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CHRONIC ALCOHOL INTOXICATION PRIMES KUPFFER CELLS AND ENDOTHELIAL CELLS FOR ENHANCED CC-CHEMOKINE PRODUCTION AND CONCOMITANTLY SUPPRESSES PHAGOCYTOSIS AND CHEMOTAXIS

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1. ABSTRACT

Chemokines are involved in the pathogenesis of alcoholic hepatitis and are considered to contribute to the migration of leukocytes into the liver during chronic ethanol intoxication. This work tests the hypothesis that chronic ethanol consumption selectively enhances chemokine release by Kupffer cells and hepatic sinusoidal endothelial cells and migration of inflammatory cells into the liver. Furthermore, enhanced hepatic chemokine secretion may induce an autocrine effect on the ability of Kupffer cells and endothelial cells to chemotax and ingest microbial particles. Male Wistar rats were fed with ethanol in agar block and water for 32 weeks, and were allowed free access to solid food. Results show that after 32 weeks of feeding, leukocyte infiltration and steatosis were observed in the livers of ethanol-fed rats. The majority of the infiltrated cells were CD8+ cells. Serum ALT, endotoxin, MIP-1α, MCP-1 and RANTES, (but not CINC and MIP-2) were also increased in the ethanol-fed rats than in the pair-fed group. Isolated Kupffer cells from ethanol-fed rats were primed for enhanced MIP-1α, MCP-1, and RANTES production in vitro, while the endothelial cells were primed for enhanced MIP-1α release only. Chronic alcohol intoxication was also associated with increased basal H_2O_2 formation, enhanced nuclear translocation and binding of NF-kB, AP-1 and MNP-1 in Kupffer Cells. Chronic ethanol feeding significantly enhanced MNP-1 binding, but not those of NF-kB and AP-1 in endothelial cells. Concomitantly, chemokine-induced chemotaxis,

E.coli phagocytosis and f-met-leu-phe-induced superoxide anion production by Kupffer cells were downregulated in the ethanol-fed group. Taken together these data demonstrate that prolonged alcohol consumption may compromise the host to hepatitis as a result of increased chemokine production and at the same time may suppress the innate immune function of hepatic non-parenchymal cells.

2. INTRODUCTION

Chemokines are involved in the pathogenesis of alcoholic liver disease. Several studies have demonstrated that serum chemokine levels correlated with the degree of leukocyte infiltration in the liver during alcoholic hepatitis and cirrhosis. Chemokines that are implicated in this pathogenesis are interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein 1 α/β(MIP-1α/β) (1-4). In experimental models of alcoholic hepatitis, the involvement of these chemotactic peptides is also demonstrated. Elevated production of cytokine-induced neutrophil chemoattractant (CINC), the rodent equivalent of human IL-8) and MIP-2 in vivo has been observed in rats at 16 weeks of ethanol feeding (5,6). IL-8 and MIP-2 belong to the CXC chemokine subfamily that is highly chemoattractant for neutrophils (PMNs) (7). It has been shown that elevated levels of IL-8 in the blood is linked to neutrophilic infiltration in the livers of patients with severe alcoholic hepatitis (1). Thus, under this condition, PMNs play an important role in hepatic injury. However, PMNs have a short half-life and are more likely to be eliminated in the liver by Kupffer cells. During alcoholic hepatitis, the involvement of other cells, i.e., lymphocytes, should be considered. Upregulation of CC-chemokines (MCP-1, MIP-1 and MIP-1b) is also observed in alcoholic hepatitis (1,2,4). CC-chemokines are chemoattractant for mononuclear cells (7). Thus in severe, alcoholic hepatitis, whereby CXC and CC chemokines are upregulated, the involvement of PMNs and lymphocytes in inflammation is significant. The impact of lymphocytes under this condition may be more relevant, because of their ability to proliferate in the liver, in contrast to PMNs with definitive life span. The presence of lymphocytes in the liver under this condition is expected to prolong the chronicity of inflammation, and may also be involved in hepatic injury. Another CC-chemokine that may play a role in hepatitis is regulated upon activation normal T-cell expressed and secreted (RANTES). RANTES is also implicated in the pathogenesis of alcoholic liver disease and SIV-induced hepatitis (8).

The major sources of proinflammatory mediators are the hepatic non-parenchymal cells, i.e., endothelial cells, Kupffer Cells and infiltrated leukocytes. Studies have shown that depletion of Kupffer Cells with gadolinium chloride or liposome-encapsulated dichloromethylene diphosphonate suppresses LPS-induced cytokine and chemokine production (9,10). During chronic ethanol intoxication, Kupffer Cells as well as endothelial cells are major sources of circulating chemokines. Despite an elevation of pro-inflammatory factors following chronic alcohol consumption, immunosuppression may also manifest. Chemokines are known to downregulate chemokine receptors by enhanced internalization of these binding sites (11). This action may also allow the internalization of other receptors (e.g., FcR) important in innate immune functions. Thus, this work tests the hypothesis that chronic ethanol consumption primes the hepatic non-parenchymal cells for enhanced CC-chemokine production and concomitantly suppresses innate immune functions, i.e., phagocytosis and chemotaxis.

