed and incubated for 4 hr, at which time RNA isolation was performed.

RNA Isolation and RNase Protection Assay (RPA).--Total RNA was extracted from the mouse N9 microglia cultures using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Chemokine and cytokine mRNA expression in mouse N9 microglia was examined by RPA with the RiboQuant RPA kit and mCK-5c and custom (mCK-2b + TNFα) multiprobe template sets (BD PharMingen). The mCK-5c and custom template sets contained probes for the housekeeping genes L32 and GAPDH, which served as internal controls for the assay. Probes were labeled with [α-33P] UTP (Perkin Elmer), resulting in an average specific activity of 5 x 10^5 cpm/μl. RPA was performed according to the manufacturer's instruction using 5 μg of total RNA per sample. Products were resolved on a 6% acrylamide gel, dried, and exposed to film (Kodak) for varying periods of time. Densitometric analysis was performed using AlphaInnotech ChemiImager software. Expression of each chemokine and cytokine was normalized to the expression of the housekeeping gene GAPDH in each sample. GAPDH mRNA levels remained constant under the treatment conditions used in this experiment.

Data Analysis.--The experiment was performed three independent times, and the results were expressed as a mean ± SEM of the three experiments. Differences in

Table 1: Chemokine mRNA expression was determined by RPA analysis. The optical density of each band was determined. Expression of each chemokine was normalized to the expression of the housekeeping gene GAPDH in the same sample. All data are presented as the mean of the normalized density ± SEM for three independent experiments. nd = not detectable.

Chemokine	12-hr Ethanol Treatment Group				24-hr Ethanol Treatment Group				48-hr Ethanol Treatment Group			
	Control	Ethanol	LPS	Ethanol + LPS	Control	Ethanol	LPS	Ethanol + LPS	Control	Ethanol	LPS	Ethanol + LPS
RANTES	nd	nd	0.44 ± 0.07	0.43 ± 0.05	nd	nd	0.55 ± 0.11	0.49 ± 0.14	nd	nd	0.35 ± 0.05	0.30 ± 0.09
MIP-1β	0.23 ± 0.05	0.45 ± 0.05	1.12 ± 0.12	1.08 ± 0.05	0.28 ± 0.04	0.40 ± 0.07	1.09 ± 0.09	1.12 ± 0.10	0.30 ± 0.04	0.55 ± 0.08	1.01 ± 0.06	1.12 ± 0.07
MIP-1α	0.52 ± 0.04	0.63 ± 0.02	1.17 ± 0.08	1.12 ± 0.07	0.58 ± 0.02	0.68 ± 0.03	1.13 ± 0.08	1.26 ± 0.05	0.61 ± 0.04	0.71 ± 0.07	1.15 ± 0.08	1.12 ± 0.00
MIP-2	nd	nd	0.55 ± 0.10	0.62 ± 0.08	nd	nd	0.67 ± 0.12	0.58 ± 0.13	nd	nd	0.60 ± 0.07	0.56 ± 0.14
IP-10	nd	nd	0.49 ± 0.05	0.38 ± 0.08	nd	nd	0.57 ± 0.06	0.48 ± 0.06	nd	nd	0.42 ± 0.04	0.40 ± 0.12
MCP-1	0.30 ± 0.02	0.42 ± 0.00	1.14 ± 0.06	1.04 ± 0.06	0.32 ± 0.08	0.40 ± 0.06	1.14 ± 0.12	1.22 ± 0.12	0.49 ± 0.06	0.63 ± 0.12	1.10 ± 0.05	1.08 ± 0.07
TCA-3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 2: Cytokine mRNA expression was determined by RPA analysis. The optical density of each band was determined. Expression of each cytokine was normalized to the expression of the housekeeping gene GAPDH in the same sample. All data are presented as the mean of the normalized density ± SEM for three independent experiments. nd = not detectable.

Cytokine	12-hr Ethanol Treatment Group				24-hr Ethanol Treatment Group				48-hr Ethanol Treatment Group			
	Control	Ethanol	LPS	Ethanol + LPS	Control	Ethanol	LPS	Ethanol + LPS	Control	Ethanol	LPS	Ethanol + LPS
TNF-α	0.04 ± 0.04	0.09 ± 0.05	0.64 ± 0.06	0.57 ± 0.02	0.09 ± 0.02	0.12 ± 0.01	0.63 ± 0.08	0.55 ± 0.08	0.15 ± 0.01	0.16 ± 0.02	0.56 ± 0.04	0.68 ± 0.30
IL-12 p35	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-12 p40	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-1α	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-1β	nd	nd	0.42 ± 0.03	0.40 ± 0.02	nd	nd	0.45 ± 0.07	0.39 ± 0.03	nd	nd	0.39 ± 0.07	0.51 ± 0.22
IL-1Ra	0.21 ± 0.05	0.23 ± 0.07	0.84 ± 0.09	0.82 ± 0.06	0.24 ± 0.01	0.24 ± 0.04	0.79 ± 0.07	0.79 ± 0.03	0.26 ± 0.06	0.24 ± 0.04	0.69 ± 0.03	0.88 ± 0.22
IL-6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
IFN-γ	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
MIF	0.44 ± 0.06	0.52 ± 0.05	0.55 ± 0.08	0.60 ± 0.08	0.57 ± 0.05	0.55 ± 0.05	0.58 ± 0.06	0.62 ± 0.09	0.54 ± 0.06	0.59 ± 0.05	0.56 ± 0.05	0.75 ± 0.19

