



2807712283

MEDICAL LIBRARY
ROYAL FREE HOSPITAL
HAMPSTEAD

CHEMOKINES AND ALCOHOLIC LIVER DISEASE

Dr. Neil C. Fisher

A thesis submitted to the University of London

1999

ProQuest Number: 10797888

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10797888

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Synopsis

Alcoholic liver disease is characterised by infiltration of the liver with leucocytes; alcoholic hepatitis is characterised by a neutrophil infiltrate whilst both hepatitis and cirrhosis are characterised by monocytic and lymphocytic infiltration. Chemokines are chemoattractant molecules that regulate leucocyte adhesion and migration at sites of tissue damage. In this thesis I studied the hypothesis that chemokines regulate the leucocyte infiltration and tissue damage characteristic of alcoholic liver disease and thereby determine the nature of the liver damage.

Immunohistochemical and in situ hybridisation demonstrated increased expression of several chemokines in alcoholic liver disease. The neutrophil chemokine IL-8 and the lymphocyte and monocyte chemokines MCP-1, MIP-1 α and MIP-1 β were present throughout the hepatic acinus in patterns corresponding to the severity of infiltration with these leucocytes. Chemokine mRNA was mainly localised to non-parenchymal cells including sinusoidal cells, fibroblasts and infiltrating leucocytes. In patients with alcoholic liver disease, circulating serum levels of the chemokine MCP-1 were raised compared to healthy controls and in proportion to histological severity of liver disease. Peripheral mononuclear cell secretion of both MCP-1 and MIP-1 α were raised in alcoholic hepatitis with evidence for both liver-derived and circulating monocyte-derived synthesis.

In vitro studies with radiolabelled chemokine protein demonstrated uptake of chemokines into isolated hepatocytes from surrounding culture medium, with uptake increased by the addition of alcohol or the pro-inflammatory cytokine TNF- α . Uptake was predominantly by low-affinity receptors but immunohistochemical studies suggest that specific receptors may nevertheless be up-regulated in alcoholic liver disease. Secretion of the chemokine MCP-1 from peripheral mononuclear cells and hepatocytes in vitro was diminished by alcohol or acetaldehyde with a dose-dependent effect. However, an ex vivo study in healthy volunteers showed that mononuclear cell secretion of MCP-1 may be increased when alcohol is ingested together with a balanced meal.

An uncontrolled pilot study of the xanthine-derivative pentoxifylline in subjects with severe alcoholic hepatitis, together with in vitro studies of its effect upon chemokine secretion, showed that this agent may have a role in the treatment of the disease.

In a controlled study examining genetic predisposition to symptomatic alcoholic liver disease, we examined two TNF- α promoter polymorphisms and HLA haplotypes DR3 and DQ2; however we failed to show any associations between these markers and the disease.

Acknowledgements

In submitting this thesis I acknowledge the generous and invaluable help and advice of many fellow workers of the Liver Research Laboratories, without whose assistance the thesis would never have been possible. First and foremost I owe a huge debt of gratitude to my supervisor Professor David Adams who not only provided the impetus to the study but has also encouraged and facilitated my endeavours whilst providing continual critical review at every stage. Secondly Dr Simon Afford, who provided invaluable supervision and practical help for many of my studies. Further gratitude is extended for practical assistance with specific studies as follows: Immunohistochemical and in situ hybridisation studies, Ann Williams and Janine Fear; chemokine measurement by ELISA, Desley Neil and Clare Morland; chemokine uptake studies, Janine Fear; cell culture techniques and DNA extraction, Mark Hathaway; provision of isolated human hepatocytes, Adrian Keogh and Laine Wallace; pentoxifylline assays, Tom Bryce and colleagues; DNA analysis, Munir Pirmohammed and colleagues. I am also grateful to my university adviser Dr. Kevin Moore for very helpful critical comments.

Other colleagues who have provided invaluable assistance include Paola Brun, Heather Crosby, Bridget Gunson, Stefan Hubscher, Sheila Jones, Norweeda Mirza, Kate Price, Matt Read, Satinder Rhandawa, Jean Shaw, Alastair Strain and Masashi Okamoto.

I am grateful to the Queen Elizabeth Hospital Liver Foundation Trust for providing my salary during this period of research. I am also grateful to Drs. Edwin Swarbrick and Suneil Kapadia for allowing me to continue and complete this work during my time at New Cross Hospital, Wolverhampton. Lastly I am grateful to my wife and family for their patience and consideration whilst I completed this thesis.

Some of this work was supported by grants from The Wellcome Trust, the Sir Jules Thorne Trust and the Endowment Fund of the Former Unit Birmingham Hospitals.

Contents

| | <u>Page</u> |
|-------------------------|-------------|
| List of tables | 5 |
| List of figures | 6 |
| Principal abbreviations | 8 |

Chapters

| | | |
|---|--|-----|
| 1 | Introduction | 9 |
| 2 | Hepatic chemokine expression in alcoholic liver disease | 26 |
| 3 | Serum levels and peripheral secretion of the beta-chemokines MCP-1 and MIP-1 α in alcoholic liver disease | 40 |
| 4 | Chemokine uptake by human hepatocytes and the role of ethanol and TNF- α | 55 |
| 5 | Secretion of MCP-1 from human hepatocytes and leucocytes | 69 |
| 6 | A pilot study of pentoxifylline in alcoholic hepatitis | 86 |
| 7 | TNF- α promoter polymorphisms and HLA-DR3 and DQ2 haplotypes in alcoholic liver disease | 100 |
| 8 | Conclusions and future directions | 110 |
| | Reference list | 119 |
| | Appendices | 135 |
| | Publications | 150 |

| <u>List of tables</u> | | <u>Page</u> |
|-----------------------|---|-------------|
| <u>Chapter 1</u> | | |
| 1.1 | Selected chemokines: Target cells and actions | 17 |
| 1.2 | Some chemokine receptors and their corresponding ligands | 18 |
| <u>Chapter 2</u> | | |
| 2.1 | Chemokine protein and mRNA expression in alcoholic liver disease | 36 |
| <u>Chapter 3</u> | | |
| 3.1 | Summary characteristics of patients with alcoholic liver disease | 45 |
| 3.2 | Correlation of laboratory indices with serum MCP-1 in alcoholic liver disease | 47 |
| <u>Chapter 4</u> | | |
| 4.1 | Expession of chemokine receptors in alcoholic liver disease | 64 |
| <u>Chapter 7</u> | | |
| 7.1 | Distribution of TNF- α -308 _{G-A} and -238 _{G-A} polymorphisms and DR3 and DQ2 haplotypes in alcoholic liver disease | 107 |

| <u>List of figures</u> | <u>Page</u> | |
|------------------------|--|----|
| <u>Chapter 1</u> | | |
| 1.1 | Ethanol metabolism and its relation to cytokines synthesis and release | 13 |
| 1.2 | Structure of the prototype alpha chemokine, IL-8, and prototype beta chemokine, MCP-1. | 15 |
| 1.3 | Illustration of steps involved in leucocyte adhesion. | 16 |
| <u>Chapter 2</u> | | |
| 2.1 | Localisation of chemokine protein and mRNA in non-inflamed liver tissue from organ donors. | 32 |
| 2.2 | Chemokine protein and mRNA expression in alcoholic hepatitis and alcoholic cirrhosis. | 33 |
| 2.3 | Immunolocalisation of TNF- α and chemokine protein in alcoholic hepatitis. | 34 |
| 2.4 | RNAase protection gel of MIP-1 α and β mRNA expression in normal and alcoholic hepatitis and cirrhosis tissue extracts. | 35 |
| <u>Chapter 3</u> | | |
| 3.1 | Serum MCP-1 levels in patients with alcoholic liver disease and comparison groups | 46 |
| 3.2. | Paired hepatic and peripheral vein MCP-1 levels in patients with alcoholic liver disease | 48 |
| 3.3. | PBMC secretion of MCP-1 and MIP-1 α in patients with alcoholic hepatitis and healthy controls. | 50 |
| 3.4. | PBMC cytospin preparation hybridised with cDNA for MIP-1 α chemokine mRNA | 51 |
| <u>Chapter 4</u> | | |
| 4.1 | Uptake of radiolabelled IL-8 and MIP-1 α by primary human hepatocytes and the effect of TNF- α and ethanol. | 61 |
| 4.2 | Uptake of radiolabelled IL-8 and MIP-1 α by primary human hepatocytes and co-incubation with acetaldehyde and ethanol. | 62 |

| <u>Chapter 4 (continued)</u> | | <u>Page</u> |
|------------------------------|---|-------------|
| 4.3 | Autoradiograph of PAGE of radiolabelled chemokines | 62 |
| 4.4 | Uptake of ¹²⁵ I IL-8 by Hep G2 cells, L190 cells and PBMC's | 62 |
| 4.5 | Assay of IL-8 binding affinity | 63 |
| 4.6 | Immunolocalisation of chemokine receptors in human liver tissue | 65 |
| 4.7 | Chemokine receptor expression on various cell types analysed by flow cytometry. | 66 |
| <u>Chapter 5</u> | | |
| 5.1 | Hepatocytes in culture as used for in vitro experiments | 73 |
| 5.2 | MCP-1 production from cultured primary human hepatocytes | 75 |
| 5.3 | MCP-1 production from cultured Hep G2 cells | 75 |
| 5.4 | MCP-1 production from cultured L190 cells | 76 |
| 5.5 | Sequential ethanol concentrations in hepatocyte culture wells | 77 |
| 5.6 | MCP-1 secretion from cultured PBMC's and the effect of ethanol and acetaldehyde | 79 |
| 5.7 | MCP-1 secretion from cultured PBMC's before and after ingestion of food only, food + alcohol, or alcohol only | 81 |
| <u>Chapter 6</u> | | |
| 6.1 | Mechanism of action of pentoxifylline | 88 |
| 6.2 | Serial laboratory indices during pentoxifylline therapy | 92 |
| 6.3 | Serial levels of pentoxifylline and its metabolite M1 | 94 |
| 6.4 | PBMC secretion of MCP-1 and IL-8 from 4 healthy volunteers with or without pentoxifylline | 96 |
| <u>Chapter 8</u> | | |
| 8.1 | Role of alcohol and systemic chemokine expression | 114 |
| 8.2 | Role of alcohol and hepatic chemokine expression | 114 |

Principal Abbreviations

| | |
|------|--|
| IL | Interleukin |
| MCP | Monocyte chemoattractant protein |
| MIP | Monocyte/ macrophage inflammatory protein |
| CXC | Cysteine-[other amino acid]-cysteine (refers to central amino acid sequence in CXC / alpha chemokine molecule) |
| CC | Cysteine-cysteine (refers to central amino acid sequence in CC / beta chemokine molecule) |
| CCR | CC chemokine receptor |
| CXCR | CXC chemokine receptor |
| TNF | Tumour necrosis factor |
| LPS | Lipopolysaccharide / endotoxin |
| PBMC | Peripheral blood mononuclear cell |

Chapter 1

Introduction

Alcoholic Liver Disease: General Considerations

Alcoholic liver disease is a major and increasing burden on healthcare resources throughout the western world. Its incidence is consistently related to *per capita* national consumption of alcohol (Rush 1986, Schmidt 1977), and in the United Kingdom the recorded incidence of deaths due to alcoholic cirrhosis has risen from approximately 30 per million in 1965 to approximately 60 per million in 1991 in keeping with a proportionate increase in overall alcohol consumption (Smart 1998). In a typical district general hospital in the United Kingdom, alcoholic liver disease is the commonest liver disease encountered by a gastroenterologist (Kingham 1997), and the healthcare burden of alcoholic liver disease is amplified by the fact that the most severe clinical complications, including ascites, variceal haemorrhage and portosystemic encephalopathy are difficult to treat and frequently require prolonged hospitalisation. The duration and amount of alcohol ingestion required to cause significant liver disease varies according to individual susceptibility and gender; a mean daily intake of around 19 units (190 mls) for men and 14 units for women over several years was recorded in patients with alcoholic cirrhosis in one study (Saunders 1981), whilst in another it was estimated that a daily intake of as little as 4-6 units may lead to cirrhosis in susceptible subjects (Anderson 1993).

Alcoholic liver disease encompasses a spectrum of characteristic pathological changes in the liver (International Group, 1981). Fatty liver is probably the earliest (and most consistent) lesion resulting from alcohol abuse (Rubin 1968), and whilst usually held to be relatively benign this may progress to cirrhosis if alcohol abuse is continued over several years following diagnosis (Teli 1995). Alcoholic hepatitis refers to hepatocyte ballooning and necrosis in association with a polymorphonuclear leucocyte infiltrate; this is usually accompanied by hyaline material in the form of Mallory bodies within hepatocytes. When present, these changes reflect recent alcohol consumption and are usually reversible with sustained withdrawal. Alcoholic fibrosis tends to develop in a characteristic pericellular pattern in the centrilobular zones and with sustained alcohol abuse this leads eventually to cirrhosis, which is characteristically micronodular and by definition irreversible. Alcoholic cirrhosis, when 'active', is accompanied by a dense mononuclear cell infiltrate which is localised to fibrous septa and portal tracts, and if alcohol is withdrawn over at least several months this frequently becomes 'inactive' with disappearance of the cellular infiltrate. It is

usually assumed that alcoholic cirrhosis develops alongside and perhaps as a sequel to hepatitis (International Group, 1981). The two conditions frequently co-exist, although the belief that alcoholic hepatitis is a necessary prerequisite to development of cirrhosis remains unproven and there is evidence from a baboon model of alcoholic liver disease that cirrhosis may develop without intervening hepatitis (Lieber 1975) which has recently been supported by human pathological studies (Reeves 1996).

The prognosis of alcoholic liver disease is clearly linked to the continuation or otherwise of alcohol consumption, although a well recognised but poorly understood feature of alcoholic hepatitis and cirrhosis is that the clinical and pathological manifestations of the disease frequently progress despite sustained withdrawal from alcohol (Powell 1968, Orrego 1987, Chedid 1991). In the latter study, 5 year survival in patients with pure alcoholic hepatitis (without cirrhosis) was 60%, with mortality being significantly worse in those who continued to consume alcohol after presentation compared to those who did not. In contrast, 5 year survival in patients with cirrhosis (with or without hepatitis) was lower (35% and 50% respectively), and in this group continued alcohol consumption was surprisingly not a significant risk factor in subsequent mortality (although level of previous alcohol consumption was), suggesting that liver injury continued despite alcohol withdrawal. Similarly, patients with clinically severe alcoholic hepatitis have a high risk of mortality that may be delayed by several weeks despite withdrawal from alcohol and intensive supportive therapy in hospital (Maddrey 1990). These observations suggest that the direct toxicity of alcohol and its metabolites cannot be solely responsible for the pathogenesis of alcoholic liver disease, and other factors must contribute. Thus there is widespread interest in the myriad of nutritional, biochemical and immunological disorders that probably contribute to its pathogenesis (Lieber 1994). In particular, in recent years, there has been considerable interest in delineating the immunological mechanisms that underlie the hepatic inflammation characteristic of alcoholic liver disease (Israel 1988, Paronetto 1993).

Alcoholic Liver Disease: Role of Inflammatory Mediators

Since alcoholic hepatitis and active alcoholic cirrhosis are characterised by marked leucocytic infiltration of the hepatic parenchyma, it follows that activation of the immune system must be an integral part of alcoholic liver disease. In recent years interest has developed firstly in the role of pro-inflammatory cytokines in alcoholic liver disease and secondly in the stimuli for expression of these cytokines. Cytokines are relatively small (< 30 kD) soluble inflammatory mediators produced from many nucleated cells, particularly monocytes (including Kupffer cells), endothelial cells and fibroblasts, and which have a diverse range of effects including 'activation' and proliferation of leucocytes with enhancement of phagocytic and cytotoxic functions. Whilst cytokines are necessary for an appropriate immunological response to tissue injury and/ or infection, and are also important in promoting regeneration of injured tissue, excess secretion may lead to tissue damage by promoting an excessive immunological response (Tracey 1995). There is now abundant evidence for raised levels of several pro-inflammatory cytokines including interleukin-1 (IL-1), IL-6, and tumour necrosis factor-alpha (TNF- α) in alcoholic liver disease and these cytokines are implicated in the pathogenesis of alcoholic liver disease (Bird 1990, Khoruts 1991, McClain 1993). The precise delineation of the pathways leading to increased expression of these cytokines in alcoholic liver disease is not clear and there are probably at least 2 major pathways involved. Firstly, lipopolysaccharide (LPS, also known as endotoxin) is a stimulus for cytokine production in many cells and circulating levels are increased as a result of alcohol ingestion (Bode 1987, Persson 1991). Secondly, the metabolism of ethanol to its highly reactive intermediary acetaldehyde, and thence acetate, results in formation of several reactive oxygen species including superoxide, peroxide and hydroxyl radicals which alter the cellular 'redox state' (Nordmann 1994, Clot 1995). Excessive intracellular reactive oxygen species leads to peroxidation of cellular lipids, whilst excessive intracellular acetaldehyde binds irreversibly to intracellular proteins. It is likely that the altered redox state and possibly the presence of altered cellular proteins and lipids stimulates transcription and expression of proinflammatory cytokines (Schreck 1991, Jaeschke 1991).

The metabolism of ethanol to acetaldehyde in the liver occurs via 2 principal metabolic pathways; firstly, alcohol dehydrogenase (ADH) which normally comprises the major route of metabolism, and secondly the microsomal ethanol oxidising system (MEOS), a

descriptive term for the part of the hepatic cytochrome P450 ('CYP') enzyme system metabolising ethanol. This enzyme has now been characterised and termed CYP 2E1 (Lasker 1987). The enzyme, unlike ADH, is inducible and with chronic alcohol abuse becomes a major route of ethanol metabolism. Metabolism with CYP 2E1 is much more likely to produce reactive oxygen intermediaries than is metabolism via ADH (Dai 1993). Thus chronic alcohol ingestion will lead to a greater propensity for oxidative injury because of induction of CYP 2E1 (Lieber 1994). The mechanisms by which ethanol metabolism may therefore lead to cytokine production are summarised below in figure 1.1.

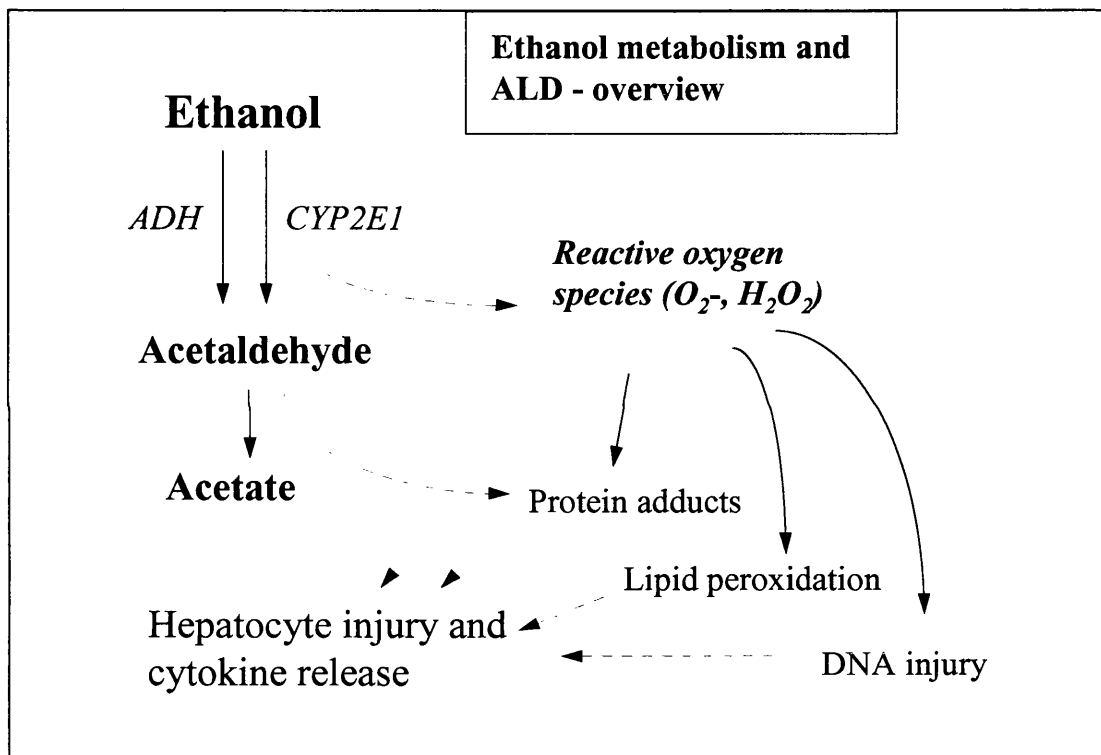


Figure 1.1 Summary of ethanol metabolism via hepatic alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1), and the ways in which this may promote hepatocyte injury and release of cytokines.

Chemokines: Categories and Characteristics

Chemokines are a family of relatively small (8-10 kD) structurally related chemotactic cytokines that are secreted by a wide range of cells including leucocytes, platelets, fibroblasts, endothelial and epithelial cells. More than 50 different human chemokines have been demonstrated, the majority of which may be divided into 2 groups according to their structure (Baggiolini 1994). These groups have a highly conserved 4 cysteine motif in the active part of the chemokine molecule: alpha (also known as CXC) chemokines contain an amino acid group between the first 2 cysteine residues in their active domain (hence the term CXC), whilst the beta (also known as CC) chemokines do not contain this group between the corresponding residues (figure 1.2). Two recently characterised chemokines do not fall into either category; lymphotactin has only 2 corresponding cysteine residues in total, without an intervening amino acid (Kelner 1994), and fractalkine has three amino acids between the cysteine residues ('CXXXC') (Bazan 1997).

The structural distinctions in the cysteine configurations are associated with the ability to act on particular leucocyte subsets. Thus, interleukin-8 (IL-8), the prototype alpha or CXC chemokine, is chemotactic for neutrophils predominantly, whilst monocyte chemoattractant protein-1 (MCP-1), the prototype beta or CC chemokine, is chemotactic for mononuclear cells such as monocytes predominantly. However, the leucocyte subset specificity within the alpha chemokine family is also dependent upon the presence or absence of a glutamine-leucine-arginine (ELR) sequence near the N-terminal, which confers specificity for neutrophil chemotaxis. Thus the prototype alpha chemokines including IL-8 and growth-related oncogene-alpha (GRO- α) possess this sequence whilst other alpha chemokines such as interferon-inducible protein of 10kD (IP-10) and monokine induced by interferon- γ (MIG) do not possess this sequence, and are chemotactic for lymphocytes. Some examples of chemokines and their target cells and actions are shown in table 1.1. Chemokines are expressed at low levels in the resting state, but are rapidly up-regulated upon activation; secretion is typically induced by pro-inflammatory cytokines such as IL-1, IL-2, TNF- α , γ -IFN and the bacterial product lipopolysaccharide (LPS) (Baggiolini 1994) and may also result from disturbances in the intracellular redox state (Schreck 1991, Xu 1996).

The discovery and characterisation of chemokines followed the discovery of adhesion molecules and cytokines in the mid-1980's; it was found that avidity of adhesion molecule binding was subject to modification by other soluble factors (Butcher 1991) which in turn lead to the discovery and characterisation of a diverse family of chemokines, so called because some chemokines had previously been described as *chemotactic cytokines*. Chemokines regulate leucocyte recruitment in at least two related ways: (i) They induce a conformational change in leucocyte adhesion molecules which enhances adhesion to other cells expressing the corresponding ligands; (ii) They induce cytoskeletal changes that result in increased motility (Luster 1998). These actions enhance leucocyte recruitment and then migration respectively; leading to 'directional migration'. More recently, further roles for chemokines have been discovered including enhancement of cytolytic activity of leucocytes, and promotion of angiogenesis (Strieter 1996). The principal steps involved in leucocyte adhesion including the role of chemokines are illustrated in figure 1.3.

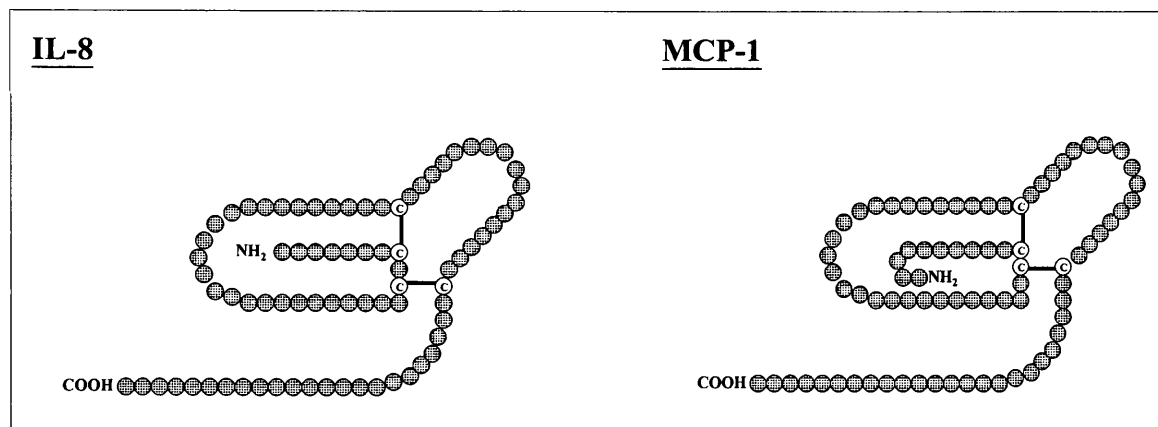


Figure 1.2 Diagrammatic representation of structure of the prototype alpha (CXC) chemokine, IL-8, and prototype beta (CC) chemokine, MCP-1. The configuration of cysteine residues in the central part of the molecule is highly conserved between different members of the chemokine family; in alpha chemokines the 2 central cysteine residues are separated by another amino acid (hence the term CXC) whilst in beta chemokines the corresponding cysteine residues are immediately adjacent (hence the term CC). The carboxyl (COOH) portion of the molecule tethers the molecule to the extracellular matrix, whilst the amine (NH₂) portion activates the chemokine receptor.

The action of chemokines is mediated via specific cell surface receptors that are members of the rhodopsin superfamily of seven transmembrane-spanning G-protein linked molecules (Kelvin 1993, Luster 1998). The intracellular domain of these receptors acts as a phosphorylation site for signal transduction. Eight human CC receptors (CCR1 to CCR8) and four CXC receptors (CXCR1 to CXCR4) have been characterised. The CXCR and CCR groups are specific for alpha and beta chemokines respectively (table 1.2). However, within these groups chemokine-receptor interactions are of variable specificity; for example the CXCR1 and CCR2 receptors are relatively specific for IL-8 and MCP-1 respectively, whilst CCR1 and CXCR2 receptors bind multiple alpha and beta chemokines respectively. A non-specific ‘promiscuous’ receptor, the Duffy antigen receptor complex (DARC) is present on red blood cells; this binds all chemokines without known physiological effect and may act as a ‘sump’ for circulating chemokines (Horuk 1993).