3. EXPERIMENTAL DESIGN AND METHODS

3.1. Experimental Animals

Male Wistar rats (Charles River Breeding Laboratories) were fed with ethanol in agar block (10-25%) and water for 32 weeks. One week after they arrived, the rats were given agar blocks without ethanol to acclimatize them to the agar blocks. Thereafter, they were given an increment of 10% ethanol every week, until 25% was reached. The rats were maintained at 25% ethanol in agar block and water for 32 weeks. Pair-fed rats were given an isocaloric equivalent of dextrin. The agar blocks (ethanol-fed and pair-fed) were also supplemented with 10% corn oil and 5% peanut butter. They were given access to solid food. This model of chronic ethanol intoxication in the rat has been described in our previous publications (5,12). The experimental protocol for the use of experimental animals in this work was approved by the Louisiana State University Health Sciences Center IACUC. All animals received humane care according to the criteria outlined in the Guide for Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985).

3.2. Measurement of serum alcohol, ALT, endotoxin, and chemokines

Serum alcohol, ALT and endotoxin were measured using diagnostic kits from Sigma and Biowhittaker. Chemokines (MIP-2, RANTES, MCP-1 and MIP-1α, RANTES) were measured using specific ELISA kits for rats from Endogen, R&D and Biosource.

3.3. Hepatic sinusoidal lavage

On the day of the experiment, rats were treated with pentobarbital (25 mg/Kg iv), and were subjected to liver perfusion with Ca++ free Hanks balanced salt solution (HBSS). Hepatic sinusoidal lavage was performed according to the method described previously. Briefly, the portal vein was cannulated with a 19-gauge catheter. The liver was perfused with HBSS at a flow rate of 3 ml/min/g per liver. As the liver was cleared of blood, the flow rate was reduced to 0.8 ml/min/g and 20 ml of perfusate was collected. The flow rate was increased to 5 ml/min/g to flush all the remaining leukocytes in the hepatic sinusoids until 200 ml of perfusate was collected in a flow-through system. The cells were identified based on morphology on cytospin preparations stained with Giemsa-Wright's stain, and flow cytometric analysis of FITC-monoclonal antibody binding to specific markers on target cells. This method has been previously described (10,13).

3.4 Isolation of Kupffer Cells, endothelial cells and infiltrated leukocyte from whole livers

After perfusion of the liver with HBSS as described above, the perfusing liquid was replaced with

HBSS containing collagenase (25 mg/10 g liver), $CaCl_2$ (55 mg/100) and bovine serum albumin (1%). The liver was perfused with collagenase for 15 min at 37°C. The liver was cut into small pieces, suspended in Geys buffer and shaken for 5 min. The cell suspension was filtered and centrifuged at 50-x g for 2 min to remove the hepatocytes. The supernatants that contained non-parenchymal cells were washed and suspended in Gey's buffer. The suspension was subjected to centrifugal elutriation, using a Beckman elutriator. Lymphocytes, endothelial cells and Kupffer Cells were collected at 11 ml/min, 23 ml/min and 45 ml/min flow rate (respectively) at 800 x g. Neutrophils were collected from the Kupffer cell fraction after differential centrifugation with Nycoprep 1.077 animal (Accurate Chemicals). Cell viability using this technique was over 99%. Cell purity was over 95% as assessed by morphology (Giemsa-Wright's stain), FITC-anti-ED2 antibody, latex beads phagocytosis and peroxidase staining.

3.5 Flow cytometry

Isolated cells were labeled with FITC-labeled monoclonal antibodies (Serotec) directed against rat CD4, CD8, NK-RP1 and CD11b/c (PMNs). Labeled cells were analyzed based on side and forward scatter and gated for specific leukocyte populations.

3.6. Primary culture of isolated cells

Endothelial cells and Kupffer Cells were suspended in Dulbecco Minimum Essential Medium (Sigma) containing 10% fetal bovine serum albumin and antibiotics. They were cultured in 6-well plates in the absence of stimuli at 37°C for 24 hr in a humidified environment containing 7.5% CO_2. The cell concentration was 1.5 x 10^6 cells/ml in total volume of 2 ml. Culture supernatants were collected after 24 hr of incubation for chemokine analysis.

3.7. Superoxide anion assay on isolated cells

Superoxide anion assay on isolated cells was performed based on superoxide dismutase-inhibitable reduction of ferricytochrome c (14). Isolated cells were suspended in phenol-red free Hank's balanced salt solution (100,000 cells/200 µL/ well in 96 well plate. Ferricytochrome c (50 µmol, Sigma) was added to the reaction mixture. Superoxide dismutase (SOD, 200 units/well) was added to the negative controls. F-met-leu-phe (0.1 µM) was added as stimulus. Superoxide anion was measured based on a change in absorbance (difference in absorbance with or without SOD) against a cell-free blank. Delta absorbance was converted in nmol using the molar extinction coefficient of 21.1 $mM^{-1}.cm^{-1}$. Superoxide anion is expressed in nmol/10^6 cells/hr.