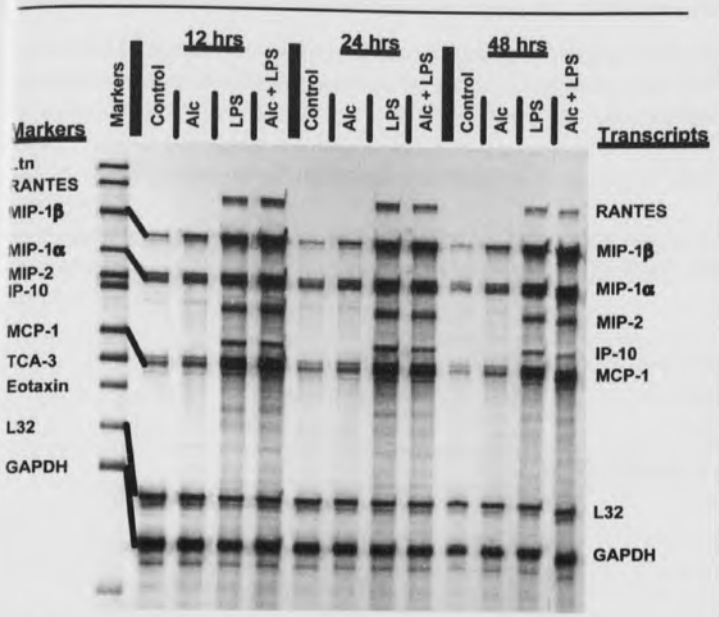

Fig. 1. Ethanol and/or LPS induce chemokine expression in mouse N9 microglial cell cultures. Microglia were treated with 0.5% (w/v) ethanol for 12, 24, or 48 hr, with or without subsequent LPS treatment for 4 hr. Expression of mRNA for the chemokines RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, and TCA-3 was analyzed by RNase protection assay (RPA).

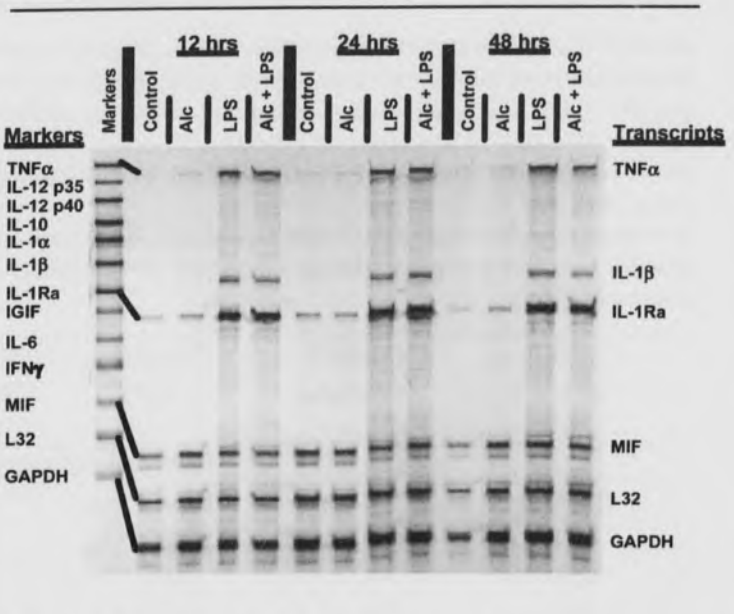

Fig. 2. Ethanol and/or LPS induce cytokine expression in mouse N9 microglial cell cultures. Microglia were treated with 0.5% (w/v) ethanol for 12, 24, or 48 hr, with or without subsequent LPS treatment for 4 hr. Expression of mRNA for the cytokines TNFα, IL12, IL10, IL1α, IL1β, IL1Ra, IL6, IFNγ, and MIF was analyzed by RNase protection assay (RPA).

expression of individual cytokines and chemokines were analyzed by treatment group and temporal duration of ethanol treatment using ANOVA (StatView, SAS Institute, Inc.) with *post hoc* analysis of variance by the Bonferroni Dunn test with significance at $P < 0.05$.

Results

The expression of mRNAs for chemokines and cytokines was determined by RPA analysis of mouse N9 microglia cell cultures under control conditions and following treatment with ethanol, LPS, or ethanol and LPS (Figs. 1 and 2). Densitometry of each mRNA band and normalization to the signal for GAPDH mRNA in the same sample allowed quantification of the relative change in chemokine (Table 1) and cytokine (Table 2) mRNA transcripts.