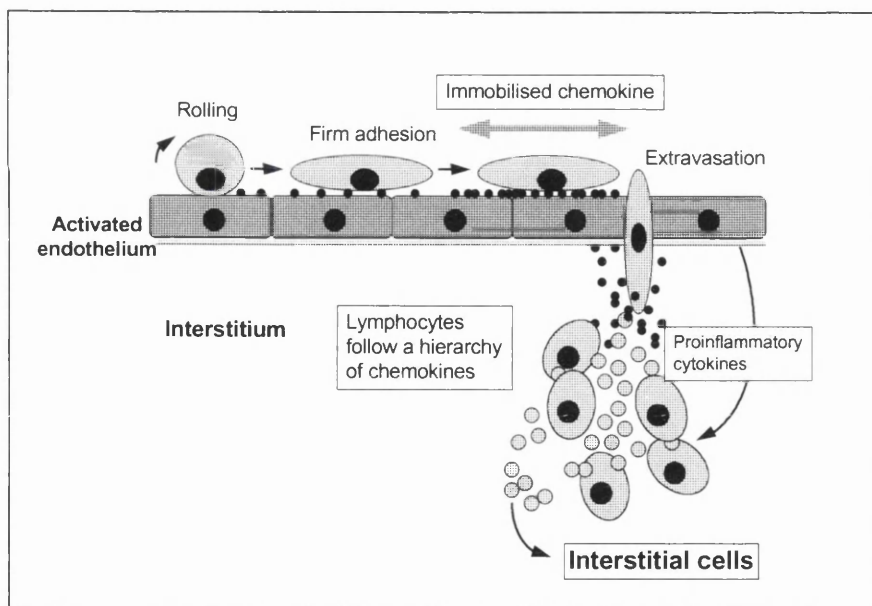


Figure 1.3 Illustration of steps involved in leucocyte adhesion. Leucocyte (in this case a lymphocyte, shown here as large circular nucleated cell) ‘rolls’ on activated endothelial surface and is tethered by selectins. Firm adhesion is then effected by integrin molecules which are ‘triggered’ by chemokines (shown here as small black circles) bound to the endothelium. The lymphocyte then migrates towards the interstitium along a gradient of chemokines, and the presence of cytokines (shown here as larger pale circles) will then help induce its effector functions. (Reproduced with kind permission of Professor Adams)

| <u>CHEMOKINE</u> | <u>TARGET CELLS</u> | <u>BIOLOGICAL ACTION IN VITRO</u> |
|---|------------------------|--|
| <u>α chemokines: containing ELR sequence</u> | | |
| IL-8 | Neutrophils | Chemotaxis, superoxide / granule release |
| | T cells, NK cells | Chemotaxis |
| | Basophils | Chemotaxis, histamine release |
| | Endothelial cells | Angiogenesis |
| GRO α,β,γ | Neutrophils | Chemotaxis, adhesion, activation |
| | Endothelial cells | Angiogenesis |
| <u>α chemokines: not containing ELR sequence</u> | | |
| IP-10 | T cells, NK cells | Chemotaxis, adhesion, cytolysis |
| | Endothelial cells | Inhibits angiogenesis |
| MIG | T cells, NK cells | Chemotaxis, adhesion, cytolysis |
| <u>β chemokines</u> | | |
| MCP-1 | Monocytes | Chemotaxis, adhesion, superoxide release |
| | T-cells | Chemotaxis |
| | Mast cells / basophils | Chemotaxis, histamine release |
| | Stem cells | Inhibits colony formation |
| MIP-1α | Monocytes | Chemotaxis |
| | T-cells | Chemotaxis, tumour cytotoxicity |
| | B cells | Chemotaxis |
| | Mast cells/ Basophils | Chemotaxis, histamine release |
| | Eosinophils | Chemotaxis, cationic protein release |
| RANTES | Monocytes | Chemotaxis |
| | T-cells | Chemotaxis, adhesion |
| | Eosinophils | Chemotaxis, cationic protein release |
| | Basophils | Chemotaxis, histamine release |

Table 1.1 Selected chemokines: Target cells and actions. Abbreviations: ELR, glutamine-leucine-arginine; IL, interleukin; GRO, growth-related oncogene, IP-10, interferon-inducible protein of 10kD; MCP, monocyte chemoattractant protein; MIG, monokine induced by interferon-γ; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T-cell expressed and secreted.

| <u>Receptor</u> | <u>Chemokine</u> |
|-----------------|---|
| CXCR1 | IL-8 |
| CXCR2 | IL-8, GRO- α |
| CXCR3 | IP-10 |
| CXCR4 | SDF (stromal cell-derived factor) |
| CCR1 | RANTES, MIP-1 α , MCP-2,3 |
| CCR2 | MCP-1,2,3,4 |
| CCR3 | Eotaxin, RANTES, MCP-2,3,4 |
| CCR4 | RANTES, MCP-1, MIP-1 α , |
| CCR5 | MIP-1 α , MIP-1 β , RANTES |

Table 1.2 Some chemokine receptors and their corresponding ligands

Chemokines and Leucocyte Recruitment

Recruitment of leucocytes to sites of tissue inflammation is critically dependent upon interactions between the leucocytes and vascular endothelium. Firstly, ‘rolling’ of leucocytes is induced by the presence of tethering molecules (principally selectins), protruding into the vascular lumen from the endothelial surface. Secondly, chemokines present on the endothelium may engage and activate the corresponding leucocyte chemokine receptors. This induces conformational change in integrin adhesion molecules and triggers ‘strong adhesion’ of leucocytes by binding of integrins their counter-receptors such as the intercellular and vascular cell adhesion molecules (ICAM and VCAM respectively) expressed on endothelial cells (Shimizu 1992). Examples of this process include the role of IL-8 in promoting binding between the integrin leucocyte function associated antigen-1 (LFA-1) on neutrophils with its counter-receptor intercellular adhesion molecule-1 (ICAM-1) on endothelial cells (Kuijpers 1992, Baggiolini 1994). Similarly, macrophage inflammatory protein-1 beta (MIP-1 β) promotes T-cell adhesion

via interactions between LFA-1 and ICAM-1 and between the integrin very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1) (Taub 1993, Tanaka 1993).

In addition to promotion of strong adhesion, chemokines induce rapid cytoskeletal changes that facilitate migration of leucocytes into the subendothelial stroma. The leucocyte may then undergo a sequence of adhesion-detachment steps influenced by local chemotactic factors, which together with rapid cytoskeletal changes allow it to traverse a concentration gradient of chemokine immobilised within the extracellular matrix; this process is known as haptotaxis (Springer 1994). The effects of chemokines in recruiting leucocytes are localised by their binding to proteoglycans in the endothelial glycocalyx, and, following migration of leucocytes, retention is promoted by chemokines bound to extracellular matrix. These mechanisms promote the concentration and retention of leucocytes at the site of chemokine secretion, which would correspond to the site of tissue injury (Ebnet 1996). These steps are summarised earlier in figure 1.3

The composition of the leucocytic infiltrate at sites of inflammation will be determined by a number of complementary factors which will include the local chemokine composition in addition to the adhesion molecules expressed on leucocytes and endothelium, as discussed above (Springer 1994). The local chemokine composition will determine the nature of the leucocytic infiltrate by virtue of chemokine receptor specificity; however the subset-specificity of most chemokines for particular leucocytes is only relative and is subject to a large degree of overlap due to promiscuity of some of the chemokine receptors (see table 1.2). Thus there is likely to be a large degree of redundancy within the chemokine families, which may be advantageous in evolutionary terms, given the essential role of chemokines in the overall inflammatory response (Strieter 1996).

Chemokines and Leucocyte Activation

In addition to promoting leucocyte recruitment, chemokines also activate leucocyte effector functions. For example, IL-8 enhances neutrophil phagocytosis, superoxide generation and granule release (Baggiolini 1994). Similarly, other leucocyte subsets may be activated by appropriate chemokines, as shown in table 1.1. This role also extends to enhancement of lymphocyte proliferation via increased production of IL-2 from activated T-cells under the influence of MIP-1 α (Taub 1996).

It follows from the discussions above that chemokines, like other cytokines, are likely to play a central role in most acute and chronic inflammatory diseases; in addition to the evidence for physiological stimuli for and modes of actions of chemokines that have been described, evidence is now also accumulating for the role of chemokines in many such diseases. Roles for beta chemokines have been shown in viral (Cook 1995) and mycobacterial (Zhang 1995) respiratory infections, in autoimmune diseases such as rheumatoid arthritis, psoriasis and glomerulonephritis (Furie 1995), allergic disorders such as asthma (Lukacs 1996), degenerative diseases such as osteoarthritis (Kunkel 1996) and atherosclerosis (Ross 1993), and in rejection of transplanted organs (Adams 1996). In some of these examples the role of chemokines will clearly be beneficial whilst in others such as autoimmune diseases or allergy, their effects will probably be unwanted. Interestingly it is also now recognised that some viruses such as HIV encode chemokine-like proteins that may engage chemokine receptors, leading to chemotaxis of leucocytes to sites of viral replication and thus contributing to the pathogenesis of the viral disease (Weissman 1997).

Chemokines in normal and diseased liver

1. Tissue expression

Some studies have recently begun to characterise the expression of chemokines in normal liver and in inflammatory liver disease. Alpha and beta chemokines can be seen in normal liver tissue by immunohistochemistry and in-situ hybridisation (Adams 1996). The alpha chemokine IL-8 is present on leucocytes, vascular endothelium and biliary epithelium, whilst the beta chemokines MCP-1, MIP-1 α and MIP-1 β are restricted to leucocytes and the vascular endothelium. Synthesis of these proteins appears to take place in leucocytes and vascular endothelial cells, as judged by the presence of mRNA. The alpha chemokines IL-8 and GRO- α , and the beta chemokines MCP-1, MIP-1 α and RANTES are all detectable in normal human liver tissue homogenates (Maltby 1996). These are the chemokines that have been studied the most in normal liver although there is likely to be synthesis and expression of other chemokines within the liver in view of the wide range of chemokines that have been discovered. The expression of chemokines in normal liver is presumably responsible for leucocyte trafficking that occurs in normal health.

In carbon tetrachloride-induced toxic liver injury in rodents there is increased expression of MCP-1 (Czaja 1994), and in rodent alcoholic liver injury there is increased expression of MIP-2 (Bautista 1997). In alcoholic hepatitis and other inflammatory liver diseases in humans there is evidence for increased expression of the alpha chemokines IL-8 and GRO- α and the beta chemokine RANTES; studies on tissue homogenates show that expression of these chemokines correlates with clinical and biochemical indicators of disease severity and histological degree of leucocyte infiltration (Sheron 1993, Maltby 1996). There is also evidence for increased expression of MCP-1, correlating closely with monocyte infiltration in chronic viral liver disease (Marra 1998) and recently there has been demonstration of increased expression of the lymphocyte chemokines Mig and IP-10 in hepatitis C cirrhosis (Shields 1999). Together these studies suggest that a range of chemokines are likely to be expressed in alcoholic and other inflammatory liver diseases in humans.

2. Circulating levels

The chemokines IL-8, MCP-1 and MCP-2 are detectable in the circulation in healthy subjects (Hill 1993, Bossink 1995). Several groups have found raised circulating levels of IL-8 in alcoholic liver disease, with serum levels reflecting the degree of active inflammation (Sheron 1993, Hill 1993, Masumoto 1993). Serum levels correlate with other laboratory indicators of disease severity such as serum bilirubin, creatinine, prothrombin time, peripheral white blood cell count, tumour necrosis factor and soluble tumour necrosis factor receptor (Sheron 1993), whilst the serum levels of IL-8 in alcoholic hepatitis slowly fall over a period of months following recovery from the acute illness (Masumoto 1993, Hill 1993). Subjects with alcohol dependency but without overt liver disease also have raised levels, and interestingly IL-8 levels have been shown to rise in the period following hospitalisation for detoxification (Masumoto 1993). There have previously been no published studies concerning circulating levels of beta chemokines in alcoholic liver disease, although levels of MCP-1 and MCP-2 are elevated in sepsis in humans (Bossink 1995). The source of circulating chemokines has not been defined in these studies but presumably results in part from release from circulating and/or tissue-bound mononuclear cells. In alcoholic liver disease this may reflect activation within the liver but may also result from the increased circulating levels of endotoxin that characterise alcoholic liver disease (Bode 1987).

Cellular Sources of Chemokines Within the Liver

The hepatocyte is the main target of cellular damage from reactive intermediaries in alcoholic and other toxic liver injury; however, evidence from in situ hybridisation studies and in vitro experiments with cultured liver cell isolates suggests that, whilst hepatocytes may produce chemokines, other 'non-parenchymal' cells are the principal sources of chemokines (and other cytokines) during hepatic inflammation (see below). These observations have been largely based on studies with rat hepatocytes, although studies in humans including in situ hybridisation suggest that similar mechanisms operate.

Several liver cell constituents have been shown to produce chemokines under appropriate conditions, including primary hepatocytes (Shiratori 1993, Maher 1995), sinusoidal lining cells (McNab 1996), biliary epithelial cells (Morland 1997), Kupffer cells (Maher 1995, Bautista 1995), and stellate (fat-storing or Ito) cells (Marra 1993, Czaja 1994). Kupffer and stellate cells are probably the most important sources during hepatic inflammation. Kupffer cells have been shown to be potent sources of TNF- α , transforming growth factor- β (TGF- β) and chemotactic intermediaries after experimental liver injury and in alcohol-fed rats (Armendariz-Barunda 1990, Maher 1995, Bautista 1997). Stellate cells were shown to be the principal source of MCP-1 after carbon tetrachloride-induced liver injury in rats (Czaja 1994). In contrast, sinusoidal lining cells were more potent sources of interleukin-8 compared to Kupffer cells and primary hepatocytes in cultured rat cells (Ohkubo 1998). It is likely that interaction between different liver cell constituents is important in enhancing production of chemokines (Maher 1995, Zickus 1998).

Chemokines and Alcoholic Liver Disease

The mechanisms governing the orchestration and release of chemotactic factors from hepatocytes and the surrounding non-parenchymal cells in inflammatory liver diseases remain poorly defined. In alcoholic liver disease, expression of chemokines may be expected to occur as a direct consequence of ethanol or its metabolites, since, like other cytokines, chemokine expression is redox-sensitive (Marui 1993, Schreck 1991) and may thus be up-regulated in response to the oxidative stress resulting from alcohol metabolism (Bachem 1992, Marra 1993). In practice, however, most workers have found that *in vitro* ethanol directly down-regulates chemokine secretion in the same way that it down-regulates cytokine secretion (Nelson 1989, Maher 1995). Thus the dynamics of ethanol-induced redox alteration and consequent stimulation of cytokine or chemokine secretion remain poorly understood. However, as described earlier, Kupffer cells are potent sources of chemokines and one possible mechanism is that the presence of acetaldehyde or hydroxyethyl-modified proteins on the membrane of hepatocytes may, in the presence of other co-stimulatory factors, engage Kupffer cells and trigger an immune reaction that could lead indirectly to release of chemokines and other cytokines. This might also lead to T-cell activation if a subset of memory T-cells were able to recognise altered proteins presented from Kupffer or dendritic cells, and studies in mice have suggested a possible model for this mechanism (Willis 1997). A further possible trigger for the release of chemokines from Kupffer cells may be the presence of increased circulating lipopolysaccharide resulting from enhanced gut permeability induced by alcohol (Persson 1991, Bautista 1997). However, the duration of this enhanced gut permeability is not known and it may be only a transient consequence of alcohol.

As discussed earlier, hepatic stellate cells are also a potent source of chemokines in inflammatory liver disease and the stimuli for release of chemokines from stellate cells may be similar to those operating for Kupffer cells, although stellate cells are not antigen presenting cells. It thus seems more likely that induction of chemokine secretion from these cells will result from soluble factors derived from either primary hepatocytes or Kupffer cells, along with perhaps a direct effect of ethanol-induced altered redox state.

Summary

Alcoholic liver disease is characterised by activation of the immune system including infiltration of the liver with leucocytes, and this is probably results directly or indirectly from the presence of reactive ethanol metabolites. Chemokines are of profound importance in regulating leucocyte recruitment and activation in inflammatory diseases. It is therefore likely that chemokine expression will play a crucial role in the pathogenesis of alcoholic liver disease.

Hypothesis

Chemokine expression is increased in alcoholic liver disease and the patterns of chemokine expression determine the inflammatory response to alcohol in this disease. Furthermore, intrinsic or extrinsic factors (other than alcohol) that influence chemokine expression may alter the risk of and natural history of alcoholic liver disease.

Aims of this Thesis

- To characterise the expression of alpha and beta chemokines in alcoholic liver disease.
- To determine the cellular source of chemokines within the liver.
- To measure circulating levels of the beta chemokines MCP-1 and MIP-1 α in relation to disease severity in alcoholic liver disease.
- To investigate directly the role of alcohol upon chemokine secretion.
- To investigate a possible role for pentoxifylline, an immunomodulatory agent, in alcoholic liver disease.
- To investigate the possibility that genetic variation in the immune response may influence risk of developing alcoholic liver disease.

Chapter 2

Hepatic Chemokine Expression in Alcoholic Liver Disease

INTRODUCTION

The development of alcohol-induced liver injury is likely to be determined, in part, by the immunological/inflammatory response that alcohol stimulates (Paronetto 1992). Alcoholic hepatitis is characterised by the presence of neutrophils amongst leucocytes infiltrating the hepatic parenchyma, whereas in alcoholic cirrhosis the leucocytic infiltration is with mononuclear cells only (International Group 1981). Although alcoholic hepatitis is frequently associated with cirrhosis, it can present as a distinct clinical syndrome that, in its most extreme form, causes liver failure and has a high mortality (Maddrey 1987). Whilst the reasons for these different responses to alcohol are not known, factors that regulate leucocyte recruitment to the liver will be crucial in determining whether patients develop acute inflammation, hepatocyte damage and alcoholic hepatitis or a chronic response with cirrhosis. Chemokines play an integral role in determining the nature of the leucocyte recruitment to sites of tissue injury. Thus the composition, distribution and duration of an inflammatory infiltrate in alcoholic liver disease are likely to depend to some extent upon the combination of chemokines present in the local microenvironment (Adams 1997).

It has been suggested that alpha chemokines are important in the recruitment of neutrophils in alcoholic hepatitis because circulating and tissue levels of IL-8 and GRO α , are elevated in acute alcoholic hepatitis (Hill 1993, Maltby 1996) and some of the histological features of alcoholic hepatitis can be reproduced in rats by transfecting hepatocytes *in vivo* with an IL-8 gene vector (Maher 1996). These studies focussed upon the role of alpha chemokines, but the expression of the beta chemokines may be equally important as these are potent chemotactic factors for monocytes and lymphocytes, and likely to be involved in establishing the chronic inflammatory response to alcohol. Furthermore, little is known about either the local distribution of chemokines or the cells responsible for their synthesis in alcoholic liver disease. It is against this background that we investigated the expression of four chemokines MCP-1, MIP1- α , MIP-1 β and IL-8 in patients with alcoholic liver disease. The aim of this study was determine whether chemokine expression is increased in alcoholic liver disease, and whether particular patterns of chemokine expression reflect the different manifestations of the disease.

METHODS

Patients

(a) Patients with histologically proven alcoholic hepatitis, who had undergone transjugular liver biopsy (N=6). Surplus biopsy material was used for this study after informed consent had been obtained from each patient. All patients had clinically severe alcoholic hepatitis and a discriminant function value > 32 (Maddrey 1978; see page 44). Four of the six patients had histological features of cirrhosis in addition to alcoholic hepatitis and the remaining 2 patients had mild-moderate fibrosis.

(b) Patients undergoing liver transplantation for end stage liver failure due to alcoholic cirrhosis (N=10). Material from the hepatectomy specimen was used for the study; all hepatectomy specimens had characteristic histological features of cirrhosis and six of these had additional features consistent with an alcoholic aetiology (i.e. Mallory's hyaline and / or ballooning degeneration of hepatocytes).

(c) Previously healthy organ donors whose liver was being used for transplant purposes (N=6). Tissue was obtained from surgical liver reduction where the liver was being used for paediatric transplantation. Liver disease in the donor liver was excluded biochemically and histologically.

Specimen processing

Tissue specimens were divided and either snap frozen in liquid nitrogen for immunohistochemistry, or fixed in 4% paraformaldehyde and prepared for in situ hybridisation as described in appendix 3. Specimens were stored at -70°C prior to analysis.

Immunohistochemistry

Immunohistochemistry was performed on snap frozen tissues as described previously by Adams (1991) and summarised in appendix 1, using polyclonal rabbit antibodies specific for IL-8, MIP-1 α , MIP-1 β , MCP-1 and TNF- α , and a monoclonal antihuman fibroblast antibody. In 3 patients with alcoholic hepatitis, 2 with cirrhosis and 2 normal donor livers,

dual staining was carried out with antibodies to TNF- α as well as anti-chemokine antibodies.

Localisation of chemokine mRNA by in situ hybridisation

In situ mRNA hybridisation was done using paraformaldehyde fixed tissue as described previously by our laboratory (Adams 1996), and summarised in appendix 2.

Histological assessment and scoring

Sections were assessed blind using a semi-quantitative system that our laboratory had previously validated by confocal microscopy (Adams 1989). Staining was scored according to intensity and distribution on a five-point scale from negative to +++++, where negative = no staining or positive hybridisation relative to control sections, + = weak positivity, and +++++ = maximum positivity. The following structures were assessed: Portal vessels (hepatic arterioles and portal venules); hepatic venules; sinusoidal cells (Kupffer cells and sinusoidal endothelium); bile ducts, hepatocytes and infiltrating leucocytes.

Measurement of chemokine mRNA by RNase protection assays

Quantitative assessment of chemokine mRNA for MIP-1 α and β was carried out using an RNase protection assay summarised in appendix 3.

RESULTS

Chemokine expression in non-diseased liver

Immunohistochemistry demonstrated that MIP-1 α , MIP-1 β , MCP-1 and IL-8 chemokine protein was present in non-diseased liver tissue. Staining was weak and confined to the vascular endothelium, sub endothelial stroma and occasional inflammatory cells (figures 2.1a and 2.1b and table 2.1), whilst hepatocytes were negative. In situ hybridisation localised chemokine mRNA to occasional inflammatory cells and areas surrounding the vascular endothelium, indicating that low level chemokine gene transcription was occurring even in the absence of active inflammation (figures 2.1c and 2.1d and table 2.1).

Chemokine protein and mRNA expression in alcoholic liver disease

Alcoholic hepatitis: All four chemokine proteins examined were detected in ballooned hepatocytes at sites of active inflammation in severe alcoholic hepatitis. Variable staining ranging from moderate to intense was also observed in vascular and sinusoidal endothelium, intrahepatic bile ducts and inflammatory cells (figure 2.2a and table 2.1). In situ hybridisation demonstrated greatly increased chemokine transcription in areas of inflammation, where positivity could be seen in inflammatory cells, vascular endothelium and sinusoidal lining cells, particularly in areas of pericellular fibrosis (figure 2.2c and table 2.1). Intrahepatic bile ducts did not show evidence of chemokine transcription. The cellular distribution of chemokine mRNA was similar for all chemokines and was associated with areas of active fibrosis as determined by immunohistochemical staining for fibroblasts (figures 2.2e and 2.2f).

Alcoholic cirrhosis: Chemokine proteins were detected in alcoholic cirrhosis without active hepatitis. All chemokines were detected in inflammatory cells, intrahepatic bile ducts, vascular endothelium and, in particular, within fibrous septa. Unlike alcoholic hepatitis, no chemokine protein was detected in either hepatocytes or sinusoids (figure 2.2b and table 2.1). mRNA for each of the chemokines studied was present throughout the fibrous septa, with MCP-1 and IL-8 transcripts also detected in vascular endothelium and vessel walls (table 2.1). As with alcoholic hepatitis, mRNA expression was strongly

associated with the presence of fibroblasts, particularly at the interface of septa and adjacent liver (figure 2.2f).

Expression of chemokine and TNF- α in alcoholic liver disease

In normal liver TNF- α protein was restricted to vascular endothelium, occasional inflammatory cells and sinusoidal lining cells, and staining was generally weak. In alcoholic cirrhosis a similar pattern was observed but the staining was stronger. In alcoholic hepatitis there was strong staining in vascular endothelium, sinusoidal lining cells and intense staining associated with inflammatory infiltrates in the parenchyma (figures 2.3a and 2.3b). Dual staining revealed co-localisation of chemokines and TNF- α to areas of active inflammation (figures 2.3c and 2.3d).

Detection of chemokine mRNA by RNase protection assays

The RNase protection assay confirmed that chemokines were present in normal and diseased tissue. Expression was low in normal tissue. In cirrhosis, MIP-1 β was modestly increased although increase in MIP-1 α was less convincing, whilst in alcoholic hepatitis expression of both MIP-1 α and MIP-1 β was increased at least 2-3 fold. These data are summarised in figure 2.4.

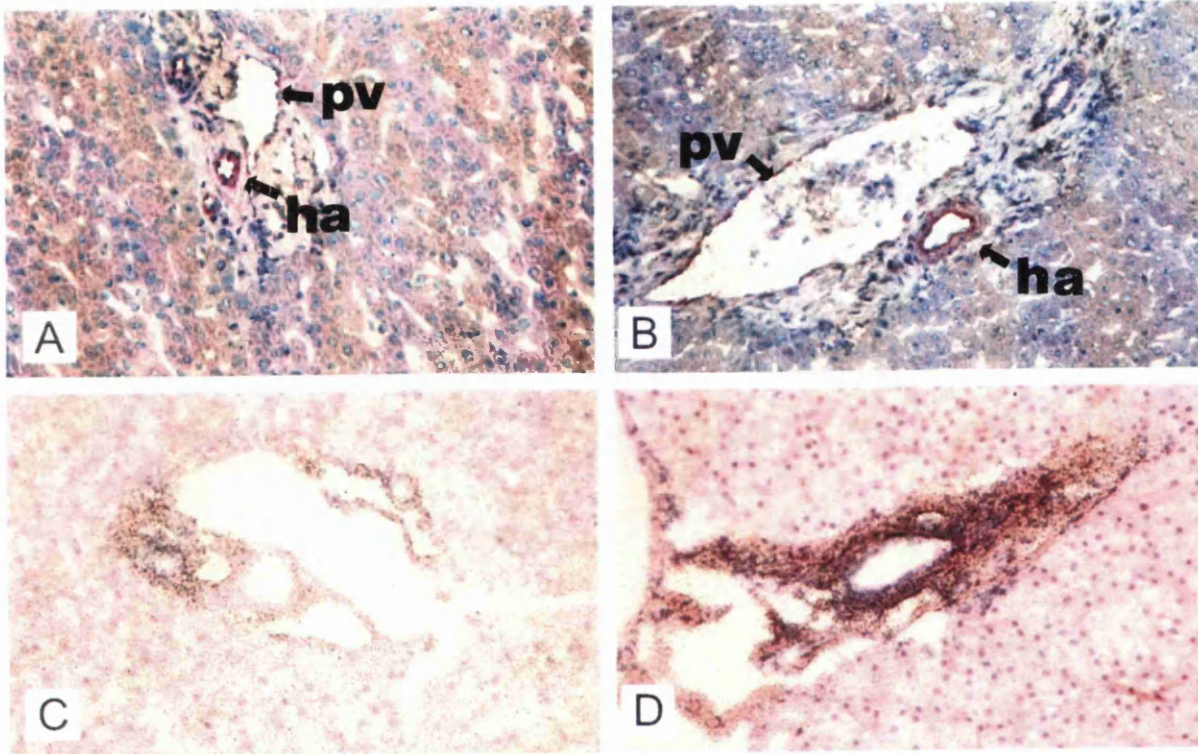


Figure 2.1 Localisation of chemokine protein and mRNA in non-inflamed liver tissue from organ donors.