3.8. Chemokine-induced chemotaxis

Chemotaxis was analyzed by fluorescence technique. Isolated Kupffer Cells and endothelial cells were labeled with 4 µM of PKH2 (Sigma) for 2.5 min. The dye was removed by centrifugation, and the labeled cells were suspended in DMEM. PKH2-labeled cells (100,000 cells/well) were placed on the upper chamber. Chemokine-induced chemotaxis was determined using a PVP-free filter (8 µm pore size) in a Boyden chamber. Chemokine (RANTES, MCP-1, or MIP-1α) was added to the lower chamber at a final concentration of 100 pg/ml. Chemotactic index is referred to as the number of cells (absorbance) that migrated across the filter in the presence of chemokines minus random migration (absence of chemokines).

3.9. Escherichia coli phagocytosis

FITC-labeled E.coli were added to a monolayer of Kupffer Cells in DMEM. After 2 hours of incubation, unphagocytosed E.coli were washed using warm PBS. The culture was treated with 100 µl of 0.2% trypan blue to quench the extracellular E.coli. Ingested E.coli were quantitated using a fluorescence reader.

3.10. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of Kupffer cells were obtained by homogenizing cell lysates with buffer A containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT and 1 mM PMSF in a Dounce homogenizer. The cell lysate was kept in ice for 10 min and centrifuged at 850 x g for 10 min at 4°C. The supernatant was removed and the cell pellet was resuspended in Buffer A containing 0.1% Triton, incubated for 10 min at 4°C, and was spun at 850 x g for 10 min. The cell pellet was washed twice with Buffer A and was resuspended in 100 µL of buffer C(20 mM HEPES, ph 7.9, 25% glycerol (v/v), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 1 mM PMSF. Nuclear proteins were recovered after centrifugation at 20,000 x g for 15 min at 4°C. ^{32}P-end-labeled double stranded MNP-1 (LSU Core laboratories, ref. # 15,16), AP-1, or NF-*k*B consensus oligonucleotide (0.1 ng/10,000 CPM, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the nuclear extracts and incubated for 20 min at 20°C.. The samples were electrophoresed on 4% polyacrylamide gel. Radioactivity was measured using an Ambis Radioactive Imaging System (San Diego CA). The gels were dried and subjected to autoradiography. These procedures were based on the methods described previously (17,18).

3.11. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs from freshly isolated Kupffer Cells and endothelial cells were extracted using TRI-chloroform mixture (Sigma). From this preparation, first single stranded cDNA was transcribed using the cDNA kit (Invitrogen). cDNA was amplified in a PCR reaction containing 2.5 U Taq DNA polymerase (Sigma), 0.08 mM dNTP, and 0.1 pM primers (RANTES, MIP-1α, MCP-1, BioSource International ,Camarillo CA). Thermocycling conditions for PCR were 94^O C (1.5 min) for denaturation, followed by 35 cycles at 94^O C for 30 s, annealing at 60^O C for 45 s plus another 45 s at 72 ^{O}C and a final 7 min at 72^{O}C (Bio-Rad GeneCycler, Bio-Rad Laboratories, Hercules CA). ß-Actin mRNA (457 bp) was used as housekeeping gene. PCR products were electrophoresed on 2% agarose (Invitrogen). The gels were stained with ethidium bromide (5 µg/ml) and analyzed using GelDoc 8000 (UVP). Units are expressed as optical density (O.D.) of chemokine mRNA/O.D. of ß-actin mRNA.

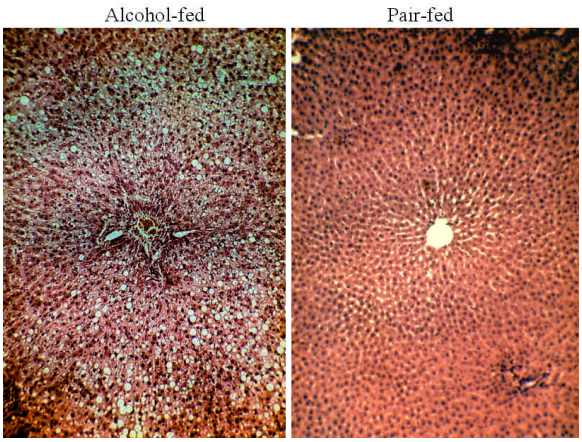

Figure 1. Histological section of livers taken from alcohol-fed and pair-fed rats. H&E stainining (10x).