Analyses revealed that N9 mouse microglia cells under control conditions constitutively express detectable quantities of mRNA transcripts for the chemokines MIP-1β, MIP-1α, MCP-1 (Fig. 1 and Table 1) as well as the cytokines TNFα, IL1Ra, and MIF (Fig. 2 and Table 2). Compared to the control groups, MIP-1β was significantly increased by 94.4% ($P < 0.05$), 45.5% ($P = 0.18$), and 84.2% ($P = 0.06$) after treatment with ethanol for 12, 24, and 48 hr, respectively (Table 1 and 3). Compared to control groups, MIP-1α showed a trend of increased expression by 23.1% ($P = 0.08$), 16.7% ($P = 0.07$), and 17.3% ($P = 0.25$) after treatment with ethanol for 12, 24, and 48 hr, respectively, although the differences were not statistically significant (Table 1 and 3). The results also showed increased expression of MCP-1 by 36.7% ($P < 0.05$), 23.8% ($P = 0.48$) and 26.9% ($P = 0.39$) after treatment with ethanol for 12, 24, and 48 hr, respectively (Table 1 and 3). In contrast, transcripts for the cytokines TNFα, IL1Ra, and MIF were not significantly changed after treatment with ethanol at any time point compared to the control group (Table 2 and 4). None of the chemokine or cytokine mRNAs analyzed showed significant differences when the control, ethanol alone, LPS alone, or ethanol and LPS groups at 12 hr time points were compared to the same treatment group at the 24 or 48 hr time points.

The N9 microglial cell cultures were treated with the endotoxin LPS to induce an activated cell phenotype with increased expression of pro-inflammatory cytokines and chemokines. Stimulation with LPS for 4 hr resulted in significant elevations of mRNA expression of the chemokines RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1 (Table 1 and 3) and the cytokines TNFα, IL1β, and IL1Ra (Table 2 and 4) compared to both the control group

Table 3. Comparison of the ratio of chemokine mRNA levels between treatment groups presented as a percentage. Difference in expression of individual chemokines was analyzed by ANOVA with *post hoc* analysis of variance by the Bonferonni Dunn test ($P < 0.05$) and was reported as percent difference of the mean ratio#.
#n = 3; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
nd = calculations could not be determined due to undetectable expression of mRNA for both treatment groups being compared.
+ = densitometric analysis showed significant expression of the chemokine in the LPS alone or ethanol and LPS treatment groups, however a percent change could not be calculated due to undetectable levels of expression in the control or ethanol treatment group.

Chemokine	Control vs Ethanol	Control vs LPS	Control vs Ethanol + LPS	Ethanol vs LPS	Ethanol vs Ethanol + LPS	LPS vs Ethanol + LPS
12-hr Ethanol Treatment Group						
RANTES	nd	+	+	+	+	-1.6
MIP-1β	94.4 *	383 **	362 ***	148 **	138 ***	-4.3
MIP-1α	23.1	127 **	117 **	84.1 **	76.2 **	-4.3
MIP-2	nd	+	+	+	+	12.6
IP-10	nd	+	+	+	+	-22.1
MCP-1	36.7 *	274 ***	240 ***	174 ***	148 ***	-9.3
TCA-3	nd	nd	nd	nd	nd	nd
24-hr Ethanol Treatment Group						
RANTES	nd	+	+	+	+	-10.8
MIP-1β	45.5	295 **	304 **	172 **	178 **	2.3
MIP-1α	16.7	94.8 **	117 ***	67.0 **	86.0 ***	11.4
MIP-2	nd	+	+	+	+	-13.0
IP-10	nd	+	+	+	+	-16.8
MCP-1	23.8	251 **	278 **	184 **	205 **	7.6
TCA-3	nd	nd	nd	nd	nd	nd
48-hr Ethanol Treatment Group						
RANTES	nd	+	+	+	+	-14.7
MIP-1β	84.2	240 ***	277 ***	84.5 *	105 **	11.0
MIP-1α	17.3	89.8 **	84.2 ***	61.7 *	57.0 **	-3.0
MIP-2	nd	+	+	+	+	-6.7
IP-10	nd	+	+	+	+	-3.8
MCP-1	26.9	123 **	118 **	75.4 *	72.1 *	-1.9
TCA-3	nd	nd	nd	nd	nd	nd

and the ethanol treatment group. Similarly, N9 microglia exposed to ethanol for 12, 24, or 48 hr with subsequent LPS treatment for 4 hr showed significant elevation of mRNA expression of RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1 (Table 1 and 3), IL1β, and IL1Ra (Table 2 and 4) compared to both the control group and the ethanol treatment group. A significant difference in cytokine or chemokine mRNA expression was not found at any time point when groups treated with both ethanol and LPS were compared to groups treated with LPS alone.