MCP 1 (panel A) and IL-8 (panel B) chemokine proteins were detected by immunohistochemistry in normal human liver tissue in portal veins (marked PV), hepatic arterioles (HA) and occasional inflammatory cells.

Panels C and D show MCP 1 and IL-8 mRNA expression respectively in normal tissue. Positive hybridisation was confined to perivascular areas (portal tracts and central vein).

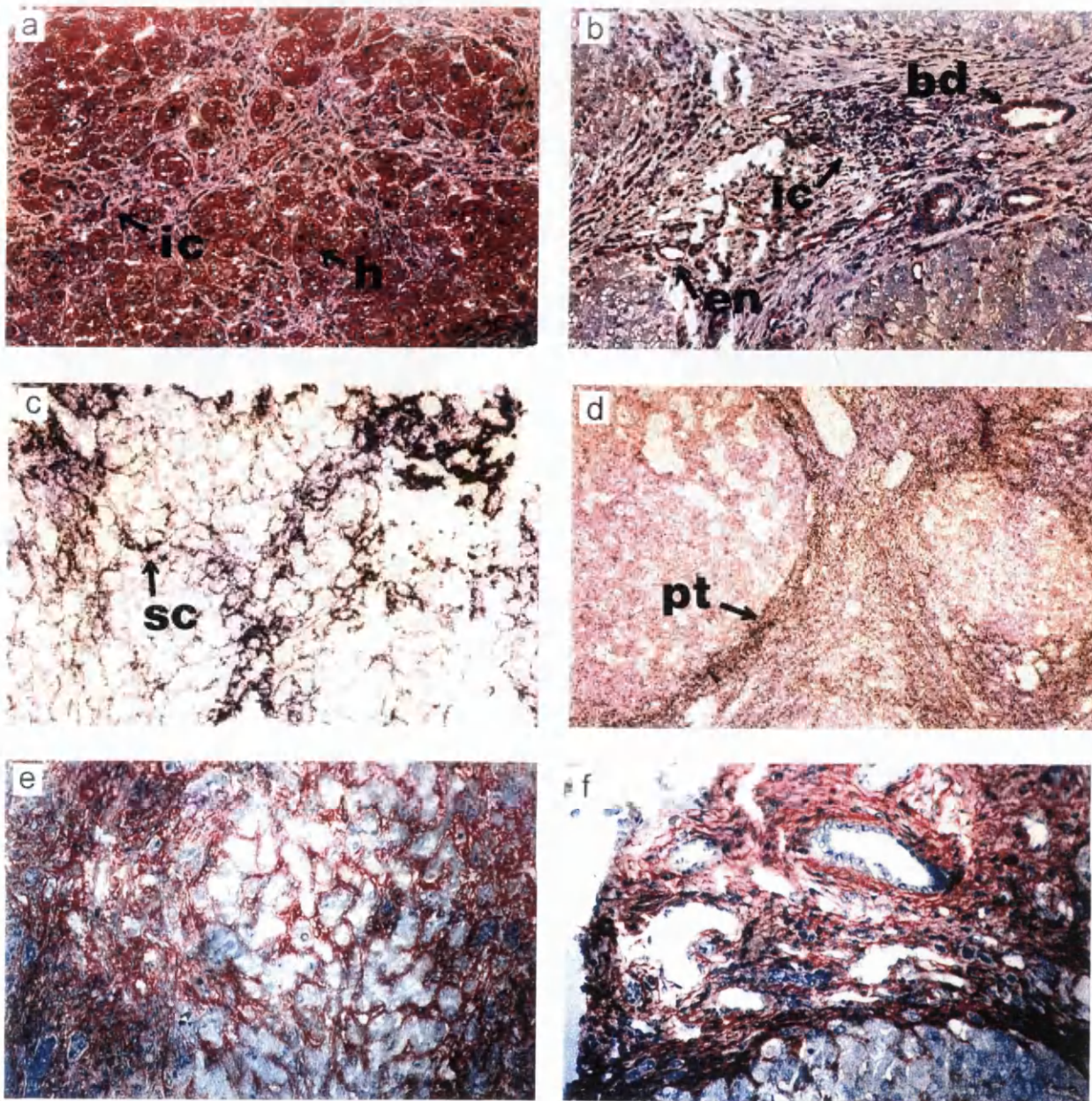


Figure 2.2 Chemokine protein and mRNA expression in alcoholic hepatitis and alcoholic cirrhosis.

The left-hand panels (a,c,e) show alcoholic hepatitis and the right hand panels (b,d,f) show alcoholic cirrhosis for direct comparison. Panel (a) and (b) show immunohistochemical staining for IL-8 chemokine protein. In alcoholic hepatitis (a) there is strong staining in hepatocytes (marked **h**) and inflammatory cells (marked **ic**) whereas in alcoholic cirrhosis (b) staining is confined to portal tract endothelium (marked **en**), intrahepatic bile ducts (marked **bd**) and inflammatory cells (marked **ic**) in portal tracts and peri-septal areas. Panels (c) and (d) show IL-8 and MIP-1 β mRNA localisation (representative results, other chemokines were similar). In alcoholic hepatitis mRNA is expressed in portal tracts and sinusoidal cells (marked **sc**) within the parenchyma whereas in alcoholic cirrhosis expression is confined to portal tracts (marked **pt**) and septal areas. Panels (e) and (f) show immunohistochemical staining for fibroblasts in patients with alcoholic hepatitis (e) and alcoholic cirrhosis (f) respectively.

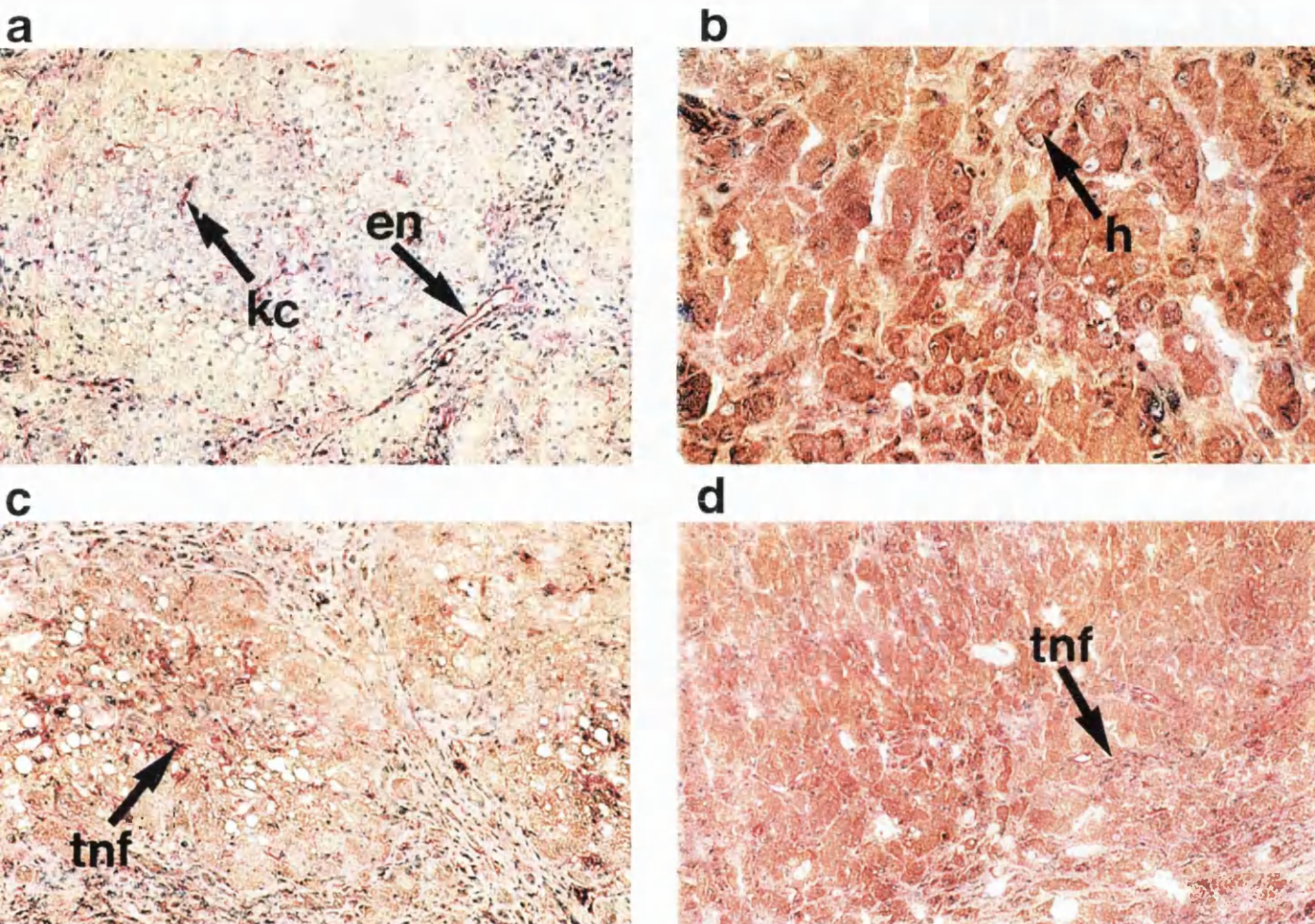
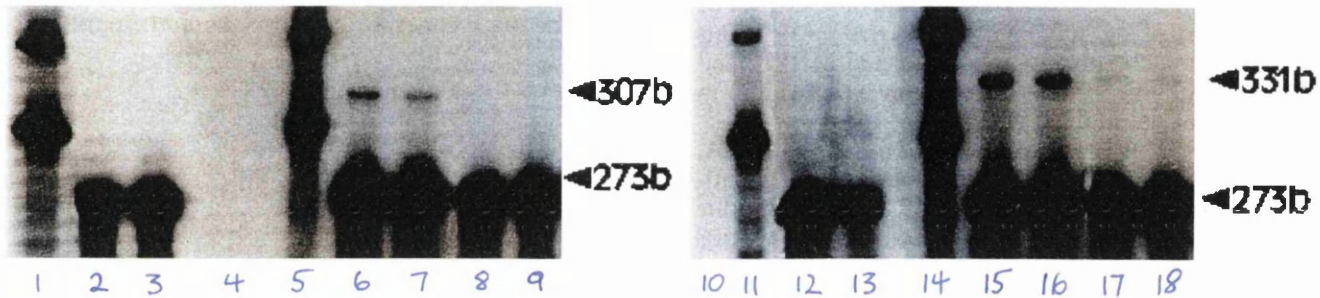


Figure 2.3 Immunolocalisation of TNF- α and chemokine protein in alcoholic hepatitis.

Panel (a) shows immunolocalisation of TNF- α using an alkaline phosphatase/fast red substrate in a patient with alcoholic hepatitis and cirrhosis demonstrating positive staining in vascular endothelium (marked **en**) and areas of inflammation in periseptal regions and within Kupffer cells in hepatic sinusoids in areas of steatohepatitis (marked **kc**). Panel (b) shows immunolocalisation of the chemokine MIP-1 β using a horseradish peroxidase/DAB substrate in severe alcoholic hepatitis. There is strong expression of chemokine protein in ballooned hepatocytes (marked **h**). Panels (c) and (d) show dual immunostaining for TNF- α (red/pink stain) and MIP-1 α (widespread parenchymal brown stain) confirming that both cytokines are present in areas of inflammation and tissue damage.

Figure 2.4 RNAase protection gel showing MIP-1 α and β mRNA expression in normal, alcoholic hepatitis and alcoholic cirrhosis tissue extracts respectively.

Gel track 1-9: MIP-1 α and β actin (protected fragment sizes 307 and 273 bases respectively) Gel track 10-18: MIP-1 β and β -actin (protected fragment sizes 331 and 273 bases respectively)



Wells

- 1 Control non hybridised MIP-1 α and β -actin probe mixture.
- 2-3 Probes hybridised with RNA from normal non inflamed liver tissue.
- 4 Control probe cocktail pre-digested with RNase.
- 5 Control non-hybridised MIP-1 α and β -actin probe mixture.
- 6-7 Probes hybridised with RNA from severe alcoholic hepatitis.
- 8-9 Probes hybridised with RNA from end stage alcoholic cirrhosis.
- 10 Control MIP-1 β and β -actin probe cocktail predigested with RNase.
- 11 Control non-hybridised MIP-1 β and β actin probe mixture.
- 12-13 Probes hybridised with RNA from normal non inflamed liver tissue.
- 14 Control non-hybridised MIP-1 β and β actin probe mixture.
- 15-16 Probes hybridised with RNA from severe alcoholic hepatitis.
- 17-18 Probes hybridised with RNA from end stage alcoholic cirrhosis.

| Chemokine | Distribution | Protein | | | mRNA | | |
|--|---------------------|----------------|----------------|-----------------|---------------|----------------|-----------------|
| | | Normal | Alc hep | Alc cirr | Normal | Alc hep | Alc cirr |
| <u>MIP-1α</u> | inflam.cells | + / ++ | +++ | +++ | + | +++ | - |
| | sinusoidal cells | - | +++ | - | - | +++ | - |
| | biliary epithelium | - | +++ | +++ | - | - | - |
| | vasc. endothelium | + / ++ | +++ | +++ | - | +++ | - |
| | hepatocytes | - | +++ | - | - | - | - |
| | fibrous septa | np | +++ | +++ | np | +++ | +++ |
| <u>MIP-1β</u> | inflam.cells | + / ++ | +++ | +++ | + | +++ | - |
| | sinusoidal cells | - | +++ | - | - | +++ | - |
| | biliary epithelium | - | +++ | +++ | - | - | - |
| | vasc. endothelium | + / ++ | +++ | +++ | - | +++ | - |
| | hepatocytes | - | +++ | - | - | - | - |
| | fibrous septa | np | +++ | +++ | np | +++ | +++ |
| <u>MCP 1</u> | inflam.cells | + / ++ | +++ | +++ | ++ | +++ | - |
| | sinusoidal cells | - | +++ | - | - | +++ | - |
| | biliary epithelium | - | +++ | +++ | - | - | - |
| | vasc. endothelium | + / ++ | +++ | +++ | ++ | +++ | +++ |
| | hepatocytes | - | +++ | - | - | - | - |
| | fibrous septa | np | +++ | +++ | np | +++ | +++ |
| <u>IL8</u> | inflam.cells | + / - | +++ | ++ | ++ | +++ | - |
| | sinusoidal cells | + | +++ | + / - | - | +++ | - |
| | biliary epithelium | + / - | +++ | + / - | - | - | - |
| | vasc. endothelium | ++ | ++++ | +++ | ++ | +++ | +++ |
| | hepatocytes | + / - | +++ | - | - | - | - |
| | fibrous septa | np | +++ | + / - | np | +++ | +++ |
| <u>TNF-α</u> | inflam.cells | + / - | +++ | ++ | | | |
| | sinusoidal cells | + / - | +++ | ++ | | | |
| | biliary epithelium | - | + | + / - | | | |
| | vasc. endothelium | + / - | +++ | ++ | | | |
| | hepatocytes | - | + | - | | | |
| | fibrous septa | - | + | + | | | |

Table 2.1 Chemokine protein and mRNA expression in normal liver, alcoholic hepatitis and alcoholic cirrhosis (TNF- α expression is also summarised for comparison). Sections were scored according to standard criteria (see methods). np = not present.

DISCUSSION

The findings of this study suggest that locally secreted chemokines are important factors in determining the nature and distribution of the inflammatory response to alcohol. Chemokines expressed in the sinusoids in alcoholic hepatitis would promote the recruitment of neutrophils, monocytes and lymphocytes to the parenchyma, whereas chemokines expressed in portal areas and fibrous septa during chronic inflammatory responses of alcoholic cirrhosis would promote recruitment of inflammatory cells at that location. Our findings also suggest that the presence of chemokine protein alone is insufficient for leucocyte recruitment *in vivo* because IL-8, which acts predominantly on neutrophils, was detected in normal liver and in alcoholic cirrhosis in the absence of a neutrophil infiltrate. This may indicate that insufficient IL-8 was present for detectable neutrophil infiltration but also suggests that other factors in addition to chemokines are probably required for leucocyte migration into tissues, including sufficient expression of particular endothelial adhesion molecules (Burra 1992, Adams 1994a).

Chemokine mRNA was expressed not only by infiltrating leucocytes but also by liver endothelium and sinusoidal cells, implying that the liver itself is involved in the regulation of leukocyte recruitment in alcohol induced liver injury. Many cell types have been shown to produce MCP-1 and IL-8 when stimulated *in vitro* including biliary epithelial cells, hepatocytes and sinusoidal endothelium, and these cells may all contribute to the local levels of chemokines within the liver (McNab 1996, Morland 1997). In severe alcoholic hepatitis, but not cirrhosis, IL-8 mRNA was expressed strongly throughout the sinusoids and lobular regions in both inflammatory and sinusoidal cells. Other studies have also shown that hepatocytes secrete chemotactic factors in response to alcohol and its metabolites, implying a central role for hepatocyte derived factors in generating the inflammatory response of alcoholic hepatitis (Maher 1995). The contribution made by hepatocytes to chemokine synthesis in humans is not known but our results suggest they contribute relatively little because, despite the presence of all four chemokine proteins in ballooned hepatocytes, no chemokine mRNA was detected. This finding suggests that hepatocytes take up or sequester chemokines synthesised and secreted by other cell types,

a process that could increase the local concentration of chemokines in the parenchyma in alcoholic hepatitis. A similar process for chemokine sequestration in endothelial cells has been proposed and confirmed; the the binding of chemokine glycosaminoglycan-binding sites to proteoglycans in the endothelial glycocalyx followed by endothelial intracellular sequestration allows chemokines to be retained at sites of inflammation *in vivo* (Tanaka 1993, Witt 1994 , Middleton 1997). This mechanism could help explain why we detected MIP-1 α and MIP-1 β chemokine protein on vascular endothelium in the absence of detectable mRNA.

Activated stellate cells or fibroblasts may be particularly important contributors to chemokine secretion in alcoholic liver disease because we found a striking co-localisation of chemokine mRNA with the distribution of fibroblasts in both alcoholic cirrhosis and alcoholic hepatitis. Smith and co-workers have proposed a role for chemokines in fibrogenesis based on their observations that the administration of antibodies to MIP-1 α decreases cellular infiltration and fibrosis in an animal model of interstitial lung fibrosis (Smith 1995). Fibrogenesis involves complex interactions between leucocytes, fibroblasts and sinusoidal cells and chemokines are likely to play a central role in this process by recruiting and maintaining inflammatory cells at sites of active fibrosis (Adams 1994b, Marra 1998).

In normal tissue, all four chemokine proteins were detected on endothelium and in inflammatory cells around blood vessels. These observations suggest that low level chemokine secretion occurs in normal liver and could be important for the regulation of leukocyte recruitment during physiological immune surveillance. Alternatively, because the “normal” liver came from organ donors who probably had an acute systemic inflammatory response by the nature of their illness, it is possible that the expression of chemokines in these patients reflected the presence of cytokines (and possibly endotoxin) in the portal blood. In normal liver IL-8 and MCP-1 mRNA were detected in vascular but not sinusoidal endothelium. Previous studies have reported differences in adhesion molecules between sinusoidal and vascular endothelium (Steinhoff 1993) and the observations reported here provide further evidence that sinusoidal endothelium differs from portal and hepatic vascular endothelium.

Because in situ hybridisation cannot quantify mRNA, we used RNAase protection assays to provide a semi-quantitative assessment of chemokine mRNA. Chemokine mRNA was markedly elevated in alcoholic hepatitis compared with cirrhosis, consistent with the increased inflammatory damage in alcoholic hepatitis. It has been proposed that increased production of TNF- α stimulates the inflammatory response in alcoholic hepatitis and TNF- α is a potent stimulus for chemokine release from several cell types in vitro including Kupffer cells and human biliary epithelial cells (Morland 1997, Ohkubo 1998). Although increased secretion of TNF- α by blood monocytes has been reported in alcoholic hepatitis, the cellular origin of TNF- α within the liver is not known (McClain 1993). We demonstrated co-localisation of chemokine and TNF- α proteins in areas of inflammation in alcoholic hepatitis suggesting the existence of a paracrine network in which TNF- α , produced by inflammatory cells, stimulates local secretion of chemokines in alcoholic hepatitis.

The findings of our study imply that leucocyte recruitment to the parenchyma and portal tracts are controlled by different mechanisms. The expression of MCP-1 on portal vascular endothelium could promote the influx of monocytes and lymphocytes into portal tracts, which, in the presence of other appropriate stimuli, activate fibrogenesis. In contrast, infiltration of the parenchyma, which is seen in alcoholic hepatitis, requires a more potent inflammatory stimulus to induce chemokine secretion from sinusoidal cells. This could be provided by high local levels of TNF- α (and possibly other pro-inflammatory cytokines) but may also result from the effect of alcohol and/ or its metabolites directly. Studies illustrated later in this thesis will examine this phenomenon further.

Chapter 3

Serum Levels and Peripheral Secretion of the Beta-Chemokines MCP-1 and MIP-1 α in Alcoholic Liver Disease

INTRODUCTION

The studies illustrated in chapter 2, along with those of other workers, showed that chemokine expression correlates with clinical disease severity and histological inflammatory activity within the liver in alcoholic and other inflammatory liver diseases (Maltby 1996, Marra 1998). Although chemokines are localised to sites of inflammation by binding to proteoglycans in the glycocalyx (Tanaka 1993) they can also be detected in the circulation, and serum levels of interleukin-8 (IL-8), the prototype alpha chemokine, correlate closely with clinical, histological and laboratory indicators of severity in alcoholic hepatitis (Hill 1993, Masumoto 1993, Sheron 1993, Huang 1996). However, alcoholic hepatitis and active cirrhosis are also associated with a marked mononuclear cell infiltrate including monocytes and lymphocytes and the studies illustrated in chapter 2 showed that hepatic expression of the beta chemokines MCP-1, MIP-1 α and MIP-1 β correlates with intensity of infiltration with these mononuclear cells in alcoholic liver disease. Serum levels of beta chemokines have not been studied in inflammatory liver diseases, although MCP-1 levels are elevated in human sepsis (Bossink 1995). For these reasons we studied circulating levels and peripheral secretion of MCP-1 and MIP-1 α in alcoholic liver disease. The aims of this study were to determine (a) whether levels of chemokines in the peripheral circulation reflect disease activity and (b) whether chemokine secretion is restricted to the liver or is part of a systemic inflammatory response.

PATIENTS AND METHODS

Patients

We studied 51 patients with biopsy-proven alcoholic liver disease. All patients had consumed around or more than 80g alcohol daily for more than 10 years and other potential contributing causes of liver disease had been carefully excluded by comprehensive clinical, radiological and serological analysis. Patients were managed with nutritional supplementation but not corticosteroids or other immunosuppressives. Patients with active sepsis (i.e. microbiologically proven or clinically suspected) or recent gastrointestinal haemorrhage (i.e. within 5 days) were excluded from the study. Patients were subdivided using histological criteria into those with severe alcoholic hepatitis (ballooning hepatocyte degeneration, Mallory's hyaline and dense leucocytic infiltration including neutrophils, N=24), mild-moderate alcoholic hepatitis (with ballooning hepatocyte degeneration and Mallory's hyaline and absent or light leucocyte infiltration but no neutrophils, N=15) and inactive cirrhosis (without histological stigmata of alcoholic hepatitis, N=12). The majority of patients with alcoholic hepatitis (18/24 with severe and 11/15 with mild-moderate hepatitis) also had cirrhosis, and all remaining patients had fibrosis of at least mild to moderate severity.

In addition to histological features of disease we recorded routine clinical and laboratory data for each patient; these are summarised in table 3.1. For comparison we also studied patients with acute seronegative hepatitis (N=8), fulminant liver failure due to paracetamol poisoning (N=11), and healthy volunteers (N=12).

Serum sampling

Serum samples obtained from peripheral venous blood were analysed for all subjects. Some of the patients with alcoholic liver disease and coagulopathy underwent transjugular biopsy for clinical indications and in these patients we took the opportunity to sample blood from the hepatic vein. In a further 5 patients with alcoholic liver disease, transjugular intrahepatic portosystemic shunts (TIPSS) were inserted radiologically for control of ascites or because of previous problematic variceal haemorrhage; in these patients we took blood samples from peripheral, hepatic and portal veins. Blood was

collected into tubes containing clot activator and serum separated within 30 minutes by centrifugation and stored at -70°C prior to analysis.

Peripheral blood mononuclear cell (PBMC) chemokine secretion

MCP-1 and MIP-1 α secretion from cultured PBMC's was measured in selected patients with severe alcoholic hepatitis (N=12) and healthy controls (N=12); isolation and culture of PBMC's is described in appendix 5. Supernatants were collected by aspiration and purified by centrifugation, then stored in aliquots at -70°C prior to analysis.

Chemokine measurement

MIP-1 α and MCP-1 levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. For measurement of PBMC secretion of MCP-1 we developed a customised ELISA which is described in appendix 6.

Analysis for PBMC chemokine mRNA

Chemokine mRNA in peripheral blood leukocytes was determined by in situ hybridisation of cytopsin preparations of PBMC's isolated as described above. Freshly isolated PBMCs were resuspended and mounted onto poly-L-lysine coated slides, fixed in 1% paraformaldehyde and stored at -70°C prior to analysis. Chemokine mRNA hybridisation was done with a probe for MIP-1 α using the technique described in appendix 3.

Statistical analysis. Tests for non-parametric data (Mann Whitney U test, Wilcoxon Rank Sum and multivariate linear regression) were done using SPSS statistical software. A p value of <0.05 was considered significant. This study was approved by the Research Ethics Committee of the University Hospital Birmingham NHS Trust and patients gave informed consent before participation in the study.

RESULTS

Serum chemokine levels in alcoholic liver disease

Peripheral vein MCP-1 levels (pg/ml, mean \pm SD) were higher in alcoholic hepatitis (severe, 640 \pm 285; mild 435 \pm 110) compared to cirrhosis (355 \pm 105) or healthy controls (325 \pm 120) (severe alcoholic hepatitis vs. healthy controls, $p < 0.01$; severe alcoholic hepatitis vs. inactive cirrhosis, $p < 0.01$; mild alcoholic hepatitis vs. healthy controls, difference not significant, Mann-Whitney U-test) (figure 3.1a). MCP-1 levels showed significant positive correlations with serum AST ($p < 0.01$), serum creatinine ($p < 0.01$) and serum bilirubin ($p < 0.01$), and a significant negative correlation with serum albumin ($p < 0.05$) (table 3.2). Linear regression analysis showed that serum AST ($p < 0.01$) and creatinine ($p < 0.05$) were independently associated with MCP-1 levels. There was a non-significant trend towards higher MCP-1 levels in non-survivors compared to survivors (mean levels 763 and 565 ng/ml respectively, $p = 0.12$, Mann-Whitney U-test). In the alcoholic hepatitis group, there was no difference in MCP-1 levels between those with and those without cirrhosis (mean levels 654 and 678 pg/ml respectively, difference not significant).