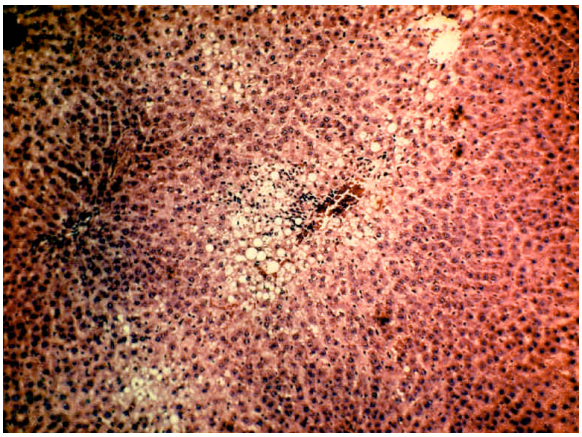

Figure 2. Histological section of a liver from an alcohol-fed rat. H&E stainining (10x).

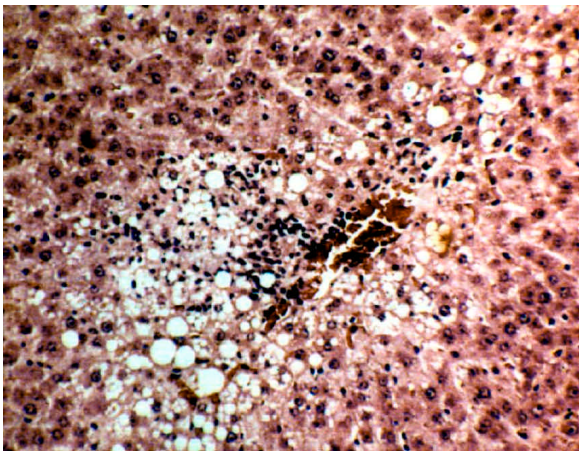

Figure 3. Histological section of a liver from an alcohol fed rat showing mononuclear cell infiltrate and steatosis. H&E stainining (40x).

3.12. Statistics

Data presented in this paper represent means ± SEM of 5-7 rats per treatment group. Statistical significance at $P < 0.05$ was assessed by ANOVA followed by Student-Neuman-Keuls multiple comparisons test.

4 RESULTS

4.1. Alcoholic hepatitis

After 32 weeks of ethanol feeding, significant histopathological changes in the livers of ethanol-fed rats were observed (Figures 1-3). Shown are histopatholgical sections of livers from alcohol-fed (Figure 1, left panel) and pair-fed rats (Figure 1, right panel). These lesions include steatosis and leukocyte infiltration. Figure 2 shows a liver section from another alcohol-fed rat. A higher magnification of a liver section showing mononuclear cell infiltrate is presented in Figure 3.

Serum ALT level was elevated in the ethanol-fed rats (110 ± 8 U/L) compared with the pair-fed rats (24 ± 5 U/L). Ethanol-fed rats (165 ± 20 pg/ml) were also more endotoxemic than the pair-fed group (10 ± 3 pg/ml). Chemokine concentrations in serum samples from ethanol-fed rats were significantly elevated: MCP-1 (560 ± 75 pg/ml), MIP-1α (425 ± 38 pg/ml) and RANTES (515 ± 66 pg/ml). In the pair-fed rats, the levels of these chemokines were less than 35 pg/ml). (Figure 4).

4.2. Leukocyte infiltration in the liver after 32 weeks of feeding

As shown above, leukocyte infiltrates were seen in the livers of ethanol-fed rats. To identify and characterize the cell type seen in tissues, leukocytes were collected from the liver by hepatic sinusoidal lavage. Cells that were in the parenchymal compartment, or those that could not be flushed out by lavage were collected after digestion of the whole liver and centrifugal elutriation at 11 ml/min flow rate. PMNs were collected in the Kupffer cell fraction and separated by Nycoprep-density gradient centrifugation. The isolated cells were characterized using monoclonal antibodies directed against CD4, CD8, NK cells and PMNs**.** Figure 5 shows that the number of cells isolated from the livers of ethanol-fed rats ($31 \pm 4.3 \times 10^6$/g liver) was significantly increased compared with the pair-fed rats ($11 \pm 1.6 \times 10^6$, $P<0.05$). The increase in leukocyte number in the liver during chronic ethanol consumption was due to numerous CD8+ cells. In the ethanol-fed rats, 85% of the CD8+ cells were in the liver tissues, while the rest are in the hepatic sinusoids. The majority of other cell types cells (90%) were flushed from the hepatic sinusoids. Ethanol-feeding was associated with reduced number of NK cells, CD4+ lymphocytes and PMNs (Figure 5). Ethanol feeding did not alter the numbers of Kupffer cell and endothelial cell populations.