Discussion

A novel finding of this study is increased mRNA levels of the chemokines MIP-1β, MIP-1α, and MCP-1 by an average of 74.7%, 19.0%, and 29.1%, respectively, upon treatment of N9 microglial cells with 0.5% (w/v) ethanol for 12 to 48 hr. These findings suggest that physiologically relevant concentrations of ethanol alter an important function of microglia, that is, the mRNA expression of

Table 4. Comparison of the ratio of cytokine mRNA levels between treatment groups presented as a percentage. Difference in expression of individual cytokines was analyzed by ANOVA with *post hoc* analysis of variance by the Bonferonni Dunn test ($P < 0.05$) and was reported as percent difference of the mean ratio#.
#n = 3; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
nd = calculations could not be determined due to undetectable expression of mRNA for both treatment groups being compared.
+ = densitometric analysis showed significant expression of the cytokine in the LPS alone or ethanol and LPS treatment groups, however a percent change could not be calculated due to undetectable levels of expression in the control or ethanol treatment group.

Cytokine	Control vs Ethanol	Control vs LPS	Control vs Ethanol + LPS	Ethanol vs LPS	Ethanol vs Ethanol + LPS	LPS vs Ethanol + LPS
12-hr Ethanol Treatment Group						
TNF-α	135	1624 **	1446 ***	633 **	558 ***	-10.3
IL-12 p35	nd	nd	nd	nd	nd	nd
IL-12 p40	nd	nd	nd	nd	nd	nd
IL-10	nd	nd	nd	nd	nd	nd
IL-1α	nd	nd	nd	nd	nd	nd
IL-1β	nd	+	+	+	+	-2.9
IL-1Ra	12.5	301 **	292 **	257 **	249 **	-2.3
IL-6	nd	nd	nd	nd	nd	nd
IFNγ	nd	nd	nd	nd	nd	nd
MIF	19.8	26.4	37.7	5.6	15.0	8.9
24-hr Ethanol Treatment Group						
TNF-α	24.5	568 **	488 **	437 **	373 **	-11.9
IL-12 p35	nd	nd	nd	nd	nd	nd
IL-12 p40	nd	nd	nd	nd	nd	nd
IL-10	nd	nd	nd	nd	nd	nd
IL-1α	nd	nd	nd	nd	nd	nd
IL-1β	nd	+	+	+	+	-11.9
IL-1Ra	2.1	230 **	229 ***	224 **	222 ***	-0.4
IL-6	nd	nd	nd	nd	nd	nd
IFNγ	nd	nd	nd	nd	nd	nd
MIF	-2.3	2.5	9.9	4.9	12.5	7.2
48-hr Ethanol Treatment Group						
TNF-α	13.0	286 ***	363	241 ***	310	20.1
IL-12 p35	nd	nd	nd	nd	nd	nd
IL-12 p40	nd	nd	nd	nd	nd	nd
IL-10	nd	nd	nd	nd	nd	nd
IL-1α	nd	nd	nd	nd	nd	nd
IL-1β	nd	+	+	+	+	29.9
IL-1Ra	-7.4	170 **	244 *	191 ***	272 *	27.7
IL-6	nd	nd	nd	nd	nd	nd
IFNγ	nd	nd	nd	nd	nd	nd
MIF	8.1	3.1	37.3	-4.6	27.0	33.2

chemokines that play an important role in neuroimmune modulation, CNS development and CNS homeostasis. Further studies will be needed to demonstrate if the relative increase in mRNA expression of MIP-1β, MIP-1α, and MCP-1 will translate into an increase in production and secretion of the corresponding proteins by microglia. Alterations in MIP-1α levels in the CNS might alter neuronal signal transduction and synaptic transmission (Meucci et al., 1998) or influence astrocyte or microglial migration in the developing brain (Tanabe et al., 1997; Rezaie et al., 2002). An increase in MCP-1 levels in the neural environment could be detrimental to astrocyte and neuronal progenitor migration (Heesen et al., 1996; Hesselgesser et al., 1997), alter CNS angiogenesis (Salcedo et al., 2000), as well as influence the maturation of Purkinje cells and their networks (Meng et al., 1999). An upregulation of MIP-1β, MIP-1α, and MCP-1 has also been found in neuroinflammatory conditions including multiple sclerosis, experimental autoimmune encephalitis, and Alzheimer's disease (Godiska et al., 1995; Berman et al., 1996; Ishizuka et al., 1997; Simpson et al., 1998; Xia et al., 1998; Balashov et al., 1999; Sorensen et al., 1999;).

Increased levels of these chemokines following ethanol exposure may lead to inflammation within the CNS. MIP-1β, MIP-1α, and MCP-1 are classified as CC chemokines, which function to attract immune competent cells, such as T lymphocytes, monocytes, basophils, and eosinophils (Bajetto et al., 2002). Activated T lymphocytes produce a variety of lymphokines that promote the activity of virtually all other cells of the immune system. For example, T lymphocytes induce humoral B lymphocyte responses as well as activate cell-mediated immune responses through macrophages, other antigen presenting cells, and natural killer cells. Elevated levels of these chemokines may attract monocytes and macrophages into the CNS parenchyma. These cells produce cytokines including IL1 and TNFα that, in excess, cause neuroinflammation, neuronal damage and cell death. Therefore, if alcohol-induced increases in MIP-1β, MIP-1α, and MCP-1 mRNA expression by microglia lead to increased levels of the corresponding proteins in the CNS, chemotaxis of T lymphocytes, monocytes, and other immune competent cells may lead to neuroinflammation.