Peripheral vein MIP-1 α levels were below the lower limit of detection of the assay (i.e. < 46 pg/ml) in all healthy controls and in all disease patients tested (N=32) except one.

Other disease groups

Serum levels of MCP-1 were also raised in fulminant liver failure due to paracetamol poisoning (2380 \pm 1355 pg/ml) and in seronegative hepatitis (1245 \pm 1425) pg/ml), confirming that MCP-1 secretion is also a feature of other inflammatory liver disease (figure 3.1b). In the group with seronegative hepatitis the highest values were recorded in two patients who developed encephalopathy. MIP-1 α levels were below the lower limit of sensitivity of the assay in all patients with seronegative hepatitis tested (N=8) except one. However, detectable levels were found in 7/ 11 patients with paracetamol poisoning (median level 69 pg/ml, range 51-102).

TABLE 3.1 Summary characteristics of patients with alcoholic liver disease, categorised as severe alcoholic hepatitis (AH), mild/ moderate AH and quiescent cirrhosis as defined using histological criteria (see text). Abbreviations: AST = aspartate transaminase; PT = prothrombin time; PSE = portosystemic encephalopathy (defined where clinically obvious).

| | | <u>Severe AH</u> | <u>Mild/ mod AH</u> | <u>Cirrhosis</u> |
|---|----------------|------------------|---------------------|------------------|
| | | (N=24) | (N=15) | (N=12) |
| <u>AST</u> | median | 64 | 50 | 36 |
| (U/L) | (range) | (21-405) | (20-113) | (13-61) |
| <u>Bilirubin</u> | median | 232 | 100 | 54 |
| (mM/L) | (range) | (45-1050) | (14-313) | (11-550) |
| <u>PT</u> | median | 23 | 21 | 19 |
| (secs) | (range) | (17-43) | (14-39) | (15-30) |
| <u>Discriminant function</u> ^{1 2} | median (range) | 65.2 (29.6-151) | 40.5 (5.6-138) | 28.4 (9.8-111) |
| PSE | | 13 | 4 | 2 |
| Transplanted ³ | | 2 | 3 | 3 |
| Deaths ³ | | 10 | 4 | 2 |

Notes

1. After Maddrey (1978), where discriminant function = [(prolongation in PT x 4.6) plus (serum bilirubin / 17)]. A discriminant function of > 32 is indicative of a risk of mortality of at least 50%.
2. Severe AH vs. mild/ mod AH, p <0.05; severe AH vs. quiescent cirrhosis, p <0.05; mild/ mod AH vs. quiescent cirrhosis, p <0.05 (Mann Whitney U-test).
3. Occurring within 3 months after serum sampling. All deaths were directly related to liver disease.

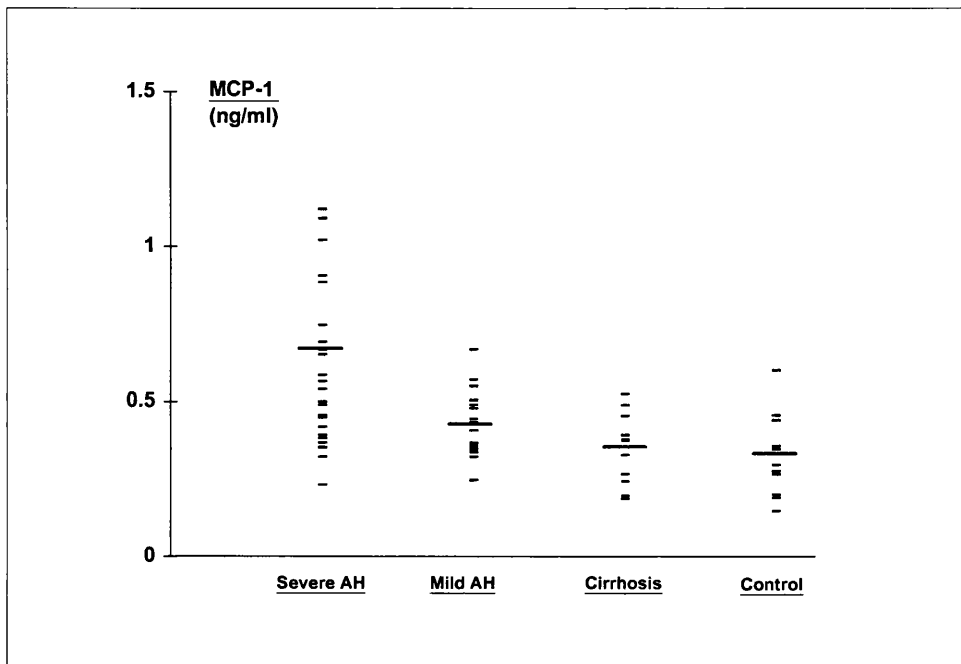


Figure 3.1 (a) Serum MCP-1 levels (ng/ml) in patients with severe alcoholic hepatitis (AH) or mild-moderate AH, inactive alcoholic cirrhosis and controls. Thick horizontal bars represent mean values. Levels in severe AH were significantly higher than cirrhotics or controls ($p < 0.01$, Mann-Whitney U-test).

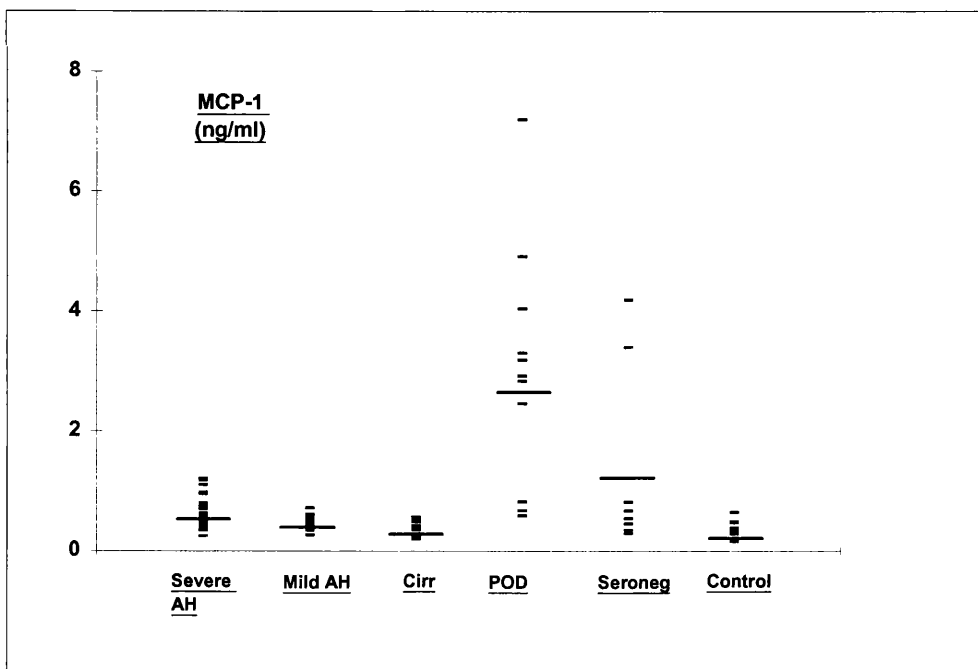


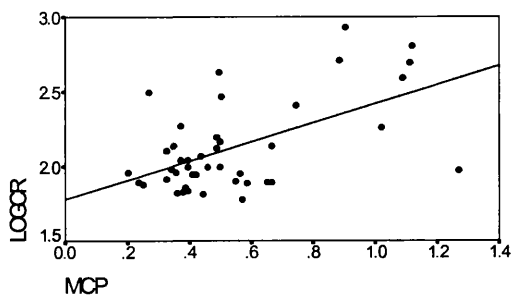
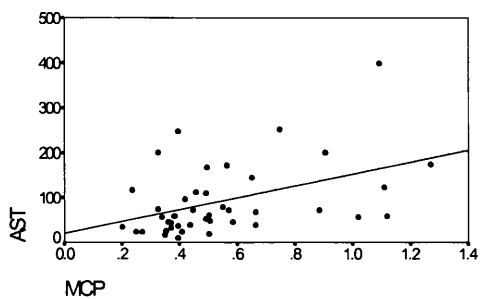
Figure 3.1 (b) Serum MCP-1 levels (ng/ml) in patients with alcoholic liver disease, as for figure 3.1(a), shown on a larger scale in comparison with patients with paracetamol overdose-induced fulminant liver failure (POD) and acute seronegative hepatitis (seroneg).

TABLE 3.2 Correlation of laboratory indices of disease severity with serum MCP-1 levels in patients with alcoholic liver disease, using Spearman's rank test. Linear regression analysis identified serum AST ($p < 0.01$) and creatinine ($p < 0.05$) as significant independent variables. Scatterplots of AST and log creatinine are shown below.

| <u>Laboratory index</u> | <u>r value</u> | <u>p value</u> |
|----------------------------|----------------|----------------|
| Serum AST | + 0.47 | <0.01 |
| Creatinine ¹ | + 0.36 | <0.01 |
| Bilirubin ¹ | + 0.35 | <0.01 |
| Albumin | - 0.33 | <0.05 |
| Prothrombin time increase | + 0.15 | NS |
| Peripheral leucocyte count | + 0.18 | NS |

Note

1. Values transformed logarithmically for statistical analysis



Peripheral and hepatic vein chemokine levels in alcoholic liver disease

In all the patients with severe alcoholic hepatitis in whom paired hepatic and peripheral veins samples were available, MCP-1 levels were higher in hepatic compared to peripheral venous blood (mean values 647 and 600 pg/ml respectively, $p < 0.01$, Wilcoxon rank sum), whilst in patients with mild to moderate alcoholic hepatitis or inactive cirrhosis the hepatic and peripheral vein levels were similar (mean values 293 and 301 pg/ml respectively, difference not significant). In the 5 patients undergoing TIPSS insertion, the highest MCP-1 levels were found in the hepatic veins, with the lowest levels occurring in the portal veins (mean levels in the hepatic, peripheral and portal veins were 437, 421 and 402 pg/ml respectively; hepatic vein vs. portal vein, $p = 0.07$, Wilcoxon rank sum). Hepatic vein MIP-1 α levels were below the lower limit of detection of the assay (as for peripheral vein levels) in all patients with alcoholic liver disease.

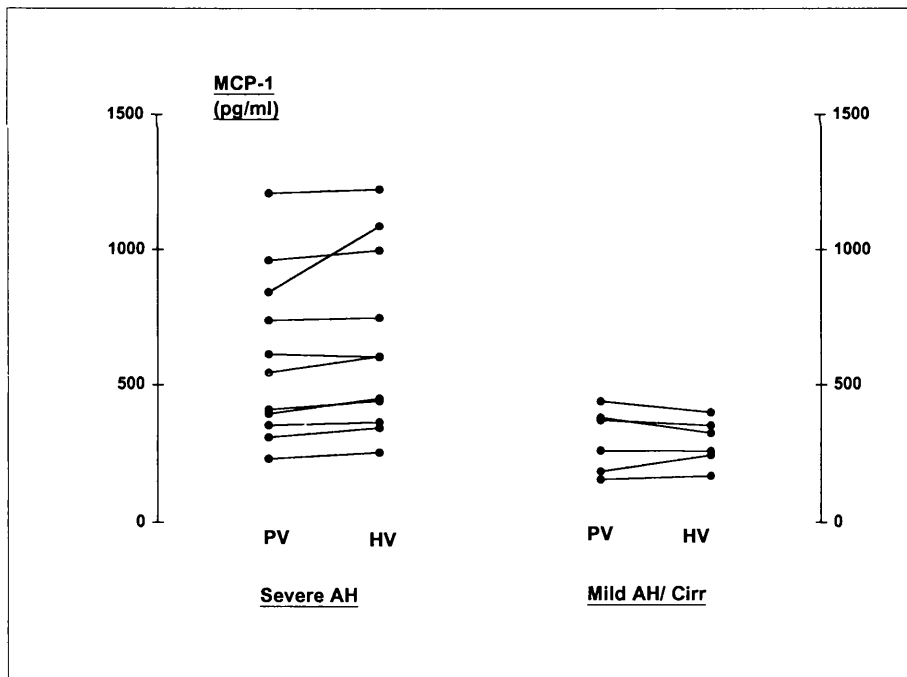


Figure 3.2

Paired hepatic and peripheral vein (HV and PV) MCP-1 levels (pg/ml) in patients with severe alcoholic hepatitis (AH) compared with mild/ moderate hepatitis or cirrhosis. For severe AH patients, HV levels were higher than PV levels ($p < 0.01$, Wilcoxon rank sum).

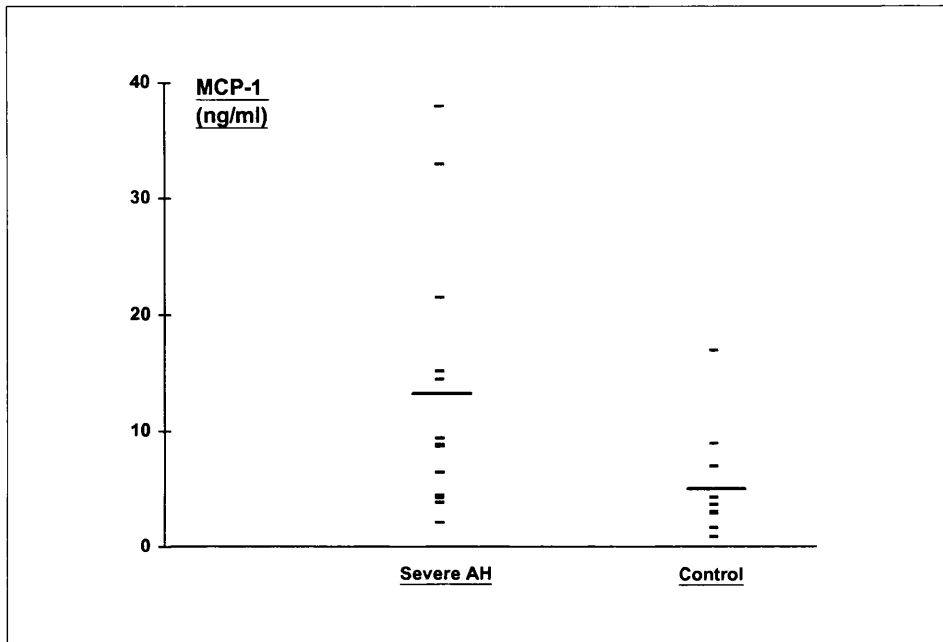
PBMC chemokine secretion

Spontaneous secretion of MCP-1 and MIP-1 α from cultured PBMC's was higher in patients with alcoholic hepatitis compared to healthy controls (figures 3.3a and 3.3b). In alcoholic hepatitis MCP-1 secretion was 13.13 ± 11.34 ng/ml and for controls 5.27 ± 4.77 ng/ml ($p=0.015$, Mann-Whitney U-test); MIP-1 α secretion was 1.98 ± 1.78 ng/ml in alcoholic hepatitis and 0.84 ± 1.10 ng/ml in controls ($p=0.04$, Mann-Whitney U-test). For patients with alcoholic hepatitis there was a significant positive correlation between PBMC secretion of and serum levels of MCP-1 ($r=0.68$, $p<0.05$, Spearman's rank test), and between PBMC secretion of MCP-1 and serum AST ($r=0.60$, $p<0.05$). However, there was no significant correlation between PBMC secretion of MIP-1 α with MCP-1 secretion or serum AST.

Identification of chemokine mRNA

In situ hybridisation of PBMC cytospin preparations using the probe for MIP-1 α mRNA showed positive staining (mainly localised to monocytes) in patients with alcoholic hepatitis (figure 3.4).

(a)



(b)

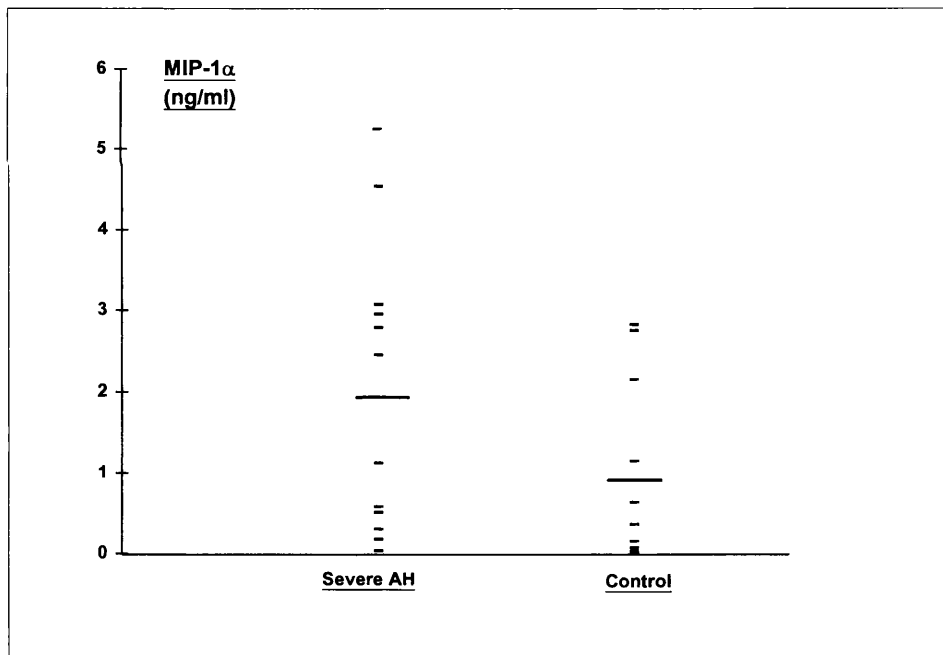
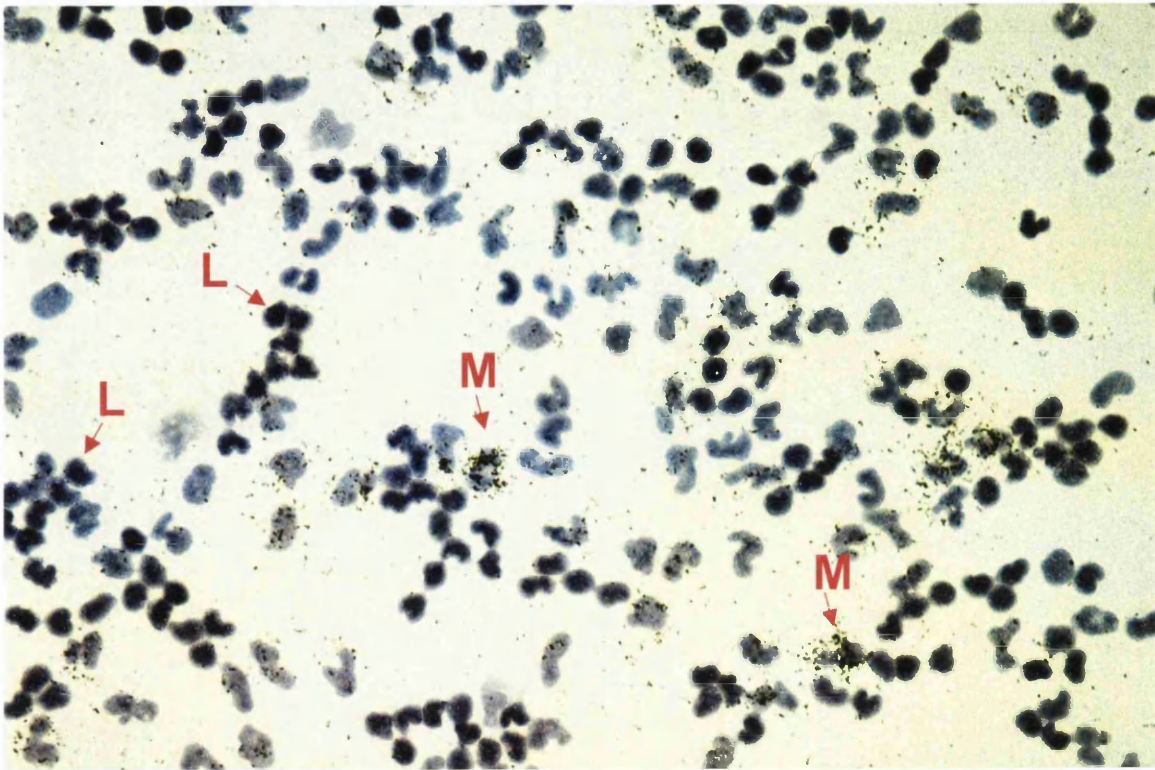


Figure 3.3 Spontaneous PBMC secretion of (a) MCP-1 and (b) MIP-1 α in patients with severe alcoholic hepatitis (AH) and healthy controls. Thick horizontal bars represent mean values. Chemokine secretion was higher in both groups compared to controls ($p < 0.05$, Mann-Whitney U-test).



B

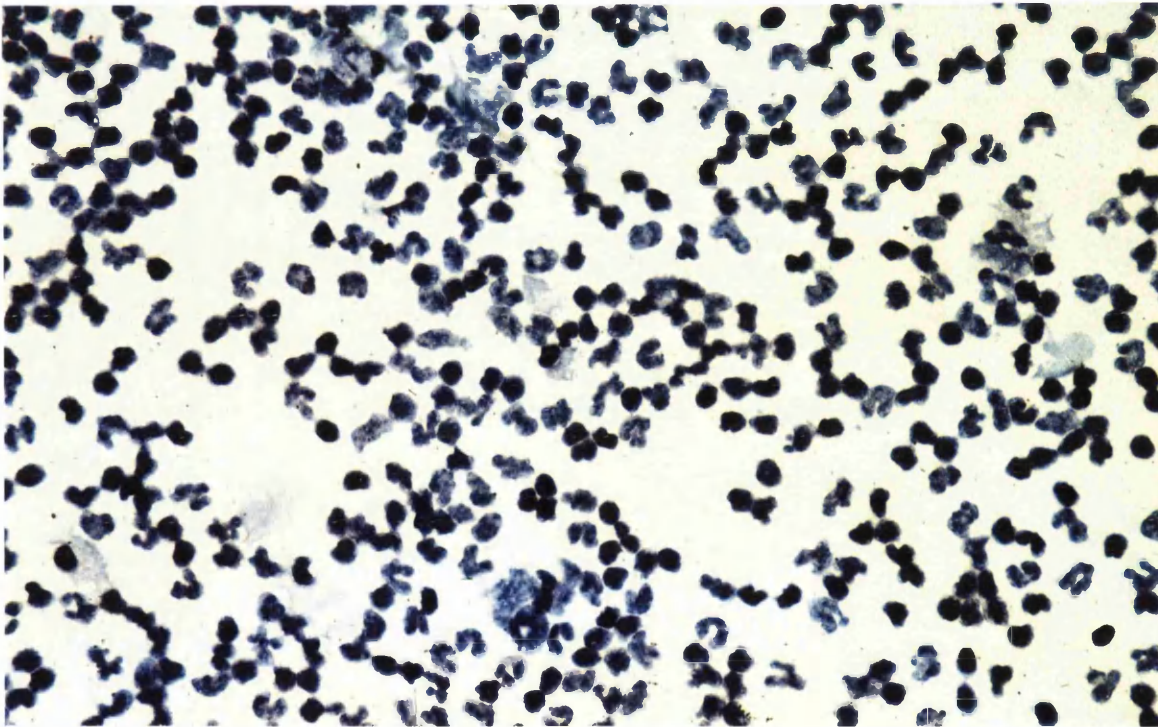


Figure 3.4 PBMC cytospin preparation hybridised with cDNA for MIP-1 α chemokine mRNA (A, antisense probe; B, sense probe). Positive hybridisation is represented by dark granules (arrow), which are seen mostly in association with monocytes (M) whilst lymphocytes (L) are mostly negative.

DISCUSSION

The results of this study suggest that secretion of MCP-1 reflects disease activity in alcoholic liver disease. These findings complement our previous observations of chemokine up-regulation in the liver in alcoholic liver disease and suggest a role for chemokines in recruiting mononuclear cells to the liver following alcohol-induced toxic injury. Previous reports of circulating IL-8 in alcoholic hepatitis emphasised the importance of neutrophils in the pathogenesis of alcoholic hepatitis. However, whilst a neutrophilic infiltrate is the characteristic hallmark of alcoholic hepatitis, the inflammatory infiltrate includes mononuclear cells which play a vital role in inflammation and fibrogenesis (Marra 1998). Thus beta chemokines such as MCP-1 are likely to play a central role in mediating these processes in alcoholic liver disease. We were unable to demonstrate increased serum levels of the beta chemokine MIP-1 α in alcoholic liver disease despite demonstrating enhanced PBMC secretion; it is possible that circulating levels were increased but below the range detectable by the assay (it is noteworthy that PBMC MIP-1 α secretion was quantitatively around fivefold lower than MCP-1 secretion). Furthermore, chemokines released into the circulation are likely to be sequestered to some extent, thus reducing detectable levels, by binding to the Duffy antigen receptor complex. The findings of significantly higher levels of MCP-1 in acute seronegative hepatitis and paracetamol-induced fulminant liver failure, and of detectable levels of MIP-1 α in the latter disease, probably reflect the more intense hepatic inflammation in these diseases. High levels of pro-inflammatory cytokines characterise fulminant liver failure (Sekiyama 1994) and thus high levels of circulating chemokines might also be expected in this disease. These findings may be deserving of further study in themselves.

The significance of raised circulating levels of MCP-1 remains unclear and whilst this may simply reflect increased tissue synthesis of MCP-1, circulating MCP-1 may also have immuno-modulatory effects including increased expression of adhesion molecules on circulating monocytes and promotion of pro-inflammatory cytokine secretion, thus amplifying the inflammatory cascade (Jiang 1992). Furthermore, circulating IL-8 has been shown to enhance neutrophil sequestration into the liver and MCP-1 may play a similar

role in enhancing hepatic uptake of mononuclear cells (van Zee 1992). However, the dynamic effects of a sustained increase in circulating chemokines are not clear and it is possible that persistent ligand occupancy of leucocyte chemokine receptors from circulating chemokines may actually inhibit transendothelial migration (Gimbrone 1989). Our finding of a positive correlation between MCP-1 levels and serum creatinine probably reflects in part the renal excretion of chemokines; by virtue of its relatively small protein size, some excretion of MCP-1 is likely to occur from the kidneys. Measurable amounts are present in the urine of most healthy subjects (data given for 37 healthy subjects in MCP-1 assay handbook, R&D Systems Inc.) and following this study we found high levels (1.34 and 1.36 ng/ml respectively) in the urine of 2 patients with severe alcoholic hepatitis (and without renal failure), corresponding to at least twice the upper limit of normal urinary values. Renal failure is therefore likely to increase circulating MCP-1 levels by a reduction in urinary excretion; however in addition renal failure itself may promote synthesis of MCP-1 and other cytokines including from intrarenal sources (Akahoshi 1995, Abdullah 1997).