4.3. Chemokine production *in vitro*

To examine the ability of freshly isolated Kupffer Cells and endothelial cells to generate CC-chemokines spontaneously in vitro, primary cultures of these cell types were established in the absence of stimuli. After 24 hr, culture supernatants were collected and assayed for MCP-1, MIP-1α and RANTES. Figure 6 shows that endothelial and Kupffer Cells spontaneously released MCP-1. Ethanol feeding did not further enhanced MCP-1 production by

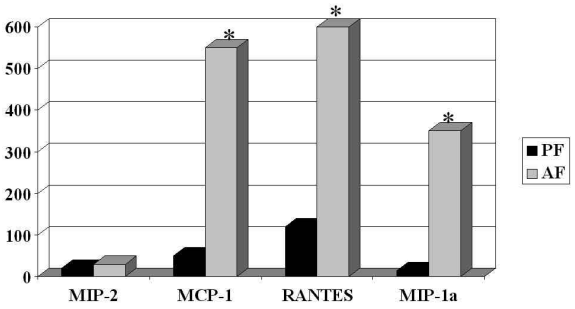

Figure 4. Serum chemokine levels after 32 weeks of feeding. Non-fasting blood samples were collected from rats fed with ethanol or dextrin. The levels of RANTES, MIP-1α, MIP-2, MCP-1 were measured in sera using commercially available ELISA kits for rats from BioSource International (Camarillo CA).

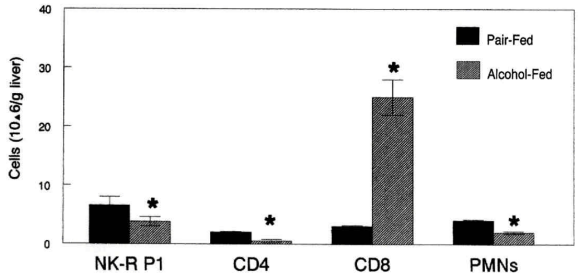

Figure 5. Characterization of leukocytes in the liver after 32 weeks of feeding. Leukocytes were collected by hepatic sinusoidal lavage and enzymatic digestion of the liver and identified by specific antibodies against CD4, CD8, NK-R, and PMNs. Flow cytometric analyses were performed based on side and forward scatter. The total number of cells positive for specific markers was calculated based on these analyses. *Indicates statistical significance between treatment groups at P<0.05 (Student t-test, N=6/group).

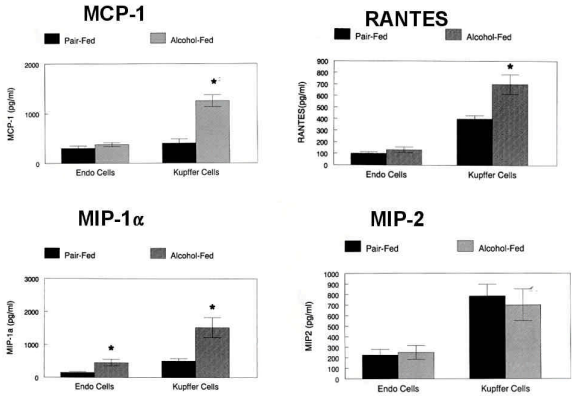

Figure 6. Basal chemokine production by endothelial cells and Kupffer cells from pair-fed and ethanol-fed rats. After 32 weeks of feeding, these cells were placed in primary cultures in the absence of stimuli for 24 hr. At the end of the incubation period, culture supernatants were assayed for MCP-1 (a), MIP-1 (b), RANTES (c) and MIP-2 (d) using commercially available ELISA kits. *Indicates statistical significance between treatment groups at P<0.05 (Student t-test, N=6/group).

endothelial cells. Ethanol consumption primed the Kupffer Cells for increased basal production of MCP-1 compared with the pair-fed rats. Figure 6 shows that ethanol feeding primed the Kupffer Cells for enhanced MIP-1α and RANTES releases in vitro. Priming for enhanced MIP-1α, but not RANTES release by endothelial cells after ethanol feeding was observed. Figure 6 shows that ethanol did not alter the ability of Kupffer Cells and endothelial cells to release MIP-2.

4.4. Nuclear transcription factors

Figure 7 shows that chronic alcohol was associated with enhanced translocation and binding of MNP-1 and NF-kB to the nuclear extracts from Kupffer cells. AP-1 binding was not significantly altered. Figure 7 further shows that treatment of cells with PMA in vitro for 1 hr variably enhanced the binding of MNP-1, NF-kB and AP-1 to Kupffer cell nuclear extracts in the pair-fed group. Such binding was further increased in the alcohol-fed group.

Figure 8 shows that MPN-1 binding to nuclear extracts from endothelial cells was also increased after chronic alcohol intoxication. AP-1 and NF-kB binding in the cell type was not altered by prolonged alcohol feeding (data not shown).

4.5. Chemokine mRNA

Total RNA from freshly isolated Kupffer Cells and endothelial cells were extracted for the determination of chemokine mRNA expression in these cell types. Figure 9 shows that endothelial cells endogenously expressed RANTES, MCP-1, and MIP-1 mRNAs. Ethanol feeding did not significantly alter mRNA expression of these chemokines, except for MIP-1α. Figure 9 shows that MIP-1α mRNA was absent, while those of MCP-1 and RANTES were expressed in the Kupffer Cells from pair-fed rats. Thirty weeks after ethanol feeding, the mRNA expressions of RANTES, MCP-1 and MIP-1a were enhanced in Kupffer cells (Figure 9).