This study has shown that stimulation of mouse N9 microglia with 1μg/ml of LPS for 4 hr results in significant elevation of expression of the following mRNA transcripts: RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TNFα, IL1β, and IL1Ra. These results are consistent with studies of purified human microglial cells which have shown an upregulation of MIP-1β, MIP-1α, MCP-1, TNFα, and IL1β mRNA transcripts via Northern blot or RT-PCR analysis after stimulation with 1ng/ml - 1μg/ml of LPS (Lee et al., 1993; McManus et al., 1998; Lee et al., 2002). The present results are also consistent with studies of purified mouse microglial cells that have shown an induction of RANTES, MIP-1α, MIP-2, MCP-1, TNFα, and IL1β protein measured with ELISA after 1ng/ml - 1μg/ml of LPS stimulation (Aloisi et al., 1999; Szczepanik et al., 2001; Hausler et al., 2002). In addition, these results are consistent with studies of expression of IP-10 in human fetal microglial cells (Hua and Lee, 2000) and induction of IL-1Ra in mixed rat glial cells (Pousset et al., 2000) after LPS stimulation. We did not observe expression of the chemokine TCA-3 or the cytokines IL1α, IL6, IL10, IL12, or IFNγ upon stimulation with LPS as might be expected from other reports (Lee et al., 1993; De Simone et al., 1998; Aloisi et al., 1999; Szczepanik et al., 2001; Hua and Lee, 2000; Hausler et al., 2002; Lee et al., 2002). The variation in these reports and the present study may be explained by differences inherent in the analysis of mRNA versus protein, the method of mRNA analysis, or the species source of microglia.

Because alcohol can suppress monocyte and macrophage activity in the peripheral immune system, it might be anticipated that pretreatment with ethanol in microglial cultures would decrease the LPS-induced increase in cytokine or chemokine mRNA expression. However, this study found no significant change in cytokine or chemokine mRNA expression in groups treated with both ethanol and LPS compared to groups treated with LPS alone. It is important to note that it would be premature to conclude that ethanol can not alter LPS-induced mRNA expression of chemokines or cytokines in microglia based on the present study which employed only a single dose of ethanol for a single temporal period and a single dose of LPS for a single temporal period.

This study demonstrates that alcohol exposure increases microglial expression of mRNA encoding MIP-1 and MCP-1 chemokines that are integral to the proper development and function of the CNS. If the observed increase in mRNA is reflected in increased production of chemokine proteins, this represents a new potential mechanism of alcohol pathogenesis mediated through microglia. We have recently realized that alcohol impairs microglial proliferation, survival and maturation in the developing brain, and the present results provide the first evidence that alcohol may interfere with interactions between neurons and microglia. Specifically, microglial production of chemokines is a central component of microglial function as the principal resident immune cells in the CNS. Increased levels of MIP-1 and MCP-1 following alcohol exposure may disrupt the development and function of neurons and glia. In addition, increased levels of MIP-1 and MCP-1 may lead to neuroinflammation, resulting in neuronal damage and cell death. These effects could lead to long-term and irreversible neural defects, such as those associated with fetal alcohol exposure. Whether alcohol exposure in the human CNS leads to upregulation of the chemokines MIP-1β, MIP-1α, and MCP-1, and further, whether the potential contribution to the neuropathology of fetal alcohol

syndrome or alcohol-related neurodevelopmental disorder is realized remains to be determined. However, given the multitude of roles that these chemokines play in the CNS and the significant neuropathology associated with fetal alcohol exposure further investigation is warranted.

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Literature Cited

Aloisi, F., R. De Simone, S. Columba-Cabezas, and G. Levi. 1999. Opposite effects of interferon-γ and prostaglandin E2 on tumor necrosis factor and interleukin-10 production in microglia: a regulatory loop controlling microglia pro- and anti-inflammatory activities. J. Neurosci. Res. 56:571-580.

Arbabi, S., I. Garcia, G. J. Bauer, and R.V. Maier. 1999. Alcohol (ethanol) inhibits IL-8 and TNF: role of the p38 pathway. J. Immunol. 162:7441-7445.

Bajetto, A., R. Bonavia, S. Barbero, and G. Schettini. 2002. Characterization of chemokines and their receptors in the central nervous system: physiopathological implications. J. Neurochem. 82:1311-1329.

Bakhiet, M., A. Tjernlund, A. Mousa, A. Gad, S. Stromblad, W. A. Kuziel, A. Seiger, and J. Anderson. 2001. RANTES promotes growth and survival of human first-trimester forebrain astrocytes. Nat. Cell Biol. 3:150-157.

Balashov, K. E., J. B. Rottman, H. L. Weiner, and W. W. Hancock. 1999. CCR5(+) and CSCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. Proc. Natl. Acad. Sci. 96:6873-6878.

Barish, M., N. B. Mansdorf, and S. S. Raissdana. 1991. Gamma-interferon promotes differentiation of cultured cortical and hippocampal neurons. Dev. Biol. 144:412-423.

Bauer-Moffett, C., and J. Altman. 1977. The effect of ethanol chronically administered to preweanling rats on cerebellum development: a morphological study. Brain Res. 119:249-268.