Increased secretion of MCP-1 from circulating monocytes, and increased MCP-1 levels in hepatic compared to peripheral (and portal) veins, suggest that MCP-1 synthesis is increased both peripherally and within the liver respectively in severe alcoholic hepatitis. Whilst we found hepatic vein levels to be only modestly higher than peripheral levels, this difference was nevertheless significant and is remarkable in that patients were sampled in a 'steady state' in the absence of any hepatic insult other than the ongoing inflammatory process. The increase in hepatic vein levels therefore suggests synthesis within the liver. These observations support a direct role for MCP-1 in disease pathogenesis, presumably by recruiting and retaining monocytes and activated lymphocytes within the liver in severe alcoholic hepatitis. The fact that circulating monocytes showed increased spontaneous secretion of MCP-1 in alcoholic hepatitis suggests that recruitment of these cells into the liver in response to hepatic MCP-1 would further elevate local levels and act as an amplifying signal to enhance liver inflammation. The circulating monocytes might have been activated within the hepatic circulation during transit through the hepatic sinusoids or directly by soluble circulating factors such as TNF- α or IL-1 (Baggiolini 1994).

The stimulus for chemokine production in alcoholic liver disease is not known but is likely to involve several signals. Proinflammatory cytokines (such as TNF- α and IL-1) and lipopolysaccharide are potent inducers of MCP-1 and all of these factors have been reported to be increased in the circulation in alcoholic hepatitis (Khoruts 1991, Bode 1987). The stimulus within the liver might be local TNF- α , the expression of which is increased in severe alcoholic hepatitis as shown by ourselves and others (Sheron 1993, McClain 1993), or the direct effects of alcohol metabolites or alcohol-induced reactive oxygen mediators. Synthesis of the chemokines is likely to occur predominantly from Kupffer and stellate cells within the liver (Czaja 1994, Armendariz-Borunda 1991). Most workers have found that alcohol itself is unlikely to act as a direct stimulus since this normally decreases cytokine or chemokine secretion (Maher 1995) although others have shown that *in vitro* rat hepatocytes may secrete homologues of the human chemokines IL-8 and GRO- α following stimulation with alcohol. Furthermore, toxic alcohol metabolites may induce a 'rebound' increase in cytokine synthesis following withdrawal of alcohol (Masumoto 1993).

The further evidence we provide of a role for chemokines in the pathogenesis of alcoholic liver disease has therapeutic implications, particularly in light of observations that mortality in severe alcoholic hepatitis remains high even after withdrawal of alcohol (Orrego 1987). If, as we propose, MCP-1 plays a central role in this process then treatment aimed at preventing MCP-1 secretion or inhibiting its effects might present an opportunity to alter the clinical course of this devastating disease.

Chapter 4

Chemokine Uptake by Human Hepatocytes and the Role of Ethanol and TNF- α

INTRODUCTION

The studies described in chapter 2 show that several chemokines are expressed in alcoholic liver disease, with the pattern of chemokine expression reflecting the nature of the leucocyte infiltrate. Histological assessment of diseased liver tissue showed that the principal site of chemokine synthesis, as determined by detection of chemokine-specific mRNA, appears to be within non-parenchymal cells (principally leucocytes and vascular endothelial cells), whereas the distribution of chemokine protein is more widespread. In alcoholic hepatitis this is principally within hepatocytes, particularly in areas of ballooning degeneration. This large discrepancy in distribution of chemokine mRNA and protein suggests that chemokines are taken up and possibly sequestered by hepatocytes. This may be relevant to the pathogenesis of alcoholic liver disease. Uptake of chemokines by epithelial cells has not been well studied previously, although similar mechanisms have been proposed for endothelial cells (Tanaka 1993, Middleton 1997), and so we examined this possibility in closer detail by further studies to address the following questions:

- (1) Are chemokine proteins taken up by hepatocytes ?
- (2) If so then is uptake mediated via specific or non-specific receptors ?
- (3) Is chemokine uptake influenced by other soluble factors, in particular alcohol or cytokines ?
- (4) Are the known (high-affinity) chemokine receptors expressed in diseased liver tissue ?

To address these questions we did *in vitro* studies using cultured hepatocytes and radiolabelled chemokine protein, and we also used monoclonal chemokine receptor antibodies to study expression in liver biopsy material and isolated liver cells.

METHODS

Isolation and culture of hepatocytes and peripheral circulating leucocytes

Primary human hepatocytes were isolated and cultured from normal human liver as described in appendices 7 and 8. Hep G2 (human hepatoblastoma) cells were cultured as described in appendix 8. L190 cells, a human liver-derived neoplastic cell line showing considerable phenotypic similarities with activated stellate cells, were cultured as described in appendix 8 (Murakami 1995). Peripheral blood mononuclear cells (PBMC's) and neutrophils were obtained from healthy donors as described in appendix 5.

Incubation of cells with radiolabelled IL-8 or MIP-1 α

Radiolabelling of recombinant human IL-8 or MIP-1 α was done with ¹²⁵Iodine as described in appendix 9. Primary hepatocytes were investigated as follows: cells were incubated for 16 hours overnight with the usual culture medium or with medium containing TNF- α (10 ng/ml) or ethanol (100mM, equivalent to 460 μ g/dL). After overnight culture, medium was refreshed with similar medium (i.e. with the corresponding concentrations of TNF- α or ethanol) but also containing additional ¹²⁵I-radiolabelled IL-8 or MIP-1 α (1×10^5 counts/ well) for 4 hours. At the end of this period culture supernatants were collected and centrifuged to remove cell debris, and the cells remaining in the culture wells were lysed with non-ionic detergent (Igepal, Sigma, UK). The incorporation of radiolabelled chemokine into the cell lysate was then calculated by radioactivity counts of cell lysates and supernatant fractions respectively using a gamma counter. In some cases the integrity of the chemokine protein in these fractions was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Similar experiments were also done with differing concentrations of ethanol (10mM and 100mM) and with acetaldehyde (500 μ M), and with Hep G2 cells, L190 cells and freshly isolated human peripheral blood leucocytes to examine chemokine uptake and the influence of TNF- α and ethanol. Experiments examining IL-8 and MIP-1 α uptake into human hepatocytes with TNF- α and ethanol were done in triplicate and on 2 different occasions; other experiments were done in triplicate on one occasion only.

Evaluation of binding affinity for IL-8 (Scatchard analysis)

Separate experiments were done to determine whether chemokine uptake was mediated by high-affinity receptors, using the method described by Scatchard (1949), which characterises the affinity of cell surface ligand binding. Briefly, hepatocytes were incubated with increasing concentrations of radiolabelled IL-8 in the presence or absence of a 1000-fold molar excess of non-labelled IL-8 for one hour at 4° C in hepatocyte culture medium containing 0.2% sodium azide and 5µg/ml cytochalasin B. Cell pellets and supernatants were then separated after centrifugation (14,000 rpm for 1 minute) and freezing for 1 hour with an equal volume of a phthalate oil mixture (3:2 butyl-phthalate/isocetyl-phthalate oil) following a technique previously described by this laboratory (Chamba 1991). The specificity and characteristics of binding were determined by analysis of binding curves, whereupon saturable binding is demonstrated if increasing concentrations of radiolabelled chemokine do not lead to linear increases in radioactivity uptake into cell pellets.

Localisation of chemokine receptors using immunohistochemistry and flow cytometry

We did immunohistochemical analysis of liver tissue sections from patients with alcoholic liver disease to study the high-affinity alpha chemokine receptors CXCR1 and CXCR2 (also known as IL-8RA and IL-8RB respectively) and the beta chemokine receptor CCR5. The alpha chemokine receptor CXCR1 is relatively specific for IL-8 whilst CXCR2 is more promiscuous and will bind other alpha chemokines. CCR5 is a receptor for MIP-1 α , MIP-1 β and RANTES. The same antibodies were used for flow cytometry analysis with the addition of the alpha chemokine receptor CXCR3 (receptor for interferon-inducible protein-10, IP-10). The antibodies used are summarised in appendix 1; the immunohistochemical and flow cytometric methods are summarised in appendices 2 and 10 respectively. Flow cytometry was done with primary hepatocytes, human biliary epithelial cells (isolated using a technique established in this laboratory, Joplin 1992) and L190 cells. These cells were analysed in comparison with 'positive controls' consisting of human lymphocytes (isolated as described in appendix 5 and stimulated by 14-day culture in the presence of human recombinant IL-2 and phytohaemagglutinin) and freshly isolated

human neutrophils (appendix 5). To examine for the possibility of induction of chemokine receptors, primary hepatocytes and L190 cells were also examined with and without pre-incubation with TNF- α (10 ng/ml), lipopolysaccharide (LPS, 1 ng/ml) ethanol (100 mM) and acetaldehyde (500 μ M), using 1 day's preincubation for primary hepatocytes and 5 days' for L190 cells.

Statistical analysis

Results of radioactivity incorporation into cells are expressed as mean +/- standard deviation for experiments that were done in triplicate and repeated on 2 different occasions; for other experiments (done in triplicate on one occasion only) results are expressed as median values. Statistical analysis was done with SPSS software, using the Wilcoxon Rank Sum test for matched pairs.

RESULTS

Uptake of radiolabelled chemokine (figures 4.1-4.4)

The uptake of IL-8 and MIP-1 α into primary human hepatocytes is summarised in figure 4.1; there was spontaneous uptake of both chemokines after 4 hours' incubation and this uptake was enhanced in the presence of ethanol ($p=0.25$ for IL-8 and $p<0.05$ for MIP-1 α , Wilcoxon rank sum) or TNF- α ($p<0.05$ for both chemokines) with further enhancement of uptake in the presence of both agents combined ($p<0.05$ for both chemokines, when compared with control wells). In a separate (single) experiment chemokine uptake into primary human hepatocytes was greater in the presence of 100mM compared to 10mM ethanol and was also slightly increased in the presence of 500 μ M acetaldehyde (figure 4.2). SDS-PAGE of cell lysates suggested that the incorporated radiolabelled chemokine was intact (figure 4.3). In further experiments we found that Hep G2 cells took up IL-8 to a similar extent to primary human hepatocytes; these also showed enhanced uptake in the presence of TNF- α or ethanol. L190 cells and isolated human peripheral leucocytes also took up chemokines spontaneously but with more variable uptake in the presence of TNF- α or ethanol (figure 4.4). Incorporation of chemokine under basal conditions appeared to vary substantially between the different cell types studied and to a certain extent between different human hepatocyte isolates; this was likely to be at least in part a manifestation of differing culture density and differing viability percentages for different isolates.

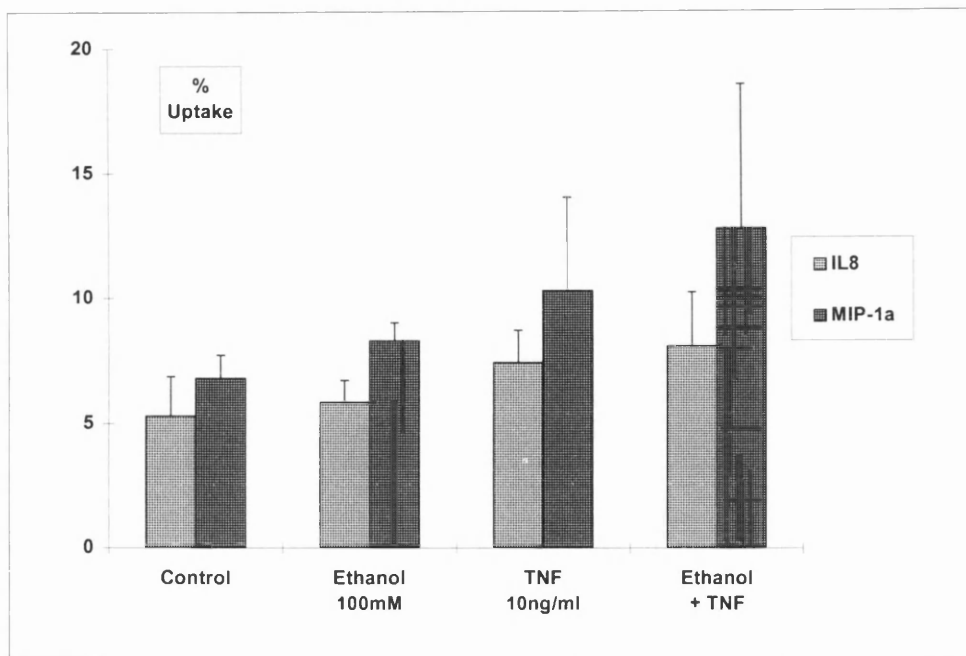


Figure 4.1 Uptake of radiolabelled IL-8 and MIP-1 α by primary human hepatocytes and the effect of co-incubation with TNF- α or ethanol. Results are expressed as a percentage of radioactivity incorporated after 4 hours incubation (mean of 6 measurements + standard deviation, from 2 triplicated experiments). Results demonstrate spontaneous uptake of radiolabelled chemokine which is enhanced in the presence of TNF- α , ethanol, or both agents combined (see text for full statistical evaluation).

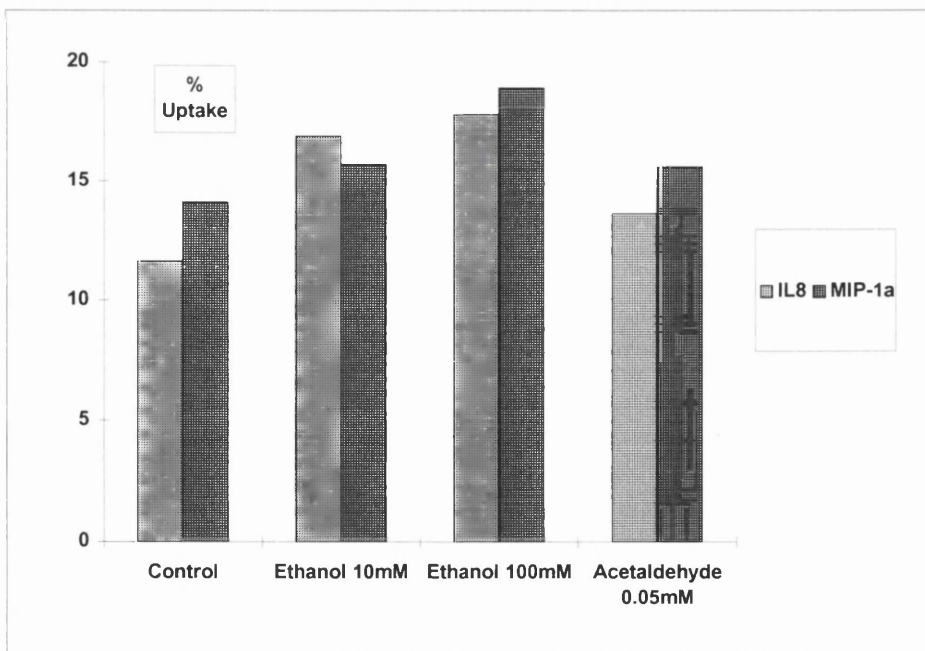


Figure 4.2 Uptake of radiolabelled IL-8 and MIP-1 α by primary human hepatocytes and the effect of co-incubation with acetaldehyde and differing concentrations of ethanol (results given are the median of 3 measurements from a single triplicated experiment).

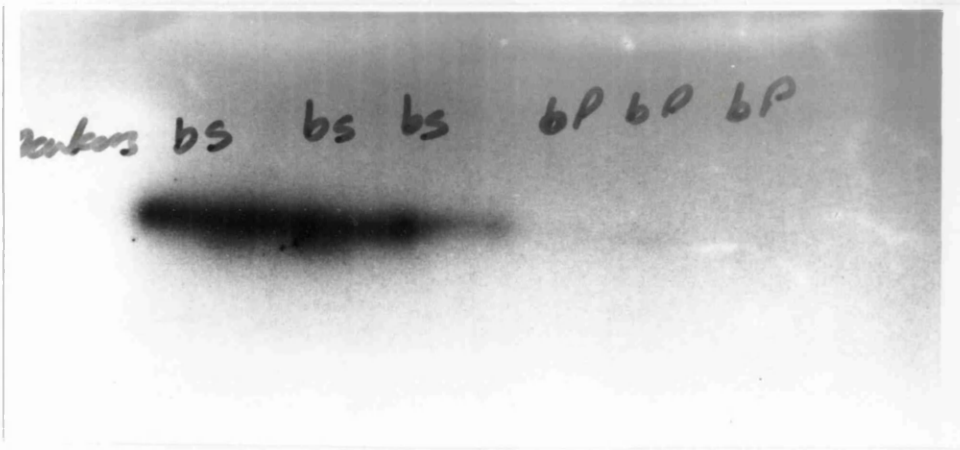


Figure 4.3 Autoradiograph of polyacrylamide gel electrophoretic strip of radiolabelled IL-8. Supernatant samples have been loaded on the left side and cell lysate pellets on the right side of the gel (3 wells each, labelled '6s' and '6p' respectively). The predominant signal is from the supernatant samples, whilst a faint but discrete band is seen for the cell lysate - this has a similar molecular weight to the supernatant band, suggesting that chemokine protein incorporated into the cell fraction was intact.

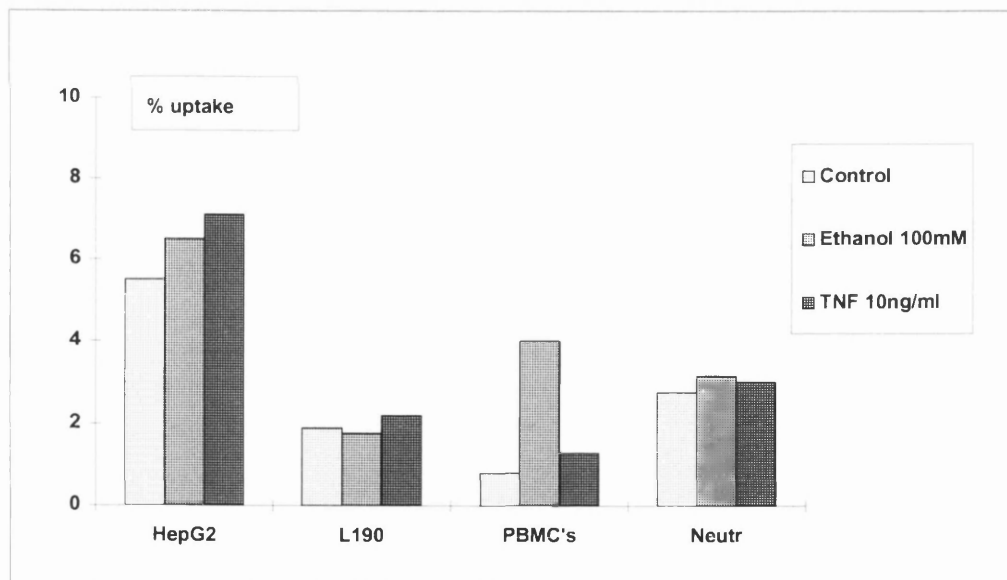


Figure 4.4 Uptake of radiolabelled IL-8 by other cell types. Hep G2 = hepatoblastoma cell line; L190 = neoplastic human cells derived from mesenchymal liver tumour and with phenotype similar to activated stellate cells; PBMC's = freshly isolated human peripheral blood mononuclear cells; Neutr. = freshly isolated human peripheral neutrophils (results given are median of 3 measurements from a single triplicated experiment).

Analysis of binding affinity

Scatchard binding curve analysis of radiolabelled IL-8 uptake into primary human hepatocytes over a range of concentrations suggested that most of the uptake was not mediated by high-affinity receptors, since uptake was not significantly diminished in the presence of a 1000-fold molar excess of non-labelled IL-8 (figure 4.5). Thus uptake was probably predominantly mediated by either non-specific ('scavenger') receptors or by passive diffusion.

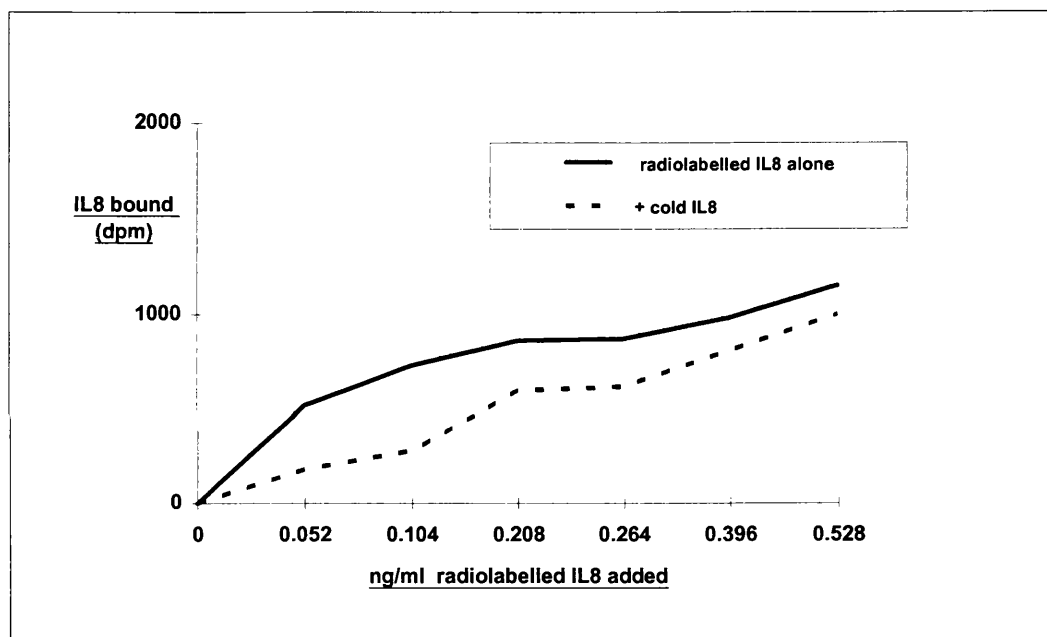


Figure 4.5 Competitive IL-8 uptake assay (Scatchard analysis); graph showing IL-8 uptake over a range of concentrations with and without 'cold' (non-radiolabelled) IL-8. Uptake was not significantly altered by the addition of a 1000-fold molar excess of 'cold' IL-8, suggesting that most of the binding was non-receptor mediated.

Immunolocalisation of chemokine receptors in human liver tissue (table 4.1 & figure 4.6)

Staining for both alpha and beta chemokine receptors was weak in normal liver tissue. CXCR1 staining was modestly enhanced in alcoholic hepatitis, mainly in areas of inflammatory cell infiltrates, biliary epithelium and venous endothelium. CXCR2 staining was strongly enhanced in both hepatitis and cirrhosis, with staining mainly localised to ballooned hepatocytes (in hepatitis), inflammatory infiltrates and biliary epithelium (the latter principally within proliferating bile ducts). Staining for the beta chemokine receptor CCR5 was modestly enhanced in cirrhosis and hepatitis, and was mainly localised to venous endothelium and areas of inflammatory cell infiltration.

1. Alcoholic Hepatitis

| Chemokine Receptor | Vascular endothelium | Biliary epithelium | Inflammatory cells | Hepatocytes |
|--------------------|----------------------|--------------------|--------------------|-------------|
| CXCR1 | + | + | + | + |
| CXCR2 | + | ++ | ++ | + |
| CXCR3 | - | - | + | - |
| CCR5 | + | + | + | + |

2. Alcoholic Cirrhosis

| Chemokine Receptor | Vascular endothelium | Biliary epithelium | Inflammatory cells | Hepatocytes |
|--------------------|----------------------|--------------------|--------------------|-------------|
| CXCR1 | - | - | + | - |
| CXCR2 | + | + | + | - |
| CXCR3 | - | - | - | - |
| CCR5 | - | - | + | - |

Table 4.1 Expression of Chemokine Receptors in Alcoholic Liver Disease

Notes

CXCR = CXC (alpha) chemokine receptor; CCR = CC (beta) chemokine receptor (see methods section for chemokine receptor specificities).

+ = weak staining; ++ = strong staining, - = no staining

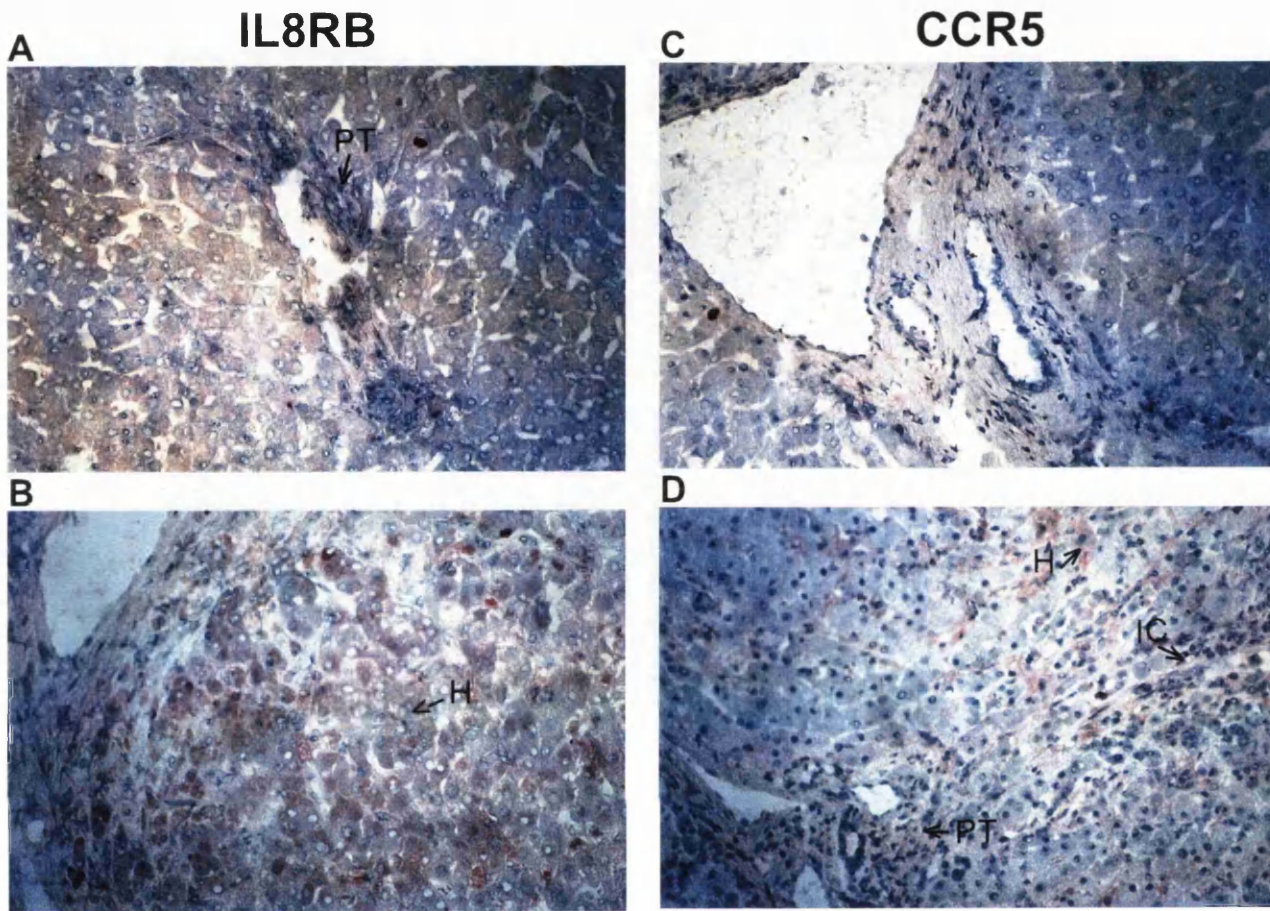


Figure 4.6 Immunolocalisation of chemokine receptors in human liver tissue (positive staining shown in red). IL8RB (CXCR2) staining is shown in the left hand column in normal liver (A) and alcoholic hepatitis (B). CCR5 staining is shown in the right hand column in normal liver (C) and alcoholic hepatitis (D). Staining patterns show that expression of these chemokine receptors is weak in normal liver tissue, with positivity mostly in portal tracts (PT), but expression is up-regulated in alcoholic hepatitis, particularly in association with inflammatory cells (IC), proliferating biliary epithelial cells and in some areas of ballooning hepatocyte degeneration (H).