4.6.Chemokine-induced chemotaxis

Chemokines are known to have an autocrine function in macrophages and lymphocytes. Figure 10 shows that Kupffer Cells were able to chemotax in response to MCP-1, MIP-1α and RANTES. Ethanol feeding downregulated chemokine-induced chemotaxis by Kupffer Cells by 70-95%.

4.7 Superoxide release

Reactive oxygen species (superoxide anion, hydrogen peroxide) are involved in antimicrobial function of phagocytic cells. For this reason, superoxide anion release by endothelial cells and Kupffer Cells in response to f-met-leu-phe was determined. Figure 11 shows that superoxide anion formation by Kupffer Cells and endothelial cells was significantly suppressed following chronic alcohol consumption. (Figure 11).

4.8. E.coli Phagocytosis

Isolated Kupffer Cells and endothelial cells were subjected to opsonized FITC-*E.coli* phagocytosis assay, to

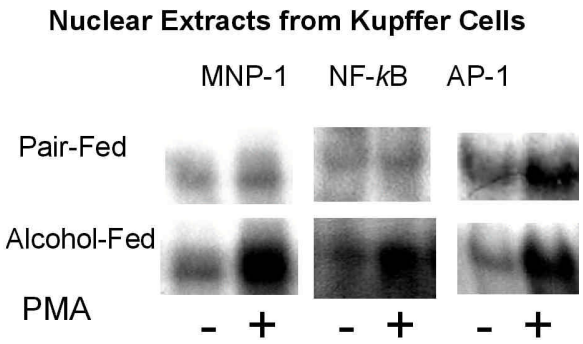

Figure 7. Translocation and binding of transcription factors to nuclear extracts from Kupffer cells. Nuclear extracts were obtained from Kupffer cells after 32 weeks of feeding with ethanol or dextrin. Before extraction, cells were treated with or without 1 μM PMA for 1 hr. Electrophoretic mobility shift assay (EMSA) was performed in these extracts using oligonucleotide probes for AP-1, MNP-1 and NF-kB. The probes were radiolabeled with ^{32}P, and analyses were performed on polyacrylamide gels and autoradiographs.

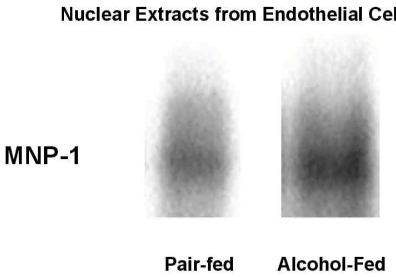

Figure 8. Translocation and binding of MNP-1 to nuclear extracts from endothelial cells. Nuclear extracts were obtained from endothelial cells after 32 weeks of feeding with ethanol or dextrin. Electrophoretic mobility shift assay (EMSA) was performed in these extracts using oligonucleotide probe for MNP-1. The probe was radiolabeled with ^{32}P, and analyses were subsequently performed on polyacrylamide gels and autoradiographs.

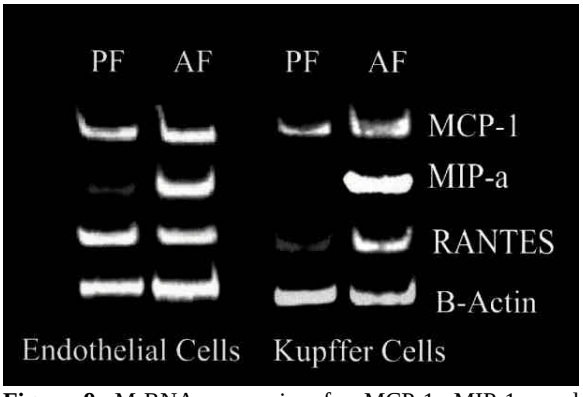

Figure 9. M-RNA expression for MCP-1, MIP-1α and RANTES in isolated Kupffer cells and endothelial cells after 32 weeks of feeding. Total RNAs from isolated cells were extracted, reverse transcribed, and subsequently subjected to PCR using commercially available primers from BioSource International.

determine their ability to ingest microbial particles after chronic alcohol feeding. Figure 12 shows that ethanol did not alter *E.coli* phagocytosis by endothelial cells. Phagocytic function of Kupffer Cells was significantly reduced by more than 70% in the ethanol-fed rats compared with the pair-fed controls (Figure 12).