Berman, J. W., M. P. Guida, J. Warren, J. Amat, and C. F. Brosnan. 1996. Localization of monocyte chemoattractant peptide-1 expression in the central nervous system in experimental autoimmune encephalomyelitis and trauma in the rat. J. Immunol. 156:3017-3023.

Bolin, L. M., R. Murray, N. W. Lukacs, R. M. Strieter, S. L. Kunkel, T. J. Schall, and K. B. Bacon. 1998. Primary sensory neurons migrate in response to the chemokine RANTES. J. Neuroimmunol. 81:49-57.

Bonthius, D. J., and J. R. West. 1991. Permanent neuronal deficits in rats exposed to alcohol during the brain growth spurt. Teratology 44:147-163.

Bukara, M., and A.P. Bautista. 1999. Acute alcohol intoxication and gadolinium chloride attenuate endotoxin-induced release of CC chemokines in the rat. Alcohol 20:193-203.

Corradin, S. B., J. Mauel, S. D. Donini, E. Quattrocchi, and P. Ricciardi-Castagnoli. 1993. Inducible nitric oxide synthase activity of cloned murine microglial cells. Glia 7:255-262.

Davis, R. L., and P. J. Syapin. 2004. Chronic ethanol inhibits CXC chemokine ligand 10 production in human A172 astroglia and astroglial-mediated leukocyte chemotaxis. Neurosci. Lett. 362:220-225.

De Simone, R., G. Levi, and F. Aloisi. 1998. Interferon gamma gene expression in rat central nervous system glial cells. Cytokine 10:418-422.

DeVito, W. J., S. Stone, and K. Mori. 1996. Low concentrations of ethanol inhibit prolactin-induced mitogenesis and cytokine expression in cultured astrocytes. Endocrinology 138:922-928.

Giovanelli, A., C. Limatola, D. Ragazzino, A. M. Mileo, A. Ruggeri, M. T. Ciotti, D. Mercanti, A. Dantoni, and F. Eusebi. 1998. CXC chemokines interleukin-8 (IL-8) and growth related gene product α (GROα) modulate purkinje neuron activity in mouse cerebellum. J. Neuoimmunol. 92:122-132.

Godiska, R., D. Chantry, G. N. Dietsch, and P. W. Gray. 1995. Chemokine expression in murine experimental allergic encephalomyelitis. J. Neuroimmunol. 58:167-176.

Hamre, K., and J. R. West. 1993. The effects of the timing of ethanol exposure during the brain growth spurt on the number of cerebellar Purkinje and granule cell nuclear profiles. Alcohol. Clin. Exp. Res. 17:610-622.

Hausler, K. G., M. Prinz, C. Nolte, J. R. Weber, R. R. Schumann, H. Kettenmann, and U. Hanisch. 2002. Inteferon-γ differentially modulates the release of cytokines and chemokines in lipopolysaccharide- and pneumococcal cell wall-stimulated mouse microglia and macrophages. Eur. J. Neurosci. 16:2113-2122.

Hayashi, M., Y. Luo, J. Laning, R. M. Strieter, and M. E. Dorf. 1995. Production and function of monocyte chemoattractant protein-1 and other beta-chemokines in murine glial cells. J. Neuroimmunol. 60:143-150.

Heesen, M., S. Tanabe, M. A. Berman, I. Yoshizawa, Y. Luo, R. J. Kim, T. W. Post, C. Gerard, and M. E. Dorf. 1996. Mouse astrocytes respond to the chemokines MCP-1 and KC, but reverse transcriptase-polymerase chain reaction does not detect mRNA for the KC or new MCP-1 receptor. J. Neurosci. Res. 45:382-391.

Hesselgesser, J., M. Halks-Miller, V. DelVecchio, S. C. Peiper, J. Hoxie, D. L. Kolson, D. Taub, and R. Horuk. 1997. CD-4 independent association between HIV-1 pg 120 and CXCR4: functional chemokine receptors are expressed in human neurons. Curr. Biol. 7:112-121.

Hickey, W. F., K. Vass, and H. Lassmann. 1992. Bone marrow-derived elements in the central nervous system: an immunohistochemical and ultrastructural survey of rat chimeras. J. Neuropathol. Exp. Neurol. 51:246-256.

Hua, L. L., and S. C. Lee. 2000. Distinct patterns of stimulus-inducible chemokine mRNA accumulation in human fetal astrocytes and microglia. Glia 30:74-81.

Ishizuka, K., T. Kumura, R. Igata-yi, S. Katsuragi, J. Takamatsu, and T. Miyakawa. 1997. Identification of monocyte chemoattractant protein-1 in senile plaques and reactive microglia of Alzheimer's disease. Psychiatry Clin. Neurosci. 51:135-138.

Kane, C. J. M. 2001. A New Cellular Target for Fetal Alcohol Exposure. Amer. Soc. Cell Biol. Press Book, 2001:8. (http://www.ascb.org/publicpolicy/pressbooks/pressbook01-10.pdf).