Analysis of chemokine receptor expression using flow cytometry

We were unable to demonstrate any significant positive staining for alpha or beta chemokine receptors on freshly isolated primary hepatocytes, biliary epithelial cells or L190 cells by flow cytometry. In contrast, human leucocytes showed positive staining as expected with lymphocytes positive for CXCR3 and CCR5, and neutrophils positive for CXCR1 and CXCR2 (figure 4.7). Further analysis of primary hepatocytes and L190 cells after pre-incubation with a variety of agents including TNF- α , LPS, ethanol or acetaldehyde in vitro did not appear to alter chemokine receptor expression.

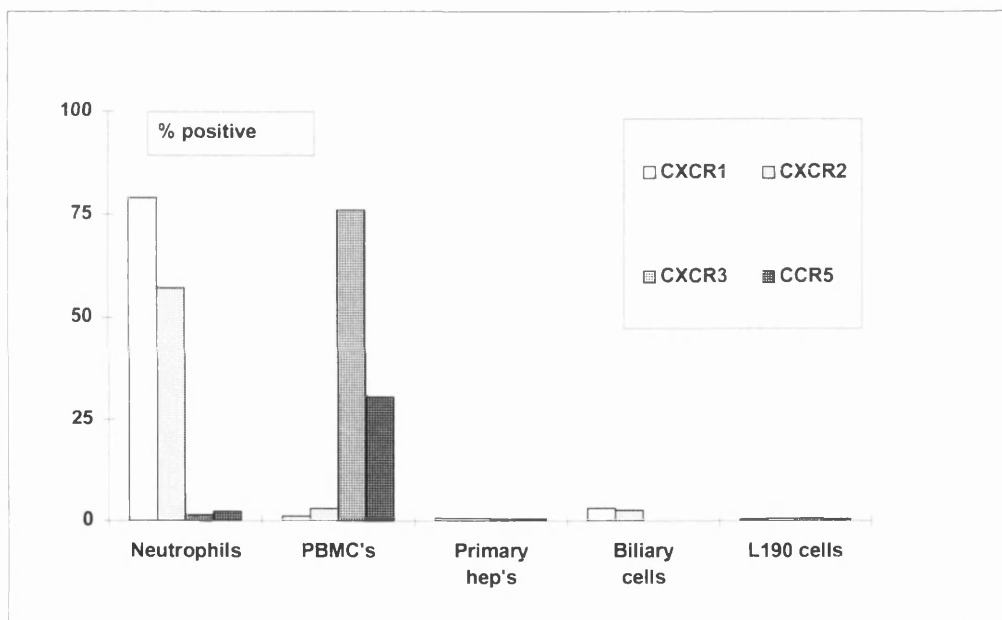


Figure 4.7 Chemokine receptor expression on various cell types analysed by flow cytometry. Results show percentage of cells staining positive for individual chemokine receptors. CXCR1-3 = CXC (alpha) chemokine receptors 1-3; CCR5 = CC (beta) chemokine receptor 5. Neutrophils = freshly isolated human peripheral neutrophils; PBMC's = human peripheral blood mononuclear cells stimulated by prolonged culture in phytohaemagglutinin; human hep's = freshly isolated primary human hepatocytes; biliary cells = freshly isolated human biliary epithelial cells. Results demonstrate that peripheral leucocytes showed positive staining as expected but none of the liver derived cells showed significant staining for any of the chemokine receptors analysed. Repeat analysis of liver derived cells after prolonged culture in TNF- α or lipopolysaccharide did not lead to any alteration in staining for chemokine receptors (data not shown). Sample flow cytometry histograms are shown in appendix 10.

DISCUSSION

Our experiments demonstrate that chemokine protein is readily taken up by hepatocytes *in vitro*, and these observations suggest a possible mechanism for the sequestration of chemokines seen within hepatocytes by histological examination in alcoholic liver disease. The failure to demonstrate high-affinity binding suggests that the majority of this uptake is non-receptor mediated and may be due to fluid-phase endocytosis or pinocytosis (Ganong, 1991). Other workers have shown that the pro-inflammatory cytokines TNF- α and IL-1 are also predominantly taken up by low-affinity but high capacity binding *in vitro* (D'Souza 1994). Our finding of enhanced chemokine uptake in the presence of TNF- α or ethanol and its metabolites may also at least partly account for sequestration of chemokines into hepatocytes in alcoholic liver disease, since increased amounts of circulating pro-inflammatory cytokines (and self-evidently alcohol) characterise this disease (Khoruts 1991). TNF- α leads to increased cell membrane permeability which would facilitate the passive diffusion of proteins (Marcus 1996) and it is likely that this may represent the principal mechanism by which TNF- α led to increased uptake of chemokines in our experiments, whilst it is also possible that there may be increased expression of 'scavenger' protein receptors (Martinez 1995). TNF- α leads to increased expression of membrane-bound regulatory G proteins and adhesion molecules (Marcus 1996, Brett 1989); thus TNF- α might also lead to increased expression of specific chemokine receptors *in vivo*; a similar increase in expression of these receptors has been observed with the cytokine IL-2 (Sozzani 1997). We were unable to demonstrate up-regulation of chemokine receptors by flow cytometry in the cells that we studied *in vitro* and this contrasts with our earlier finding of significant uptake of chemokines into cultured hepatocytes and leucocytes; nevertheless histological assessment of biopsy material did suggest increased expression of chemokine receptors on hepatocytes (particularly in areas of ballooning degeneration) in ALD. Therefore increased expression may only be apparent after protracted hepatocyte injury or alternatively may be more difficult to stimulate under *in vitro* conditions.

Enhancement of chemokine uptake in the presence of ethanol or acetaldehyde is probably a reflection of facilitated passive diffusion in the presence of these agents rather than induction of chemokine receptors, since ethanol (at least) leads to increased membrane

permeability in epithelial cells (Goates 1994, Worthington 1978). The possibility of an osmotic effect of ethanol contributing to increased chemokine uptake remains but other workers have reported that hyperosmolality, when induced by sucrose, does not alter such 'fluid phase' endocytosis of proteins by hepatocytes (Casey 1995).

Prolonged alcoholic injury *in vivo* leads to impairment of receptor-mediated uptake of proteins (including cytokines) by hepatocytes in animal models (Camacho 1993, Fawcett 1993, Tuma 1991) and it has been suggested that impaired hepatic clearance may therefore lead to increased circulating levels of cytokines such as TNF- α in ALD (Tuma 1996). This suggestion contrasts with our own observations. However, other workers have found that an acute alcohol load will increase clearance of radiolabelled cytokines *in vivo*, including enhancement of uptake into the liver (Deaciuc 1996). Whilst our own experiments only included short-term exposure to ethanol under *in vitro* conditions, our findings are consistent with the latter study and would suggest that *in vivo* chemokine distribution may still be considerably influenced by (non-receptor-mediated) hepatic uptake.

It remains unclear whether non-receptor mediated or low-affinity sequestration of chemokines is of any physiological consequence, or is merely a reflection of passive uptake of locally released chemokine protein. The liver may act as a clearing site for circulating or 'free' chemokines as occurs for other cytokines (Andus 1991); thus the tissue distribution of secreted chemokine protein in ALD is likely to be influenced by several dynamic factors. Since the biological activity of chemokines is principally mediated by interaction with specific membrane-bound receptors, it is possible that non-receptor mediated uptake of chemokines, and their presence within the cytosol, will have no biological effects. However, it is also possible that this may be a mechanism for local concentration and sequestration of chemokines; a similar mechanism has recently been demonstrated for endothelial cells (Middleton 1997). Furthermore it has previously been recognised that Mallory bodies, which characterise alcoholic liver injury and contain aggregated intracellular protein, are chemotactic (Jensen 1994). Thus our demonstration of intracellular sequestration of intact chemokine protein suggests that chemotactic activity of Mallory bodies may be at least partly attributable to the presence of chemokines.

The role of non-receptor mediated internalisation of chemokines is deserving of further study, and it may also be helpful to examine whether upregulation of chemokine receptor expression can be demonstrated *in vitro* under appropriate conditions.

Chapter 5

Secretion of MCP-1 from Human Hepatocytes and Leucocytes

INTRODUCTION

The histopathological studies described in chapter 2 demonstrated increased expression of alpha and beta chemokines in tissue sections of human liver in alcoholic liver disease, and studies described in chapter 3 demonstrated increased circulating levels of the prototype beta-chemokine MCP-1 in proportion to histological severity of disease. Several types of cells within the liver may produce chemokines in response to an appropriate stimulus. Histological and cell culture studies suggest that non-parenchymal cells including stellate cells, Kupffer cells and infiltrating leucocytes are the principal sources of chemokine secretion in toxic liver injury (Marra 1993, Czaja 1994, Adachi 1994, Bautista 1995). However, hepatocytes, the main target of alcoholic liver injury, also secrete a range of chemokines *in vitro* with appropriate stimulation (Shiratori 1993, Maher 1995, Rowell 1997) and may therefore contribute to recruitment of leucocytes in alcoholic liver disease. Whilst increased expression of chemokines in ALD has been demonstrated convincingly, the precise mechanisms leading to increased expression are not understood. It is known that pro-inflammatory cytokines, such as TNF- α and IL-1, and bacterial lipopolysaccharide (LPS) are potent stimuli for chemokine secretion (Sylvester 1993, Morland 1997, Rowell 1997), but it is not clear whether ethanol or its metabolites are direct stimuli for chemokine secretion since some workers have found direct release of chemokines from hepatocytes exposed to alcohol (Shiratori 1993) whilst others have not (Maher 1995, Ohkubo 1998). We therefore conducted experiments with cultured hepatocytes and leucocytes to determine the effect, if any, of ethanol and its metabolite acetaldehyde on chemokine secretion from these cells. We also conducted experiments to assess MCP-1 secretion from leucocytes isolated from human volunteers before and after alcohol ingestion. The hepatocytes studied were primary human hepatocytes (from fresh human liver tissue) and the Hep G2 cell line (derived from a human hepatoblastoma). We also studied L190 cells, a human liver-derived neoplastic cell line which has considerable phenotypic similarities to activated stellate cells (Murakami 1995). We studied these cells because there is considerable evidence that activated stellate cells play a central role in chemokine release and fibrogenesis in alcoholic liver disease (Mak 1988, Maher 1994a, Sprenger 1997). We studied isolated human peripheral blood mononuclear cells (PBMC's)

because circulating monocytes might reflect the behaviour of hepatic Kupffer cells, which themselves play a central role in pathogenesis of alcoholic liver disease as outlined above.

METHODS

Culture of hepatocytes and leucocytes

Primary human hepatocytes were isolated and cultured from surplus normal human donor liver tissue as described in appendices 7 and 8 respectively. We also used Hep G2 cells and L190 cells as described in appendix 8. All cultures were subconfluent at the time of experimentation (figure 5.1). Freshly isolated human peripheral blood mononuclear cells (PBMC's) were obtained from 5 healthy donors and cultured as described in appendix 5. Cells were cultured for 24 hrs. prior to collection and separation of supernatants.

Preliminary experiments were done by adding TNF- α (10ng/ml) or LPS (1 μ g/ml) to culture medium for 24 hours in order to verify whether MCP-1 production could be stimulated in these cells. Thereafter experiments were done with or without ethanol (1-1000 mM; Fisher Scientific Ltd, UK) or acetaldehyde (0.05 - 50 mM; Sigma, UK) added to culture medium for 24 hours. Cell culture supernatants were collected by aspiration, purified by centrifugation and then stored at -70⁰ C prior to MCP-1 level measurement. Cell viability was assessed at the end of experiments by trypan blue exclusion (hepatocytes were separated by trypsinisation prior to this analysis) and by measurement of lactate dehydrogenase (LDH) levels in supernatants using a commercial colorimetric assay (Sigma, UK) based on LDH-dependent reduction of NAD. In order to examine the rate of metabolism and/or evaporation of ethanol from tissue culture medium, we measured ethanol concentrations at intervals during the 24-hour incubation period, using similar well plates with the same quantity of culture medium but without hepatocytes as a control. Ethanol levels were measured by Dr. R. Cramb in the department of biochemistry, Selly Oak Hospital, by a commercial colorimetric alcohol dehydrogenase-based assay.

The effect of alcohol and food ingestion on PBMC chemokine secretion

Four human volunteers were studied before and after (i) a heavy meal consisting of approx. 1800 kcal (of which 35% of the calories were derived from fat, 45% from

carbohydrate and 20% from protein), (ii) a similar meal taken with 1ml/ kg alcohol (i.e. 1 unit per 10 kg bodyweight), and (iii) ingestion of 1 ml/ kg alcohol alone. In each case, the meal and/or alcohol were taken over a period of approximately 1 hour after a period of at least 6 hours' fasting. Blood samples were taken for isolation of PBMC's immediately before the meal and/or alcohol and 2 hours (for alcohol alone) or 4 hours (for food +/- alcohol) after ingestion. PBMC's were isolated and cultured as described in appendix 5, with separation of culture medium after 24 hours for MCP-1 measurement. We also cultured some of the PBMC's separately in the presence of lipopolysaccharide (LPS, *Salmonella abortus equi*, Sigma, UK) at 1µg/ml.

In the experiment involving alcohol ingestion alone we measured serum endotoxin levels before and after alcohol at the same time as drawing other blood samples. Endotoxin was measured by Dr Tom Worthington in the Department of Microbiology, Queen Elizabeth Hospital, using a commercial assay (Limulus Amoebocyte Lysate assay, Chromogenix AB, Sweden).

MCP-1 assays were done using a customised ELISA as described in appendix 6. All hepatocyte and PBMC culture experiments were done in triplicate, and MCP-1 assays were done in duplicate. MCP-1 levels were taken as the mean of the 2 (duplicated) values obtained by ELISA, and the final result for each cell culture was taken as the median of the 3 wells. In vivo experiments assessing the direct effect of alcohol and acetaldehyde on secretion of MCP-1 from cultured PBMC's are expressed as the mean \pm SD for the 5 subjects studied, and ex vivo experiments assessing the effect of prior food and/ or alcohol ingestion on cultured PBMC's are expressed as the mean \pm SD for the 4 subjects studied.

Statistical analysis of results was done using SPSS statistical software; for experiments measuring the effect of progressive concentrations of ethanol or acetaldehyde on hepatocytes the one-factor repeated measures ANOVA test was used; for similar experiments with cultured PBMC's Friedman's ANOVA test (for non-parametric data) was used and for ex vivo experiments assessing the effect of prior food and/ or alcohol ingestion on cultured PBMC's the Wilcoxon rank sum test was used. This study was approved by the local research ethics committee and informed consent was obtained from each volunteer before participation.

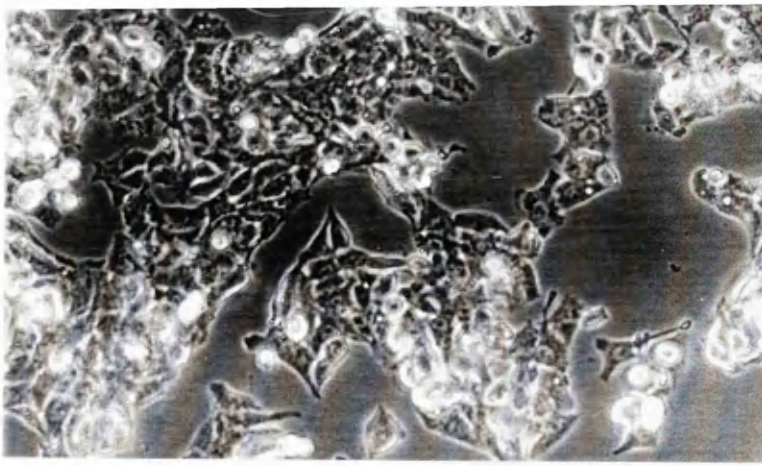
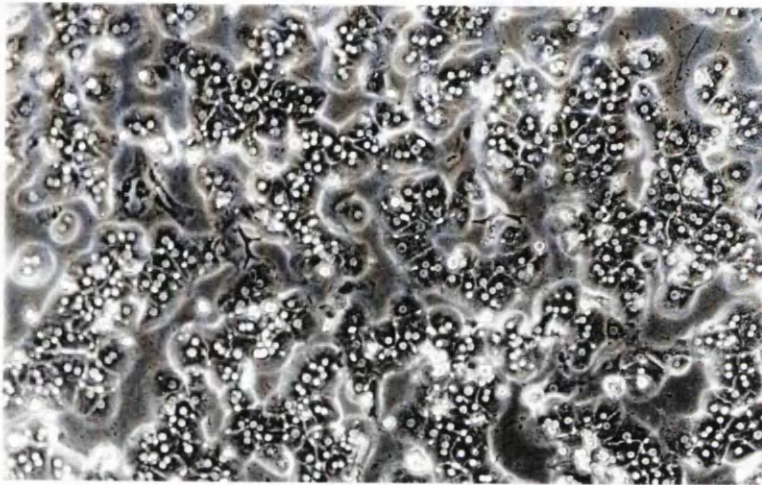
A**B****C**

Figure 5.1 Hepatocytes in culture as used for in vitro experiments. Panel A = Hep G2 cells; panel B = primary human hepatocytes; panel C = L190 cells. Hep G2 cells and primary human hepatocytes are shown at subconfluent growth, whilst L190 cells are shown following recent passage.

RESULTS

MCP-1 secretion from hepatocytes

There was spontaneous secretion of MCP-1 from primary human hepatocytes under basal conditions and this increased with TNF- α or LPS, but not with ethanol or acetaldehyde at concentrations of 100 mM or 500 μ M respectively (figure 5.2). There was also spontaneous secretion of MCP-1 from HepG2 under basal conditions (figure 5.3); secretion was reduced with ethanol in a dose-dependent manner in the range of 1-1000 mM ($p < 0.01$, one-factor ANOVA) whilst acetaldehyde led to a slight increase in secretion at doses of 0.05 and 0.5 mM but reduced secretion at doses of 5 and 50 mM ($p < 0.01$, one-factor ANOVA). Trypan blue analysis suggested that Hep G2 cells were near 100% viable under basal conditions and without discernible alteration in the presence of 1000 mM ethanol or 5 mM acetaldehyde. LDH levels in culture supernatants from hepatocytes showed a possible toxic effect of ethanol at 100mM (LDH level in control specimen, 55 ng/ml and with ethanol, 77 ng/ml). However (in a separate experiment) there was no apparent effect of acetaldehyde (LDH level in control specimen, 114 ng/ml and with 50, 500 and 5,000 μ M acetaldehyde respectively levels were 84, 109 and 129 ng/ml).

L190 cells also secreted MCP-1 under basal conditions (figure 5.4). There was no apparent response to ethanol in the ranges of 10-100mM, but reduced secretion at 1M (differences not significant). There was progressive reduction in MCP-1 with increasing doses of acetaldehyde in the ranges of 500 μ M - 50 mM ($p < 0.05$, one-factor ANOVA).

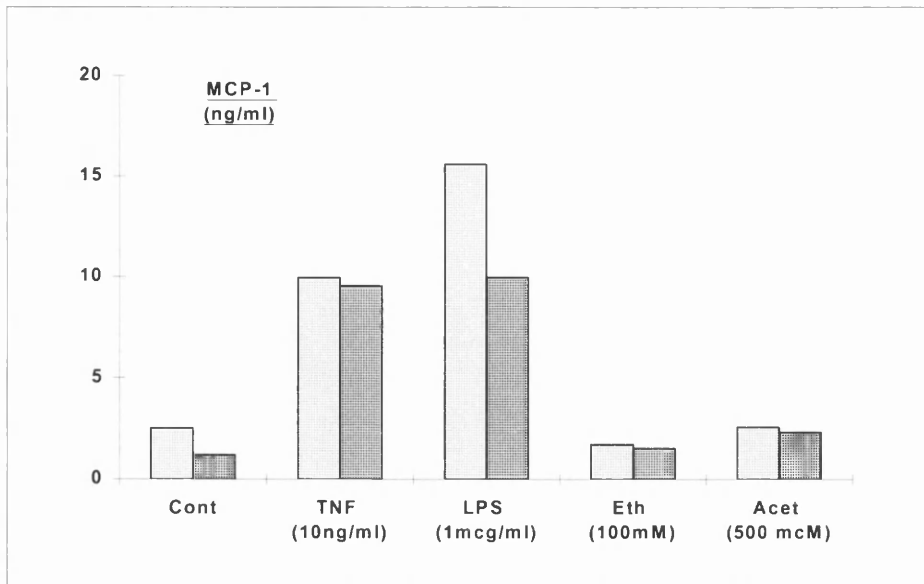


Figure 5.2 MCP-1 production from cultured primary human hepatocytes and the effect of TNF- α (TNF), LPS, ethanol (Eth) and acetaldehyde (Acet). Values shown are median of 3; different shaded columns correspond to 2 different experiments.

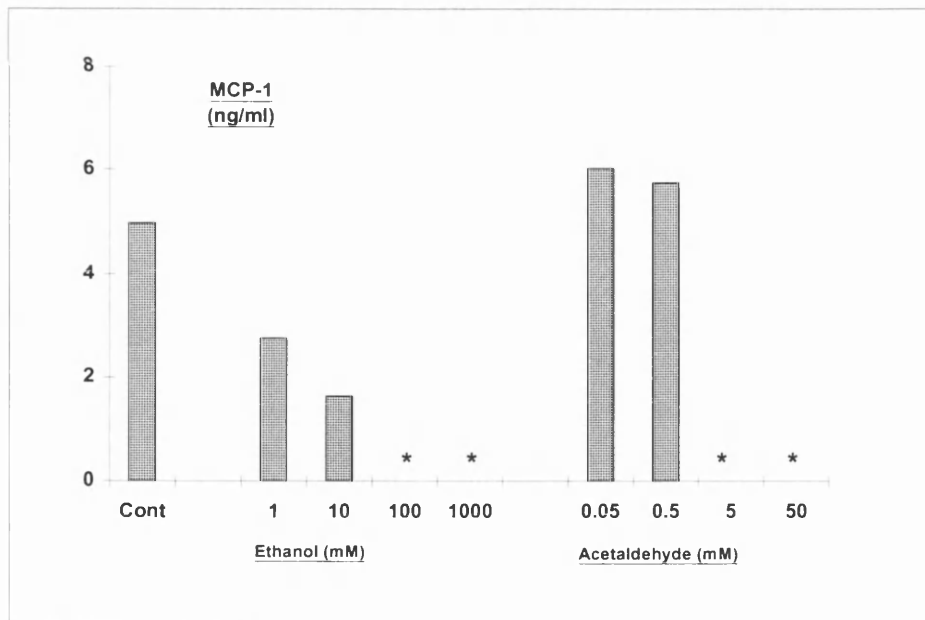


Figure 5.3 MCP-1 production from cultured Hep G2 cells and the effect of ethanol and acetaldehyde. Values shown are median of 3. Lower limit of sensitivity for MCP-1 assay was 0.125 ng/ml (* = result below lower limit of sensitivity of assay). Effect of both ethanol and acetaldehyde, $p < 0.01$, one-factor ANOVA.

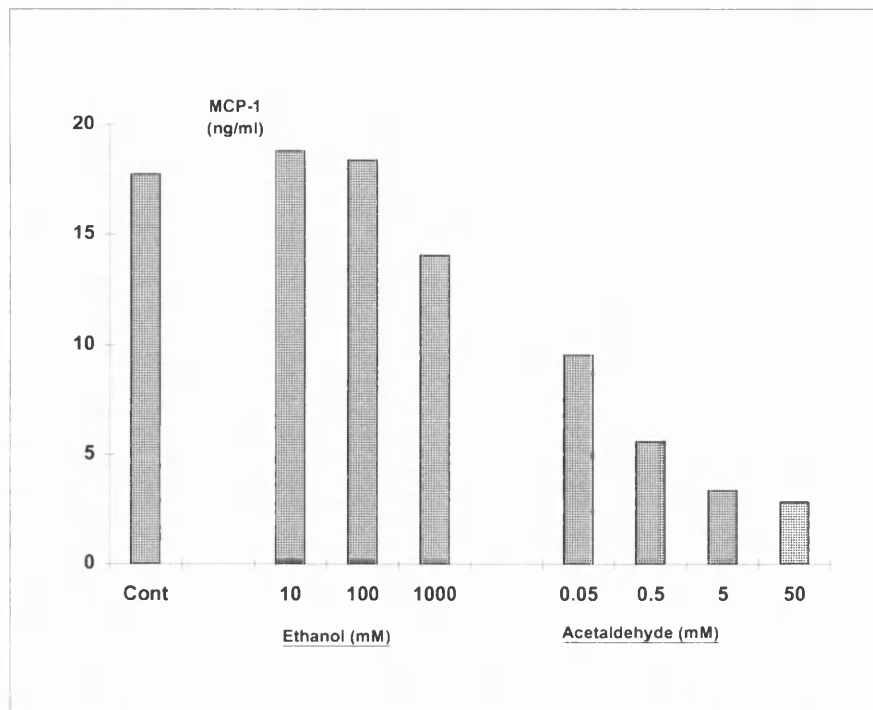


Figure 5.4 MCP-1 production from cultured L190 cells and the effect of ethanol and acetaldehyde. Values shown are median of 3. Effect of alcohol; differences not significant, effect of acetaldehyde; $p < 0.05$, one-factor ANOVA.

Alcohol levels in culture supernatants

Analysis of sequential alcohol concentrations in hepatocyte tissue culture medium showed a progressive fall over 24 hours that was similar for both hepatocyte culture supernatants and control samples (figure 5.5), suggesting that evaporation (rather than metabolism from hepatocytes) was the principal reason for progressive reduction in levels.