5. DISCUSSION

Migration of inflammatory leukocytes to the site of inflammation is mediated by chemokines and cytokines produced by resident macrophages and endothelial cells in response to immunological stimuli. In chronic alcohol intoxication, gut-derived endotoxin is the factor implicated in the activation of resident macrophages in the liver for enhanced generation of pro-inflammatory mediators. Results presented in this study show that chemokine production by isolated Kupffer Cells taken from alcohol-fed rats was significantly enhanced compared with the pair-fed rats. The upregulation of chemokine release by Kupffer Cells correlated with elevated serum levels of chemokines. In human subjects with alcoholic hepatitis elevated serum levels of chemokines correlate with the degree of hepatitis and expression of chemokine protein and mRNA in liver tissues (1). In this work, CC-chemokine releases in vivo and in vitro were elevated following prolonged alcohol consumption for 32 weeks. These are MCP-1, MIP-1α and RANTES. These chemokines are chemotaxins for mononuclear cells. Results also show that alcohol feeding was associated with enhanced influx of mononuclear cells into the liver. CD8+ cell were the predominant cell type sequestered in the liver. The role of CD8+ in the induction of hepatotoxicity during alcoholic hepatitis has not been thoroughly investigated. In ethanol-fed rats fed treated with concanavalin A or LPS enhanced influx of lymphocytes is also observed. (19). This is associated with an induction of lesions with T lymphocytes that can mediate liver injury after polyclonal mitogen activation (19). Furthermore, CD8 lymphocytes may also participate in the development of an autoimmune component of alcoholic hepatitis. In experimental models of alcohol intoxication, this aspect needs to be investigated further. Enhanced influx of mononuclear cells in the liver may include those cells that are undergoing apoptosis for removal by Kupffer Cells. Despite a significant increase in mononuclear cell infiltration in the liver, the numbers of CD4+ and NK cells were reduced in the alcohol-fed rats than in the pair-fed rats. The reduction in NK cell number may contribute to the attenuation of the immunosurveillance capacity of the liver to attack and destroy tumor cells. This may compromise susceptible alcoholics to hepatocarcinoma.

We have previously shown that at 16 weeks of ethanol feeding, MIP-2 production in vivo and in vitro by Kupffer Cells is enhanced. Mild neutrophilic infiltration is also observed (5). At similar time interval of ethanol feeding significant elevations in serum CC-chemokines are not observed (6). In the present study, alterations in MIP-2 after 32 weeks of ethanol feeding for 32 weeks were not observed. Profound neutrophilic infiltration was not observed either. Others have observed the time-dependent production in cytokines and chemokines (20). In the present study, however, generalized chemokine up-

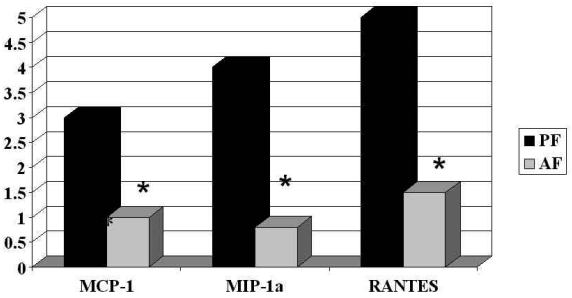

Figure 10. Chemokine-induced chemotaxis by Kupffer Cells from pair-fed and ethanol-fed rats. After 32 weeks of feeding, these cells were labeled with fluorescent PKH2 and exposed to 100 pg/ml of MCP-1 (a), MIP-1α (b) and RANTES (c) for 8 hr in a Boyden chemotactic chamber. Chemotaxis was determined based on the absorbance of chemokine-induced migration minus random migration (without chemokines). Values for random migration have been deducted from the above data. . *Indicates statistical significance between treatment groups at P<0.05 (Student t-test, N=6/group).

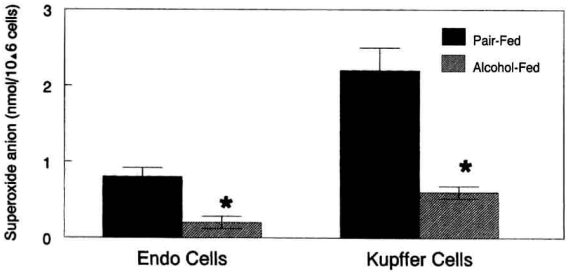

Figure 11. Formyl-methionine-leucine-phenylalanine-(f-met-leu-phe)-induced superoxide anion formation by endothelial cells and Kupffer Cells. Cells were exposed to 0.1 µM f-met-leu-phe and assayed for superoxide anion based on SOD-inhibitable reduction of cytochrome c. *Indicates statistical significance between treatment groups at P<0.05 (Student t-test, N=6/group).

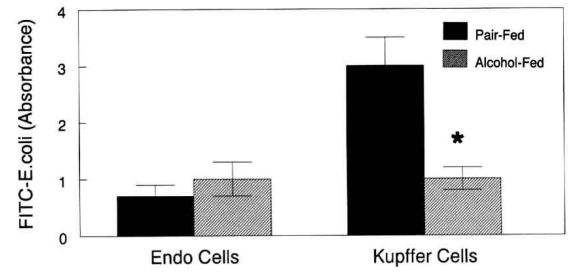

Figure 12. Opsonized FITC-*E.coli* phagocytosis by endothelial cells and Kupffer Cells from pair-fed and ethanol-fed rats. Cells were exposed to 1 mg/E.coli/10^6 cells for 1 hr. Uningested *E.coli* from cell monolayers were removed by washing the monolayers with PBS. Bacteria attached on the surface of target cells were quenched by 4% trypan blue. Intracellular fluorescent bacteria were measured using Cytofluor II. *Indicates statistical significance between treatment groups at P<0.05 (Student t-test, N=6/group).

regulation was not observed. This suggests different signaling mechanisms are involved in the synthesis of CC or CXC chemokines in the liver at different time points during chronic alcohol intoxication.