Kane, C. J. M., D. R. Pierce, N. N. Nyamweya, H. Yang, Y. Kasmi, R. Mosby, D. C. Serbus, and K. E. Light. 1997. Nutritional factors modify the inhibition of CNS development by combined exposure to methadone and ethanol in neonatal rats. Pharmacol. Biochem. Behav. 56:399-407.

Korner, H., D. S. Riminton, D. H. Strickland, F. A. Lemckert, J. D. Pollard, and J. D. Sedgwick. 1997. Critical points of tumor necrosis factor action in central nervous system autoimmune inflammation defined by gene targeting. J. Exp. Med. 186:1585-1590.

Lee, S. C., W. Liu, D. W. Dickson, C. F. Brosnan, and J. W. Berman. 1993. Cytokine production by human fetal microglia and astrocytes. J. Immunol. 150:2659-2667.

Lee, Y. B., A. Nagai, and S. U. Kim. 2002. Cytokines, chemokines, and cytokine receptors in human microglia. J. Neurosci. Res. 69:94-103.

Liao, S. L., W. Y. Chen, S. L. Raung, and C. J. Chen. 2003. Ethanol attenuates ischemic and hypoxic injury in rat brain and cultured neurons. Neuroreport 14:2089-2094.

Light, K. E., D. P. Brown, B. W. Newton, S. M. Belcher, and C. J. M. Kane. 2002. Ethanol-induced alteration of neurotrophin receptor expression on purkinje cells in the neonatal rat cerebellum. Brain Res. 924:71-81.

Ling, E. A., and W. C. Wong. 1993. The origin and nature of ramified and amoieboid microglia: a historical review and current concept. Glia 7:9-18.

Mattson, S. N., E. P. Riley, T. L. Jernigan, A. Garcia, W. M. Kaneko, C. L. Ehlers, and K. L. Jones. 1994. A decrease in the size of the basal ganglia following prenatal alcohol exposure: a preliminary report. Neurotoxicol. Teratol. 16:283-289.

McManus, C. M., C. F. Brosnan, and J. W. Berman. 1998. Cytokine induction of MIP-1α and MIP-1β in human fetal microglia. J. Immunol. 160:1449-1455.

Mehler, M. F., and J. A. Kessler. 1994. Growth factor regulation of neuronal development. Dev. Neurosci. 16:180-195.

Mehler, M. F., and J. A. Kessler. 1995. Cytokines and neuronal differentiation. Crit. Rev. Neurobiol. 9:419-446.

Meng, S. Z., A. Oka, and S. Takashima. 1999. Developmental expression of monocyte chemoattractant protein-1 in the human cerebellum and brainstem. Brain Dev. 21:30-35.

Meucci, O., A. Fatatis, A. A. Simen, T. J. Bushell, P. W. Gray, and R. J. Miller. 1998. Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. Neurobiology 95:14500-14505.

Meyer, L. S., L. E. Kotch, and E. P. Riley. 1990. Neonatal ethanol exposure: functional alterations associated with cerebellar growth retardation. Neurotoxicol. Teratol. 12:15-22.

Napper, R. M. A., and J. R. West. 1995. Permanent neuronal cell loss in the cerebellum of rats exposed to continuous low blood alcohol levels during the brain growth spurt: a stereological investigation. J. Comp. Neurol. 362:283-292.

Oppenhheim, J. J., and M. Feldman. 2001. Introduction to the role of cytokines in innate defense and adaptive immunity. In: Cytokine reference (Oppenhheim, J. J., Feldman, M., eds) Academic, New York, pp 3-20.

Patterson, P. H. 1992. The emerging neuropoietic cytokine family: first CDF/LIF, CNTF and IL-6; next ONC, MGF, GCSF? Curr. Opin. Neurobiol. 2:94-97.

Pierce, D. R., C. R. Goodlett, and J. R. West. 1989. Differential neuronal loss following early postnatal alcohol exposure. Teratology 40:113-126.

Pierce, D.R., C. J. M. Kane, D. C. Serbus, and K. E. Light. 1997. Microencephaly and selective decreases in cerebellar Purkinje cell numbers following combined exposure to ethanol and methadone during rat brain development. Dev. Neurosci.19:438-445.

Plata-Salaman, C. R. 1991. Immunoregulators in the nervous system. Neurosci. Biobehav. Rev. 15:185-215.

Pousset, F., K. Palin, D. Verrier, A. Bristow, R. Dantzer, P. Parnet, and J. Lestage. 2000. Production of interleukin-1 receptor antagonist isoforms by microglia in mixed rat glial cells stimulated by lipopolysaccharide. Eur. Cytokine Netw. 11:682-689.

Probert, L., K. Akassoglou, G. Kassiotis, M. Pasparakis, L. Alexopoulou, and G. Kollias. 1997. TNF-alpha transgenic and knockout models of CNS inflammation and degeneration. J. Neuroimmunol. 72:137-141.

Ransohoff, R. M., and E. N. Benveniste. 1996. Cytokines and the CNS. CRC Press, Boca Raton, FL. 339 pp.