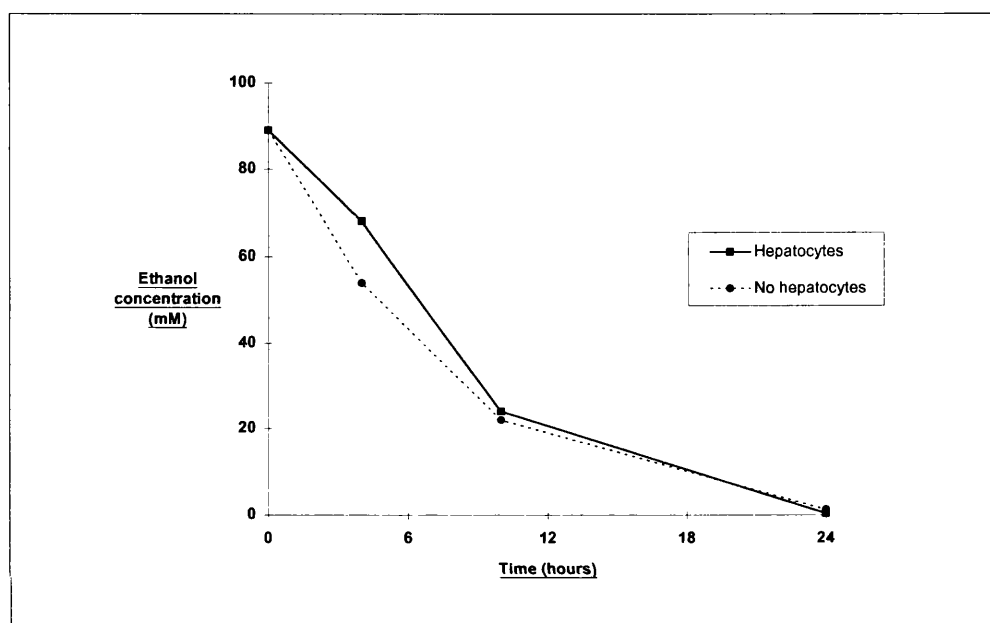


Figure 5.5 Sequential ethanol concentrations in wells containing cultured hepatocytes and control wells.

MCP-1 secretion from PBMC's

A preliminary experiment confirmed spontaneous secretion of MCP-1 from cultured PBMC's from 2 healthy volunteers under basal conditions, with measurable MCP-1 in culture supernatant from 6 hours onwards (but not before); mean levels at 6, 12 and 24 hours were 1.75, 2.35 and 5.68 ng/ml respectively. The substantive experiments then showed that secretion of MCP-1 after 24 hours was suppressed by ethanol in a dose-dependent manner (figure 5.6); the mean level for 5 subjects was 7.66 +/- 4.50 ng/ml, and mean levels with 1, 10, 100 and 1000mM ethanol were 6.80, 5.97, 4.71 and 0.16 ng/ml respectively ($p < 0.01$, Friedman's test). There was a trend towards increased MCP-1 secretion in the presence of 0.05 mM acetaldehyde (mean level 8.35 ng/ml, difference not significant), but at higher doses there was progressive reduction in secretion (mean levels with 0.5, 5 and 50mM were 5.1, 1.54 and 0.16 ng/ml respectively, $p < 0.01$, Friedman's test). Trypan blue exclusion analysis suggested that the PBMC's remained near 100% viable in the presence of 10 or 100 mM ethanol, but only 5% viable with 1000 mM ethanol. They were near 100% viable in the presence of 0.05 or 0.5 mM acetaldehyde, but only 50% viable with 5 mM and 25% viable with 50 mM. However, LDH levels in culture supernatants were below the lower limit of sensitivity of the assay (< 25 ng/ml) in all samples.

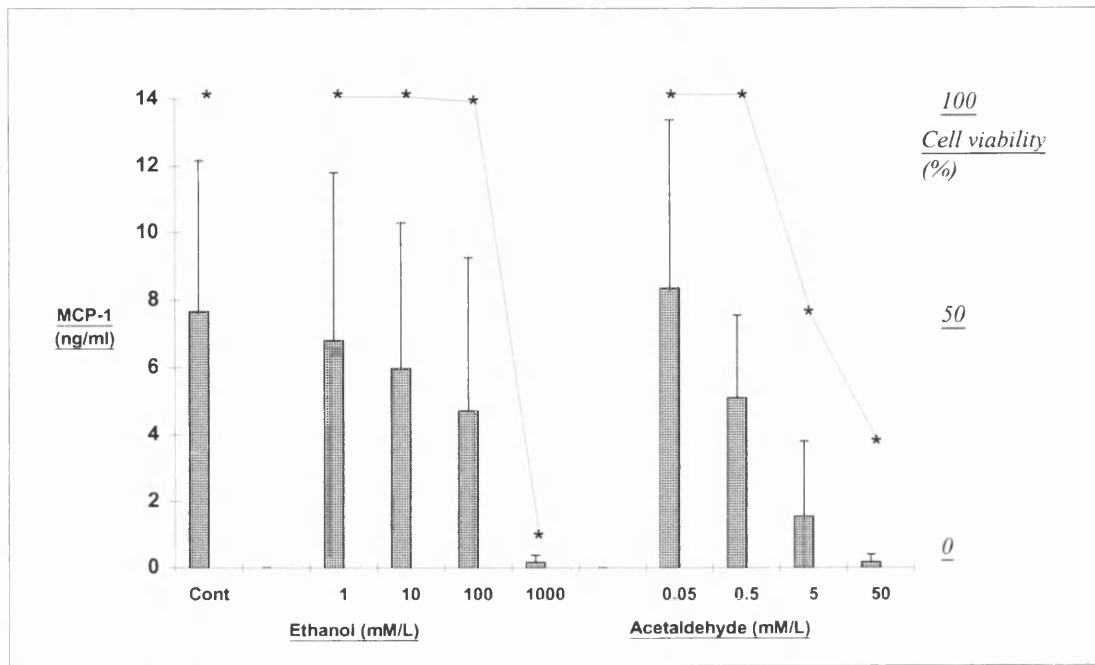


Figure 5.6 MCP-1 secretion from cultured PBMC's from 5 healthy volunteers and the effect of ethanol and acetaldehyde (values shown are mean +/- SD) (effects of both ethanol and acetaldehyde, $p < 0.01$, Friedman's test). Asterisks above columns indicate cell viability, expressed as percentage using scale on right hand y axis, as assessed by trypan blue exclusion at end of 24 hour incubation period.

MCP-1 secretion from PBMC's and the effect of food and/ or alcohol ingestion

Analysis of levels of MCP-1 secretion from cultured PBMC's before and 2 hours after ingestion of a heavy meal did not show any apparent effect of the meal ingestion upon MCP-1 secretion (mean MCP-1 secretion 10.1 ng/ml before vs. 10.8 ng/ml after, difference not significant). When a similar meal was taken with alcohol however, there was a clear trend towards increased secretion following ingestion (mean MCP-1 secretion 7.9 ng/ml before vs. 15.2 ng/ml after, $p=0.07$, Wilcoxon rank sum). In contrast, when a similar quantity of alcohol was taken alone, there was a clear trend towards decreased secretion after ingestion (mean MCP-1 secretion 10.2 ng/ml before vs. 5.4 ng/ml after, $p=0.14$, Wilcoxon rank sum). Each of these findings was similar when PBMC's were cultured in the presence of 1 $\mu\text{g/ml}$ LPS (figure 5.7).

Serum endotoxin levels before and after ingestion of alcohol alone gave values below the limit of detection for the assay (<0.2 endotoxin units/ml) in all 4 subjects. We did not measure endotoxin levels following ingestion of food and alcohol taken together.

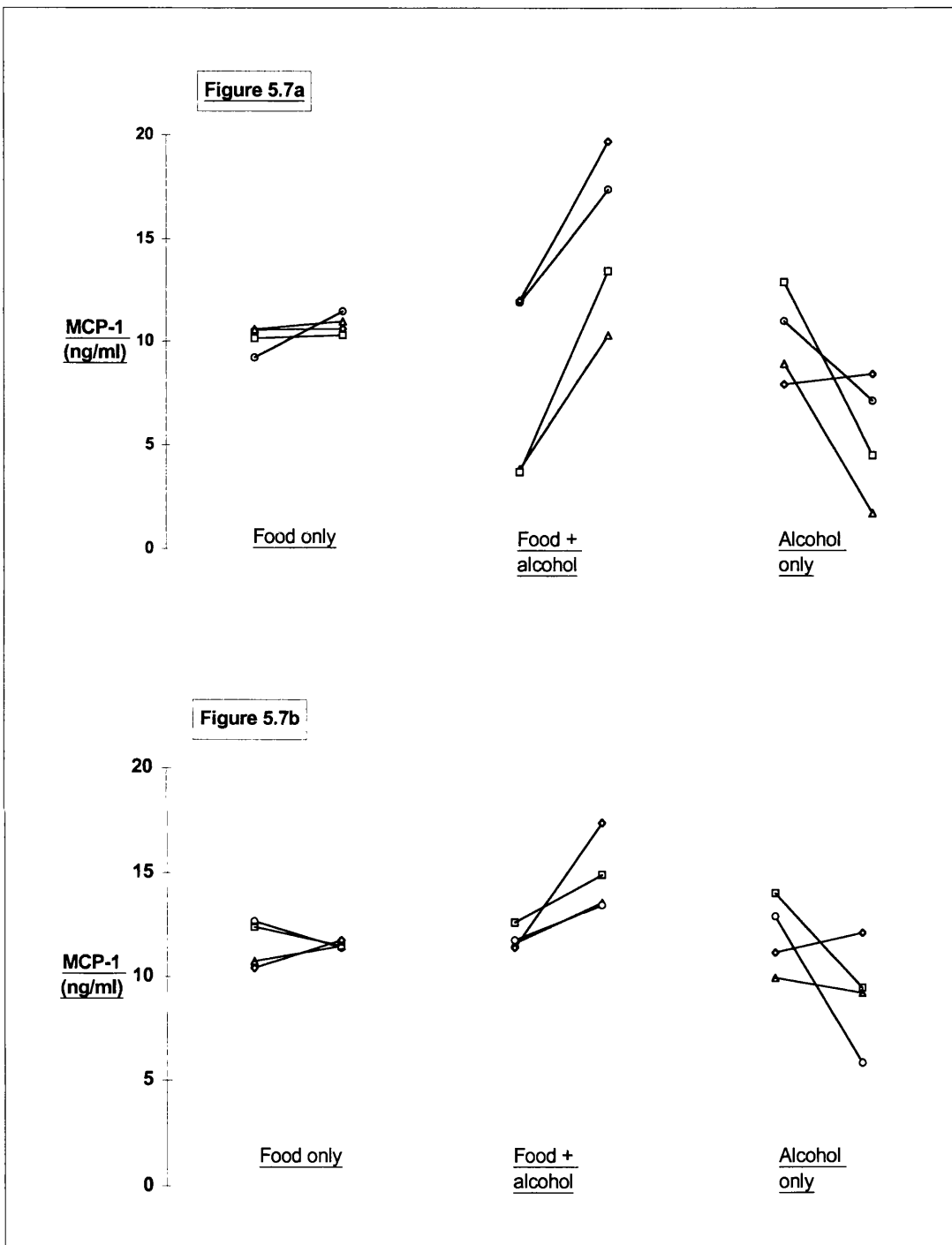


Figure 5.7 MCP-1 secretion from cultured PBMC's from 4 healthy volunteers before and after ingestion of food (1800 kCal meal) only, food + alcohol (1 ml/kg), or alcohol only (**a**, unstimulated culture; **b**, LPS-stimulated culture). The left-hand point of each line represents values immediately before ingestion, and the right-hand point 2 hours after ingestion (food alone; differences not significant, food + alcohol; $p=0.07$, alcohol alone; $p=0.14$, Wilcoxon rank sum).

DISCUSSION

Whilst expression of MCP-1 and other chemokines is increased in alcoholic liver disease, this expression (as demonstrated histologically) persists long after alcohol has been withdrawn, suggesting that factors other than alcohol itself are necessary to sustain hepatic injury. The present studies suggest that alcohol is unlikely to act as a direct stimulant for chemokine synthesis or release, raising the questions of how these experiments relate to the pathogenesis of alcoholic liver disease *in vivo* and how alcoholic injury leads to amplification of the inflammatory cascade. Chemokine synthesis is stimulated by oxidative injury and is thus likely to be increased in the presence of acetaldehyde since this alters the cellular redox state (Nordmann 1994, Maher 1994b, Xu 1996). Our own experiments suggested that whereas small concentrations of acetaldehyde may lead to enhanced MCP-1 secretion (although this increase did not reach statistical significance), higher concentrations suppress secretion. It is likely that acetaldehyde will have toxic effects including interference with protein trafficking (Zhang 1992), and this may help explain the reduced MCP-1 secretion seen with increasing concentrations of acetaldehyde. The concentrations of alcohol encountered *in vivo* will be nearer to the lower range of concentrations that we used *in vitro* and it is unlikely that the higher concentrations of alcohol or acetaldehyde that we used will ever be encountered in the circulation *in vivo*, although tissue levels reached within the liver are unknown and we studied the higher concentrations to verify whether or not direct toxicity could be confirmed. The response of the different cell types to escalating doses of ethanol appeared broadly similar except that the MCP-1 secretion from L190 cells was less affected by ethanol than were the other cells studied. Likewise the response to acetaldehyde was similar for different cells except that L190 cells appeared more sensitive to the effects of low concentrations of acetaldehyde in reduction of MCP-1 secretion. Our observations on the effects of alcohol on hepatocytes are similar to those of others (Maher 1995, Ohkubo 1998) although similar experiments with acetaldehyde or using L190 cells have not been reported previously.

Trypan blue analysis confirmed toxicity leucocytes at high concentrations of ethanol or acetaldehyde but appeared an unreliable guide to hepatocyte toxicity, and likewise LDH analysis appeared to be an unreliable guide to toxicity with our experimental protocol and

assay. However the lack of any apparent direct toxicity at lower concentrations suggests that the reduction in chemokine secretion at these concentrations would result from altered chemokine synthesis or release rather than cell death. Other workers have also found that ethanol reduces MIP-1 α secretion *in vitro* (Standiford 1997). It is uncertain why ethanol itself should reduce chemokine secretion; several potential mechanisms have been proposed including alterations of signal transduction, altered membrane structure, altered G-protein activity and decreased binding of the nuclear transcription factor NF κ B (Mandrekar 1997, Spitzer 1998). Some of these inhibiting effects may be due to ethanol metabolites including acetaldehyde (Zhang 1992, Clot 1995), although the observed decreases may reverse within a 24-hour period after exposure to ethanol (Spitzer 1998). We did not determine whether acetaldehyde was produced from ethanol in our experiments. Metabolism of ethanol would require the presence of alcohol dehydrogenase (ADH) which was not measured and whilst serial concentrations of ethanol in tissue culture medium did not suggest any substantive ethanol metabolism from hepatocytes, ADH is likely to have been expressed at least in small quantities in this culture system (Maher 1995).

The experiments confirmed that each of the cell types studied secreted chemokines with appropriate stimulation, and the increase in secretion observed with TNF- α and LPS is relevant to alcoholic liver disease since serum levels of both are raised in this disease (Bode 1987, McClain 1989). It is possible that some of the chemokine secretion measured from primary human hepatocytes was actually derived from contaminating non-parenchymal cells, although quantitatively this contribution will have been small since the hepatocyte preparations are consistently > 95% pure (Strain 1991). *In vivo* it is likely that interactions between these cell types leads to amplification of chemokine secretion and leucocyte recruitment (Thornton 1991, Armendiaz-Borunda 1991, Maher 1995). It is interesting to compare our results with rodent experiments done by other workers, who have found that isolated liver cell constituents from repeated alcohol-fed rats with liver injury show clear enhancement of expression of activation markers and cytokine secretion (Bautista 1995, Pennington 1997). These studies would support the notion that hepatic inflammation is perhaps inhibited during the presence of alcohol itself but delayed

stimulation may then occur after withdrawal of alcohol; further evidence for this notion is provided by observations in alcohol-abusing humans who had a transient rise in serum IL-8 levels immediately after hospitalisation and withdrawal of alcohol (Masumoto 1993).

Since release of chemokines *in vivo* following alcohol ingestion is likely to depend on factors other than the prevailing plasma or tissue concentration of ethanol encountered, we extended our studies to human volunteers in an attempt to replicate the actual net effect of alcohol ingestion. The results of these studies are nevertheless difficult to interpret because different effects on PBMC secretion of MCP-1 were seen according to whether or not food was taken along with alcohol. The reduction in MCP-1 secretion observed after alcohol was taken alone would be in keeping with our other (*in vitro*) data showing reduced secretion from isolated cells in the presence of alcohol. Furthermore, this also supports other studies showing reduction in LPS-induced cytokine secretion following infusion of alcohol in rat models of alcoholic injury (D'Souza 1989, Nelson 1989). However it was noteworthy that, in contrast, MCP-1 secretion was increased when the alcohol was taken with food and there are several possible reasons for this: (i) chemokine secretion may vary at different timepoints after alcohol ingestion and food will delay the kinetics of alcohol absorption (Persson 1991); (ii) the dietary fat intake is an important factor in governing the severity of alcoholic liver injury in human and experimental alcoholic liver disease (Lieber 1970, Matsuoka 1990). Thus the presence of circulating lipids after a large meal may potentiate the secretion of chemokines, perhaps via the presence of oxidised lipids (Matsuoka 1990); (iii) finally endotoxin levels may have been raised after the alcohol had been taken with food; we were unable to confirm this possibility as we did not make the appropriate measurements; however we did confirm the absence of any rise when the alcohol was taken alone.

It appears unlikely from our experiments that food alone would have altered chemokine secretion significantly in the absence of alcohol. However, it is recognised that alcohol ingestion itself leads to decreased secretion of TNF- α and IL-1 from PBMC's *ex vivo* (Nair 1994, Szabo 1996). This is presumably due to a direct effect of alcohol rather than via other mediators such as lipopolysaccharide, because alcohol leads to increased intestinal permeability which in turn leads to increased plasma lipopolysaccharide

(Bjarnason 1984, Persson 1991) and the latter would presumably act as a stimulus to cytokine or chemokine secretion. Another group has reported the presence of circulating cytotoxic proteins generated by alcohol ingestion that could presumably influence the spontaneous secretion of cytokines from circulating leucocytes (Wickramasinghe 1987). Thus the net effect of alcohol ingestion on chemokine secretion is likely to be the result of several interacting factors, and it is noteworthy that one group has found that after an initial suppression of cytokine secretion, alcohol ingestion leads to a delayed increase (Szabo 1994).

The possibility of enhanced chemokine secretion from hepatocytes and other cells following exposure to low concentrations of acetaldehyde is deserving of further study since this is likely to have implications for the pathogenesis of alcoholic liver disease. Furthermore, the possibility of increased chemokine secretion from circulating leucocytes after exposure to alcohol and food is of potential importance since these leucocytes are likely to enhance hepatic inflammation on passage through the liver. Further studies should aim to confirm these findings and perhaps examine the possibility of altered levels of chemokine secretion at different timepoints following alcohol ingestion.

Chapter 6

A Pilot Study of Pentoxifylline in Severe Alcoholic Hepatitis

INTRODUCTION

Pentoxifylline (PTX, also known as oxpentifylline) is a xanthine derivative which was initially characterised as a haemorrheological agent (i.e. altering blood viscosity), and is of demonstrated value in the treatment of peripheral vascular disease (Aviado 1984, Ciuffetti 1991). However, PTX also inhibits the intracellular enzyme phosphodiesterase, resulting in increased levels of the intracellular 'second messenger' cyclic AMP. In recent years this mode of action has been found to modify release of cytokines via inhibition of mRNA transcription (Bessler 1986, Navarro 1996, Doherty 1991). This inhibitory effect appears particularly pronounced for the pro-inflammatory cytokine TNF- α , with a lesser effect upon IL-1 β and IL-2 secretion, and little or no effect upon IL-6 and IL-8 (Bienvenu 1995, Benbernou 1995, van Furth 1995, Rieneck 1993). The mechanism of action of PTX is summarised in figure 6.1.

Clinical studies with PTX at oral dosages of 1200-2000mg daily show a significant effect in suppressing TNF- α release from cultured mononuclear cells (Neuner 1994, Bauditz 1997, Bianco 1991). These studies have also shown that PTX inhibits production of the immuno-modulatory cytokines interferon- γ , IL-4 and IL-10, which may theoretically counteract the anti-inflammatory effect of PTX in clinical usage. Nevertheless, the net effect of PTX administration appears to be an immunosuppressive one and PTX is of value in a range of disease states which are characterised by increased levels of circulating TNF- α including graft versus host disease (Attal 1993, Bianco 1991), cancer-related cachexia (Dezube 1993) and AIDS-related cachexia (Landman 1994). However, some studies have failed to show any effect where suppression of TNF- α might be expected to be beneficial including septic shock (Zeni 1996) and severe Crohn's disease (Bauditz 1997).

PTX therapy is well tolerated in most subjects. Potential side effects include nausea, vomiting, diarrhoea, agitation, headache, sleep disturbance, flushing or hypotension. Hypersensitivity reactions such as pruritus or rash are said to be rare (data contained in product information leaflet, Hoechst Marion Roussel Ltd.).

Since alcoholic hepatitis is characterised by increased levels of circulating and hepatic TNF- α and other proinflammatory cytokines (Bird 1990, Ohlinger 1993, Khoruts 1991), we hypothesised that PTX therapy may be beneficial for this disease. Furthermore, we

hypothesised that PTX may suppress chemokine secretion directly, since transcription of mRNA for chemokines such as MCP-1 and MIP-1 α is sensitive to NF- κ B activation (Tsukamoto 1996) which may be inhibited by PTX (Navarro 1996). We therefore carried out an uncontrolled pilot study of PTX in severe alcoholic hepatitis and did in vitro studies to determine the direct effect of PTX on PBMC chemokine secretion.

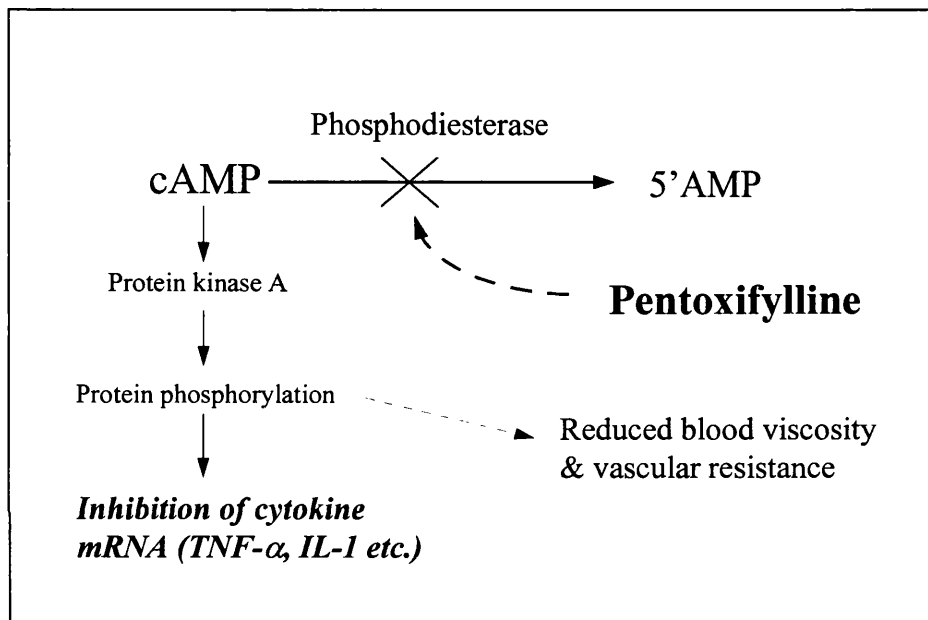


Figure 6.1 Mechanism of action of pentoxifylline. Inhibition of phosphodiesterase leads to accumulation of cyclic AMP which in turn leads to protein phosphorylation via protein kinase A. Further 'downstream' effects are poorly understood but this leads to inhibition of cytokine mRNA transcription.

PATIENTS AND METHODS

Patients

We studied patients with clinically validated and biopsy-proven severe alcoholic hepatitis as defined by (i) a history of sustained substantial alcohol consumption (around or more than 80g daily) over several years corroborated by close relatives, (ii) characteristic histological features of active alcoholic hepatitis on liver biopsy (International Group 1981) and (iii) a discriminant function [i.e. (increase in prothrombin time in seconds x 4.6) + (serum bilirubin in μM divided by 17)] approaching or greater than 32 (a value of 32 or more indicates a 50% risk of mortality within 2 months, Maddrey 1978). All patients had consumed alcohol within 3 months of inclusion into this study and were referred to our unit for specialist management of their liver disease. All patients had biopsies (percutaneous or transjugular) for clinical purposes within 14 days of commencing PTX, and we excluded patients who had serological or histological evidence of other contributing causes of liver disease (e.g viral or autoimmune hepatitis, alpha-1-antitrypsin deficiency or haemochromatosis). We also excluded any patients with active microbiologically-proven sepsis or recent gastrointestinal haemorrhage. Patients were otherwise managed conventionally with nutritional support, thiamine and other B vitamin supplementation, but without corticosteroids. Portosystemic encephalopathy occurring during the study was managed with lactulose and empirical antibiotic therapy, and variceal haemorrhage was managed with sclerotherapy.

Pentoxifylline dosage and monitoring

Since PTX is metabolised within the liver and its half-life is increased at least two-fold in the presence of severe hepatic impairment (Rames 1990), we selected a lower dosage than has previously been used in clinical studies of PTX therapy for non-hepatic disease. Thus patients were given 400mg twice daily by mouth for 4 weeks, commencing therapy on an in-patient basis and then continuing as an out-patient if appropriate. Compliance was then verified by asking patients to return their medications and counting the remaining tablets. In all patients we measured 'trough' concentrations of PTX in serum 24 hours after the first dose (i.e. 12 hours after the second dose) in order to determine the early levels of PTX.

Thereafter in 2 patients, after 14 days of therapy, we also monitored serial serum concentrations of PTX and its principal metabolites pre-dosage and then 1, 2, 3, 4, 8 and 12 hours after PTX was given at the usual dosage. This was done to evaluate the pharmacokinetic profile of PTX after treatment had been fully established and stabilised. Measurement of PTX and its metabolites was kindly done by Dr.T.Bryce at the Hoechst Marion Roussel Drug Development Laboratories, Milton Keynes, UK, using a gas chromatographic method established in that laboratory (Bryce 1989). We recorded serum levels of PTX and of its principal metabolite M1 or hydroxy-pentoxifylline, which has similar pharmacological activity to the parent compound (Ambrus 1995).

Clinical and laboratory follow-up

During therapy we monitored the following clinical and laboratory variables on a weekly basis: Haemoglobin concentration, peripheral white cell count, platelet count, serum bilirubin, aspartate transaminase (AST), albumin, electrolytes and creatinine, prothrombin time; mean arterial blood pressure, presence of ascites and presence of encephalopathy. We also aimed to measure MCP-1 secretion from PBMC's (isolated and cultured as described in appendix 5) in study patients prior to and on completion of 4 weeks' therapy. We also assessed for any recognised or other potential side-effects of therapy and recorded any other major clinical events during the study period.

Pentoxifylline and PBMC chemokine secretion in vitro

To examine the effect of PTX upon chemokine secretion *in vitro*, we did experiments using PBMC's from 4 healthy volunteers isolated and cultured for 24 hours as described in appendix 5. PBMC's were cultured in the presence or absence of lipopolysaccharide (LPS, 1 µg/ml and with or without PTX (kindly provided by Hoechst Marion Roussel Ltd.) at concentrations from 1 to 100 µg/ml. Culture supernatants were purified by centrifugation, aliquoted and stored at -70° C until analysis. MCP-1 and IL-8 were measured using customised ELISA's described in appendix 6. Statistical analysis of these results was done with SPSS software using the Wilcoxon rank sum and Friedman's ANOVA test.