Results show that chemokine gene expression in Kupffer Cells was upregulated and correlated with enhanced protein release in vitro. In the pair-fed rats, chemokine mRNA expression in Kupffer Cells was not detected, but was induced after ethanol consumption. CC-chemokine mRNA induction during chronic alcohol intoxication may have resulted from enhanced LPS-induced influx of endotoxin from the gut (21). Kupffer Cells and endothelial cells are the likely targets of LPS. Furthermore, in vivo Kupffer Cells are the major sources for pro-inflammatory factors in response to endotoxin (21). We have previously demonstrated that depletion of Kupffer Cells by gadolinium chloride or liposome-encapsulated dichloromethylene diphosphonate suppresses LPS-induced ROS, cytokine and chemokine release [9,10]. Endothelial cells and splenocytes are likely to play a minor role. In the present study, endothelial cells were selectively primed for enhanced chemokine production. For example, ethanol intoxication altered MIP-1α mRNA expression, but not those of MCP-1 and RANTES in the endothelial cells. These observations suggest that intracellular signaling pathways for chemokine gene expression and protein release are different from that of Kupffer Cells. Others have demonstrated differential regulation of RANTES and IL-8 production by endothelial cells and epithelial cells in response to H_2O_2 and TNF (22). The mechanism for enhanced CC-chemokine production by Kupffer Cells during chronic alcohol intoxication may result from the activation of nuclear transcription factors, i.e., AP-1, NF-kB, MNP-1. LPS-induced ROS and TNF are involved in the activation of these factors for enhanced chemokine gene expression and protein release. Others have demonstrated that enhanced oxidative stress is observed following alcohol consumption (23,24). Activation of NF-kB in liver tissues following alcohol intoxication has also been demonstrated (25). LPS-responsive nuclear transcription factors, i.e., AP-1, NF-kB, MNP-1 regulates gene expression of CC and CXC chemokines (15,16,26). Thus, activation of one or more of these factors during chronic alcohol intoxication, may explain in part the selective upregulation of MCP-1, MIP-1α and RANTES (but not MIP-2) by hepatic non-parenchymal cells. Enhanced translocation and binding of MNP-1 in endothelial cell nuclear extracts may contribute to the upregulation of MIP-1α secretion by this cell type. MNP-1 is involved in the regulation of MIP-1α secretion (15,16).

Upregulation of chemokine secretion is expected to downregulate the expression of chemokine receptors. Cross regulation between CCR and CXCR receptors have also been shown (11). LPS has the capacity to directly downregulate chemokine receptors in vitro (27). This is likely to occur during chronic alcohol intoxication, because alcohol-fed rats were endotoxemic. As a result, downregulation of chemokine receptors may manifest, and this could contribute to suppressed chemokine-mediated

chemotaxis by Kupffer Cells and endothelial cells. These observations also reinforce the importance of the autocrine functions of these chemokines. Local production of these factors in the liver are likely to induce profound effects on immune functions by non-parenchymal cells. Chemotaxis by Kupffer Cells is an important aspect of innate immune function. Although they are resident in the liver, it is essential that they migrate to different areas in the liver in response to chemotactic stimuli. This migration within the liver facilitates the elimination of infectious pathogens and effete leukocytes. Furthermore, results show that phagocytosis of opsonized *E.coli* and f-met-leu-phe-induced superoxide release by Kupffer Cells and endothelial cells were downregulated. Such effects may be due in part to the reduction in cell surface receptors for Fcγ II/III, complement and f-met-leu-phe. The mechanism for reduced phagocytosis and superoxide release may also involve downregulation of cell surface receptors required in the recognition of antigens by Kupffer Cells. Gut-derived endotoxin due to chronic alcohol use and locally produced chemokines in the liver may contribute to enhanced internalization of cell surface receptors required for phagocytosis and respiratory burst in hepatic non-parenchymal cells.

In conclusion, prolonged alcohol consumption elicits sequestration of lymphocytes into the liver by stimulating local production of CC-chemokines. Concomitantly, innate immune functions are suppressed which may be due in part to the locally released chemokines and endotoxin. Upregulation of CC chemokines may allow the persistence of chronic alcoholic hepatitis, while reduced phagocytosis may permit dissemination of microbial pathogens to other tissues. These results further demonstrate that despite an exaggerated chemokine secretion by these cell types, the basic innate immune functions of hepatic non-parenchymal cells are suppressed,

6. ACKNOWLEDGEMENT

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