Ren, L. Q., D. K. Garrett, and P. J. Syapin. 1999. Ethanol decreases LPS-induced expression of IP-10 mRNA in C6 glioma cells. Alcohol. Clin. Exp. Res. 23(Suppl):95A.

Rezaie, P., G. Trillo-Pazos, J. Greenwood, I. P. Everall, and D. K. Male. 2002. Motility and ramificaton of human fetal microglia in culture: an investigation using time-lapse video microscopy and image analysis. Exp. Cell Res. 274:68-82.

Righi, M., L. Mori, G. De Libero, M. Sironi, A. Biondi, A. Mantovani, S. D. Donini, and P. Ricciardi-Castagnoli. 1989. Monokine production by microglial cell clones. Eur. J. Immunol. 19:1443-1448.

Riley, E. P., S. N. Mattson, E. R. Sowell, T. L. Jernigan, D. F. Sobel, and K. L. Jones. 1995. Abnormalities of the corpus callosum in children prenatally exposed to alcohol. Alcohol. Clin. Exp. Res. 19:1198-1202.

Ryabinin, A. E., M. Cole, F. E. Bloom, and M. C. Wilson. 1995. Exposure of neonatal rats to alcohol by vapor inhalation demonstrates specificity of microcephaly and Purkinje cell loss but not astrogliosis. Alcohol. Clin. Exp. Res. 19:784-791.

Salcedo, R., M. L. Ponce, H. A. Young, K. Wasserman, J. M. Ward, H. K. Kleinman, J. Oppenheim, and W. J. Murphy. 2000. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood 96:34-40.

Simpson, J. E., J. Newcombe, M. L. Cuzner, and M. N. Woodroofe. 1998. Expression of monocyte chemoattractant protein-1 and other beta-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions. J. Neuroimmunol. 84:238-249.

Sorensen, T. L, M. Tani, J. Jensen, V. Pierce, C. Lucchinetti, V. A. Folcik, S. Qin, J. Rottman, F. Sellebjerg, R. M. Strieter, J. L. Frideriksen, and R. M. Ransohoff. 1999. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. J. Clin. Invest. 103:807-815.

Stratton, K., C. Howe, and F. Battaglia. 1996. Fetal alcohol syndrome: diagnosis, epidemiology, prevention, and treatment. Institute of Medicine, National Academy Press, Washington, D.C., 213 pp.

Szabo, G., L. Girouard, P. Mandrekar, and D. Catalano. 1998. Regulation of monocyte IL-12 production: augmentation by lymphocyte contact and acute ethanol treatment, inhibition by elevated intracellular cAMP. Int. J. Immunopharmacol. 20:491-503.

Szabo, G., P. Mandrekar, and D. Catalano. 1995. Inhibition of superantigen-induced T cell proliferation and monocyte IL-1β, TNFα, and IL-6 production by acute ethanol treatment. J. Leukoc. Biol. 58:342-350.

Szczepanik, A. M., S. Funes, W. Petko, and G. E. Ringheim. 2001. IL-4, IL-10 and IL-13 modulate Aβ(1-42)-induced cytokine and chemokine production in primary murine microglia and human monocyte cell line. J. Neuroimmunol. 113:49-62.

Tanabe, S., M. Heesen, M. A. Berman, M. B. Fischer, I. Yoshizawa, Y. Luo, and M. E. Dorf. 1997. Murine astrocytes express a functional chemokine receptor. J. Neurosci. 17:6522-6528.

Thibault, C., C. Lai, N. Wilke, B. Duong, M. F. Olive, S. Rahman, H. Dong, C. W. Hodge, D. J. Lockhart, and M. F. Miles. 2000. Expression profiling of neural cells reveals specific patterns of ethanol-responsive gene expression. Mol. Pharmacol. 58:1593-1600.

Xia, M. Q., B. J. Bacskai, R. B. Knowles, S. X. Qin, and B. T. Hyman. 2000. Expression of the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10 in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease. J. Neuroimmunol. 108:227-235.

Xia, M. Q., S. X. Qin, L. J. Wu, C. R. Mackay, and B. T. Hyman. 1998. Immunohistochemical study of the beta-chemokine receptors CCR3 and CCR5 and their ligands in normal and Alzheimer's disease brains. Am. J. Pathol. 153:31-37.

Yang, J., and R. T. Zoeller. 2002. Differential display identifies neuroendocrine-specific protein-A (NSP-A) and interferon-inducible protein 10 (IP-10) as ethanol-responsive genes in the fetal rat brain. Dev. Brain Res. 138:117-133.

Yang, Z. Q., W. Chen, and C. J. M. Kane. 1999. Ethanol impairs the survival and proliferation of rat microglia *in vitro*. J. Health Toxicol. 13:12-15.

Zhang, Z., G. J. Bagby, D. Stoltz, P. Oliver, P. O. Schwarzenberger, and J. K. Kolls. 2001. Prolonged ethanol treatment enhances lipopolysaccharide/phorbol myristate acetate-induced tumor necrosis factor-α production in human monocytic cells. Alcohol. Clin. Exp. Res. 25:444-449.