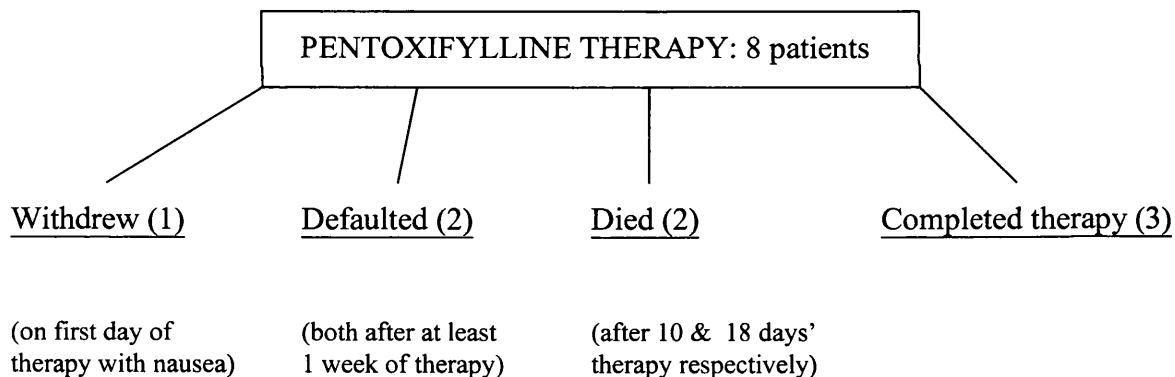
This study was approved by the Research Ethics Committee of the University Hospital Birmingham NHS Trust and patients gave informed consent before participation.

RESULTS

Patients and clinical course during therapy

Eight patients were recruited to the study; 4 male, 4 female; median age 46 (range 35 to 55). Alcoholic hepatitis was verified histologically by transjugular biopsy in 7/8 patients and by percutaneous biopsy in the remaining patient. All patients except one had associated cirrhosis; the remaining patient had severe fibrosis. At the time of recruitment into the study, median values for liver function tests (with range in brackets) were as follows: serum bilirubin 328 μ M (52-850), AST 113 U/L (31-235), albumin 26 g/L (18-35), prothrombin time 18s (15-20). Median serum creatinine value was 104 μ M (80-202) and median discriminant function value was 48.2 (24.7 - 68.4). All patients except 2 had clinically detectable ascites but only one patient had clinically detectable hepatic encephalopathy which was mild and fluctuant.

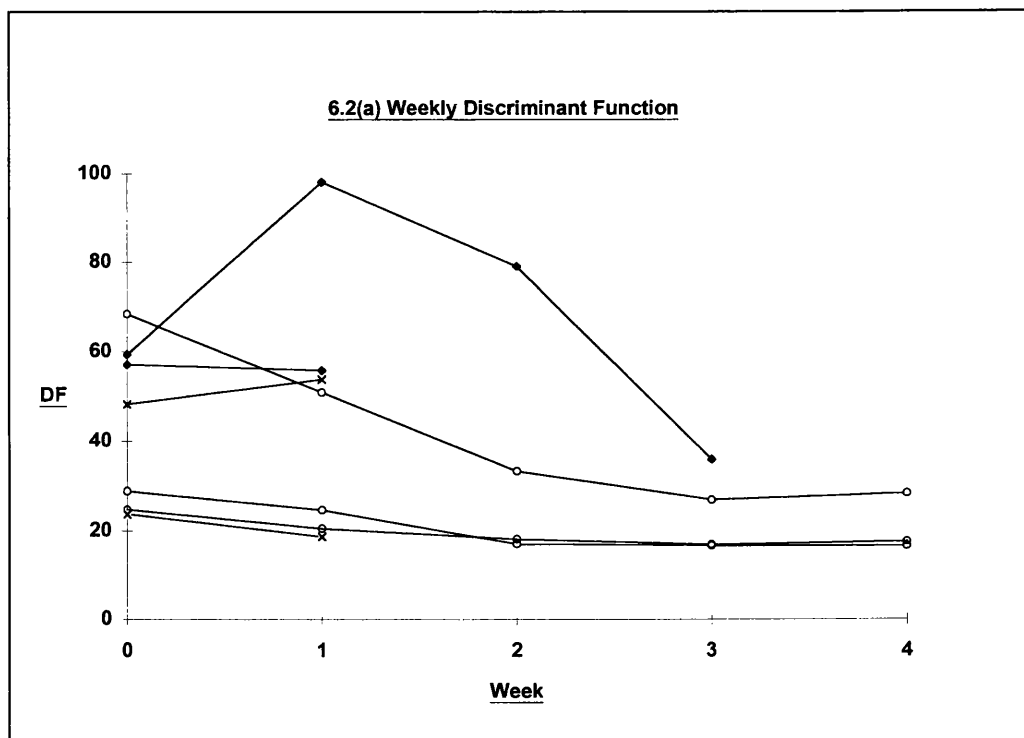
The course of therapy following commencement into the study is summarised below:



The patient who withdrew did so because of nausea after the second dose of PTX. Of the 2 defaulting patients, the first was a recently bereaved woman who was discharged from hospital after commencing therapy and did not appear to be experiencing any adverse effect on review after one week of therapy; however she did not attend for follow-up review the following week and did not respond to our attempts to contact her thereafter. Some weeks after the end of the study period she was found to have relapsed into alcoholism and was found dead in her own home. The second patient was also discharged

from hospital (to an alcohol rehabilitation clinic) after commencing therapy and was progressing well at the first weekly follow-up but did not return thereafter; he was admitted 3 weeks later in an intoxicated state after relapsing into alcoholism. The 2 deaths in the study period both occurred in patients who developed variceal haemorrhage during the study period; in both cases this was complicated by circulatory failure and death within 3 days; both patients were complying with therapy at the time of variceal haemorrhage (one was an in-patient throughout the study). The 2 patients who died during therapy had amongst the highest discriminant function values at entry into the study (57.1 and 59.4 respectively) of all patients. The 3 remaining patients completed therapy without apparent complication and all survived for at least 6 months after the study period.

Serial weekly recordings of discriminant function, serum AST and creatinine during therapy are illustrated in figures 6.2a - 6.2c. There were no significant alterations in full blood count indices during therapy. No patient developed new encephalopathy whilst taking PTX therapy other than the 2 patients who died after variceal haemorrhage, both of whom became encephalopathic only after haemorrhage. There was no apparent exacerbation of ascites during therapy in any patient and no discernible effect upon mean arterial pressure (median 80 mmHg at onset and 85 mmHg at completion of therapy).



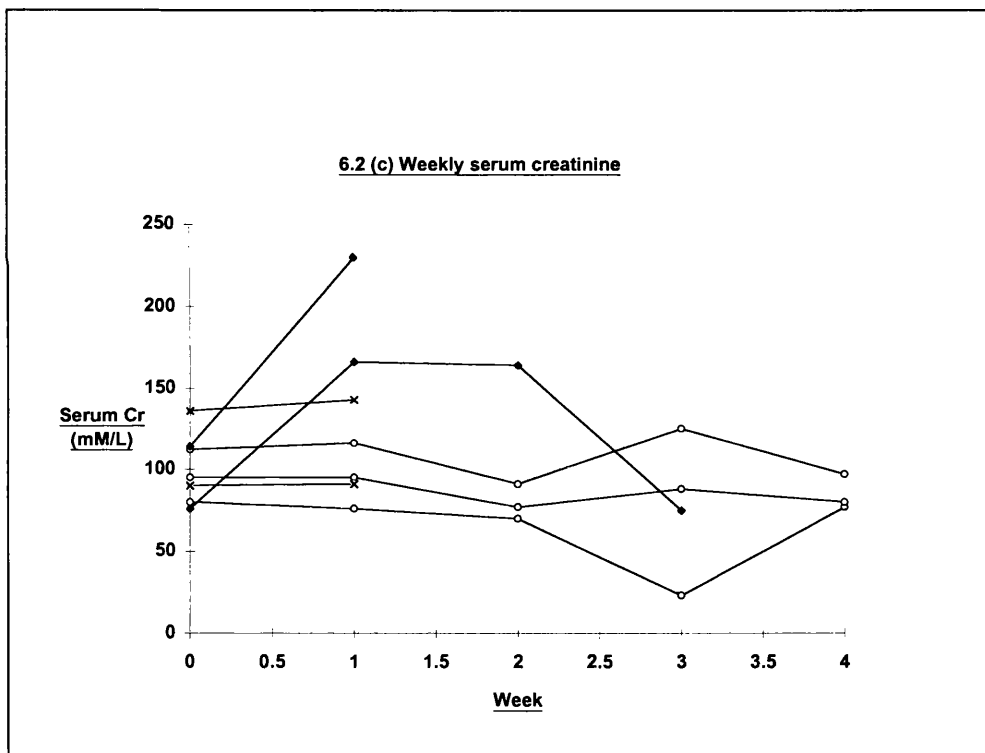
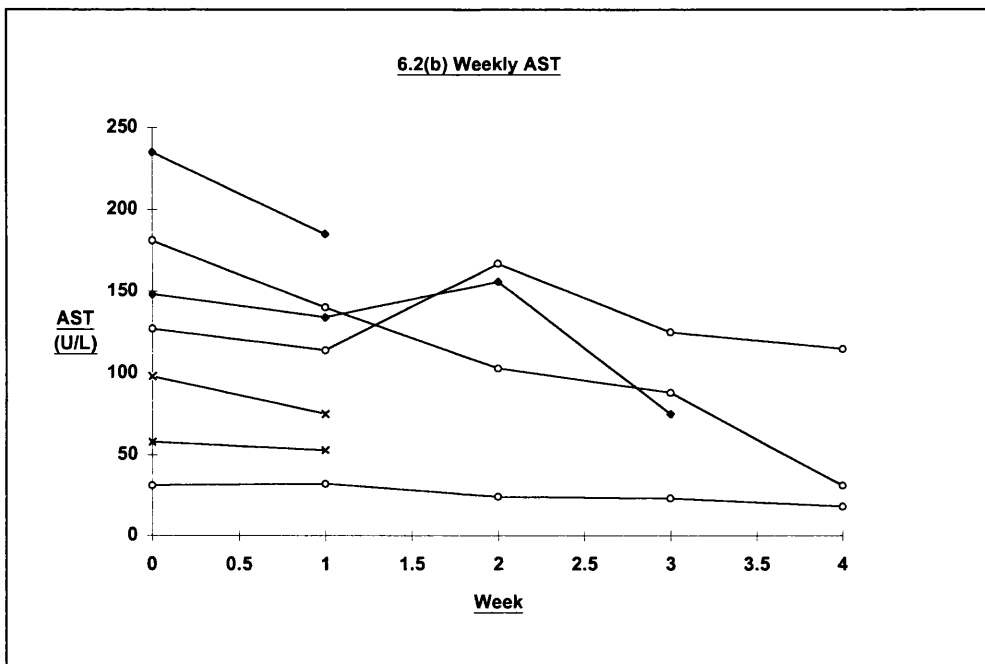


Figure 6.2 Serial laboratory indices during the 4 week study period of pentoxifylline therapy in 7 patients who continued therapy for at least 1 week (N=7). (a) Discriminant function (DF) values; (b) serum aspartate transaminase (AST) values; (c) serum creatinine values (see page 41 for explanation of DF value).

Keys: O = patients completing 4 weeks of therapy; X = patients defaulting during the study period; ♦ = patients dying during the study period.

Ex vivo PBMC secretion of MCP-1 following PTX therapy

We analysed ex vivo production of MCP-1 from PBMC's before and after 4 weeks of PTX therapy in 2 patients who completed the study. There was no significant reduction in secretion; levels at the onset of therapy were 3.24 and 2.62 ng/ml respectively and corresponding levels at the end of the study were 3.10 and 2.54 ng/ml.

Pharmacokinetic monitoring

After the first 24 hours of therapy the median serum PTX level in 7 patients analysed was 125 ng/ml (range 106-280) and median level of the metabolite M1 was 524 ng/ml (range 272-857). The serial levels of PTX and M1 measured inbetween 2 doses midway during the course of the study period are illustrated in figure 6.3.

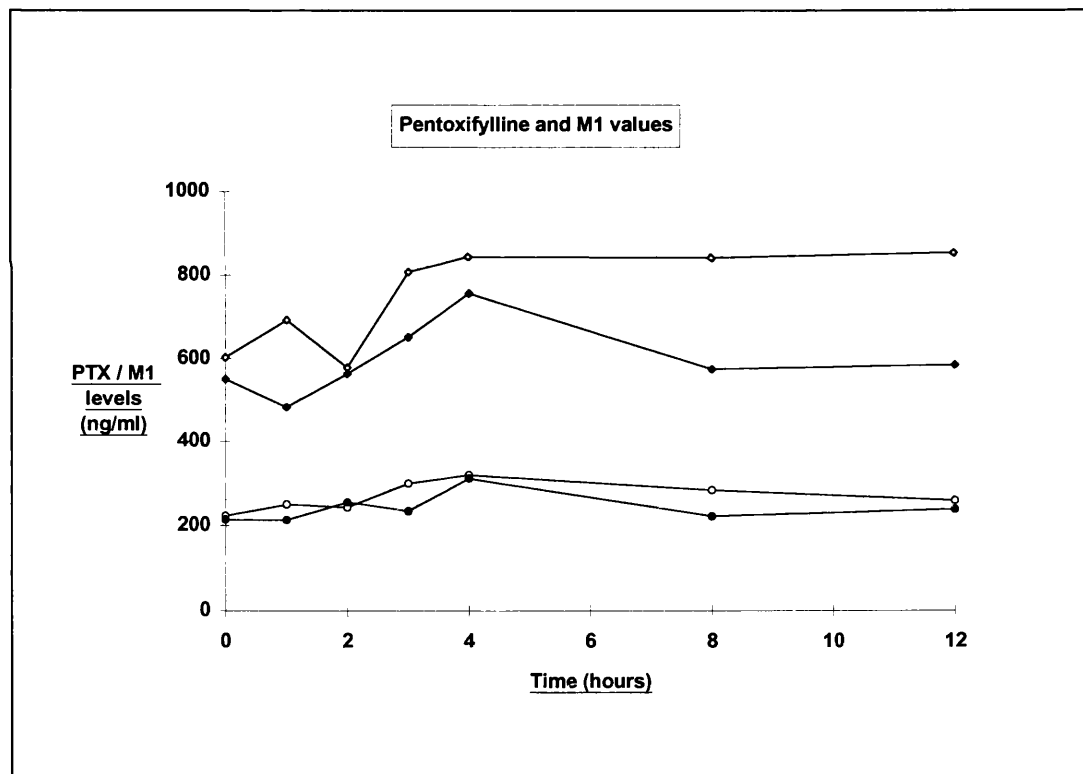


Figure 6.3 Serial levels of pentoxifylline (PTX) and its principal active metabolite hydroxypentoxifylline (M1) over a 12-hour period midway during the 4-week study period in 2 patients

Key: O = PTX; \diamond = M1; shaded data series = patient 1; unshaded data series = patient 2.

Note; PTX concentrations of 1 μ g/ml have previously been shown to reduce inflammatory cytokine secretion in vitro (Reimund 1997). The pharmacological potency of PTX and M1 is similar (Ambrus 1995).

In vitro PBMC chemokine secretion and PTX

The effect of PTX on secretion of MCP-1 and IL-8 from PBMC's is illustrated in figures 6.4a and 6.4b. There was a serial reduction in MCP-1 secretion in both unstimulated and LPS-stimulated culture with increasing dosage of PTX (figure 6.4a). With unstimulated culture mean MCP-1 secretion (\pm SD) was 5.57 ± 0.43 ng/ml, reducing to 4.61 ± 1.00 ng/ml with PTX at a dosage of 1 μ g/ml ($p=0.07$, Wilcoxon rank sum). With higher doses of PTX the reduction in secretion became more marked ($p<0.01$, Friedman's test). The findings were similar with LPS-stimulated culture.

In contrast, there was no consistent effect of PTX upon IL-8 secretion with unstimulated or LPS- stimulated culture (figure 6.4b). With unstimulated culture, mean IL-8 secretion (\pm SD) was 8.98 ± 1.36 ng/ml, which actually rose to 11.86 ± 2.93 ng/ml with PTX at a dosage of 1 μ g/ml ($p=0.14$, Wilcoxon rank sum). With higher doses of PTX, however, IL-8 secretion was reduced (difference not significant, Friedman's test). Likewise there was no apparent alteration of IL-8 secretion with PTX in LPS-stimulated culture.

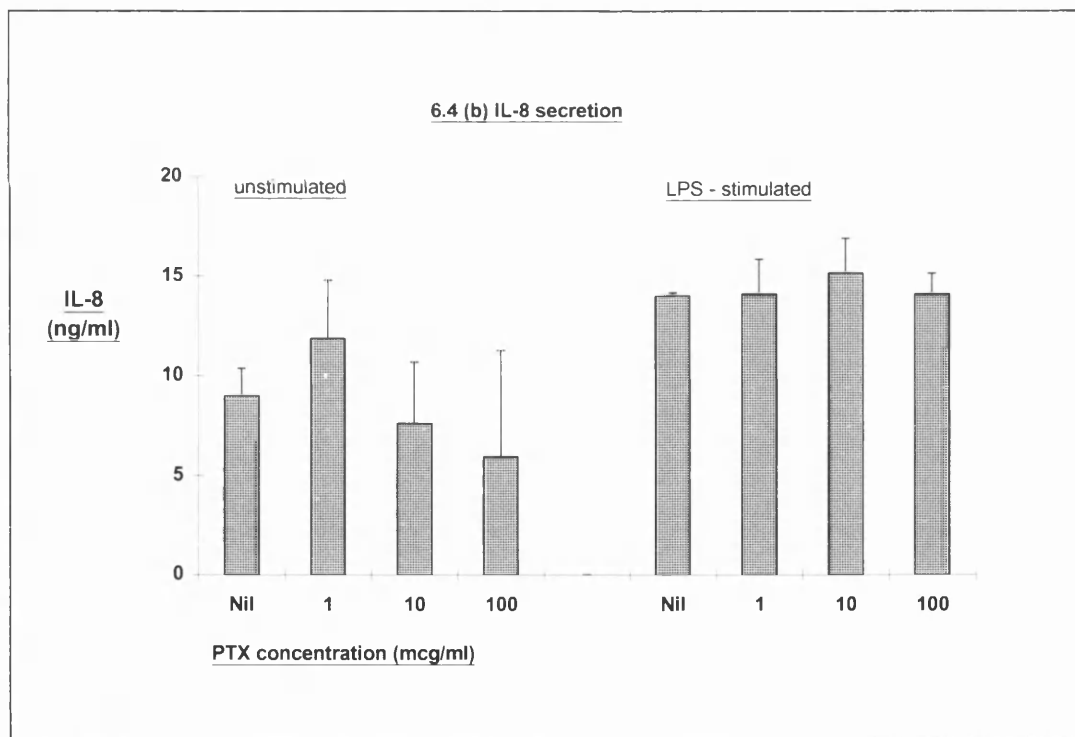
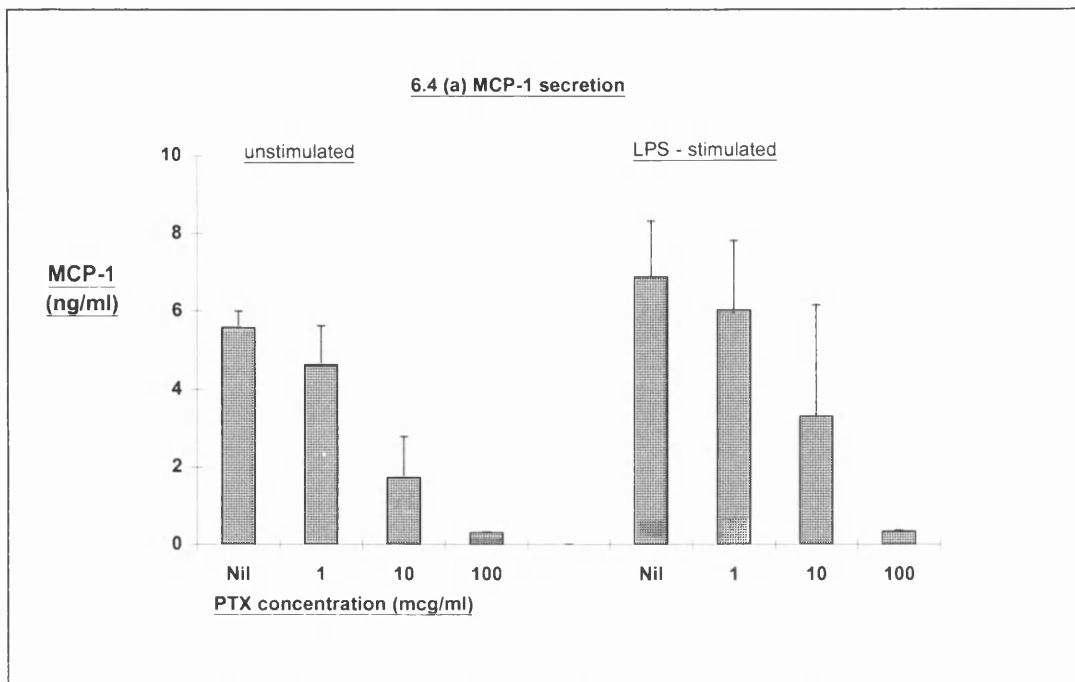


Figure 6.4 Peripheral blood mononuclear cell (PBMC) secretion of (a) MCP-1 and (b) IL-8 from 4 healthy volunteers with or without lipopolysaccharide (LPS) at 1 $\mu\text{g/ml}$ and with or without pentoxifylline (PTX) at serial concentrations of 1, 10 and 100 $\mu\text{g/ml}$; values shown are mean \pm SD (effect of PTX on MCP-1 secretion; $p < 0.01$, effect of PTX on IL-8 secretion, difference not significant, Friedman's ANOVA test).

DISCUSSION

Our pilot study suggests that further studies of the role of PTX in treatment of severe alcoholic hepatitis are merited. One such study was in fact presented whilst our own was in progress; this was a large randomised controlled trial of PTX in alcoholic hepatitis (Akriviadis 1997). The study had similar entry criteria to our own but used PTX at a larger dosage of 400mg by mouth thrice daily. Forty-eight patients were treated in each (treatment and placebo) arm, and the groups appeared well-matched at randomisation with mean discriminant function values of 44.0 and 43.6 respectively. After therapy a clear but non-significant trend towards reduced mortality was seen in the active treatment group (11/48 vs. 20/48); this reduction appeared largely attributable to a significant reduction in the incidence of deaths due to hepatorenal syndrome (5/48 vs. 18/48, $p < 0.01$). These findings may suggest a beneficial role for PTX in preventing the onset of hepatorenal syndrome, perhaps by reducing secretion of the vasoactive mediators (including pro-inflammatory cytokines) that are implicated in the pathogenesis of the syndrome (Moore 1997).

Our own study was too small to draw any meaningful conclusions regarding clinical benefit of PTX in alcoholic hepatitis, and was designed to assess the tolerability of this agent whilst ensuring that the clinical outcome in treated patients was at least no worse than would have been expected otherwise. No patient refused to take part in our study although it was disappointing that 1 patient withdrew at any early stage because of nausea, a recognised side-effect of PTX. It was my impression that this patient had some difficulty coming to terms with the potentially stigmatising disease label of alcoholic hepatitis; no other patient reported onset of nausea during the study period. It was also disappointing that 2 patients defaulted from follow-up and relapsed into alcoholism; this highlights the difficulties of securing full compliance with therapy in alcoholic liver disease patients generally. The 2 deaths that occurred during compliant therapy were entirely consistent with the expected mortality rate and were partly predictable by poor prognostic indices at entry into the study.

The pharmacokinetic studies suggested that sustained 'steady state' levels of PTX were achieved in plasma within a few days of commencing therapy, and the relative lack of inter-dose variability in levels presumably reflects the prolonged half-life of PTX and its metabolites in hepatic insufficiency (Rames 1990). Whether a more frequent or higher dosage regime than the one we used would be clinically advantageous is uncertain.

The *in vitro* studies of PTX supported our hypothesis that this agent may alter secretion of chemokines. The effects of PTX observed *in vitro* at a concentration of 1 µg/ml may correlate roughly with the effects of PTX *in vivo* at the dosage used in our patients, since the sum of the concentrations of PTX and its metabolite M1 (which are of roughly equivalent potency) tended to approximate to 1 µg/ml after steady state concentrations had been reached. However, if tolerable, it seems likely that higher dosages would be required to exert a more potent 'immunosuppressive' effect. Interestingly, *in vitro* secretion of MCP-1 was reduced in a clear dose-dependent fashion but there was no clear effect upon IL-8 secretion. Reduced secretion of MIP-1α with PTX has been reported previously (Burgmann 1995), whilst reports of its influence on IL-8 are conflicting (Neuner 1994, Reimund 1997). Thus the effect of cyclic AMP accumulation upon chemokine secretion may differ for alpha and beta chemokines although it is also possible that PTX may influence chemokine secretion in other ways.

Whilst PTX may lead to reduced secretion of some chemokines, the absolute benefit of this reduction in the setting of alcoholic hepatitis and the possible diminution of this effect by counter-balancing actions upon 'immuno-modulatory' cytokines remains speculative. An analogous situation may be drawn with corticosteroid therapy, which, despite reducing TNF-α secretion (Vane 1987), is of debatable value in alcoholic hepatitis (Christensen 1995).

PTX may additionally have a potentially beneficial role in alcoholic hepatitis (and other liver diseases) via haemorrhheological effects; a significant increase in hepatic blood flow was reported in patients with cirrhosis following an intravenous bolus of 200mg PTX (Kopenhagen 1979) and PTX was reported to reduce portal venous pressure in a rat model of cirrhosis (Sanchez 1997). The latter study contrasts with the effects of steroids in the study by Maddrey (1978) in which no reduction in wedged hepatic venous pressure

gradients was found in patients given corticosteroids compared to those given placebo for severe alcoholic hepatitis in a 4-week randomised controlled study.

In summary, we found that PTX therapy did not cause apparent symptomatic side-effects in most patients with alcoholic hepatitis at the dosage that we used, although tolerability was difficult to assess because of small numbers of patients completing therapy. The dosage that we used led to sustained levels of parent compound and its active metabolite in vivo which could be expected to have beneficial effects in alcoholic hepatitis via reduction of cytokine and chemokine synthesis, and perhaps via other vascular effects. Other workers have shown a beneficial role for PTX in alcoholic hepatitis when used at a higher dosage and further independent confirmation of this benefit could establish a clear role for PTX in this disease.

Chapter 7

TNF- α Promoter Polymorphisms and HLA-DR3 and DQ2 Haplotypes in Alcoholic Liver Disease

INTRODUCTION

It is well recognised that genetic factors play an important role in determining individual susceptibility to alcoholic liver disease (Devor 1989, Lumeng 1994), and these are assumed to account for at least some of the wide variation in severity of liver injury in individuals consuming excessive amounts of alcohol (Derr 1990). Genetic factors that influence the development of the disease include polymorphisms for the enzymes metabolising alcohol that lead to accelerated formation of its reactive metabolites, including alcohol dehydrogenase (Sherman 1993), aldehyde dehydrogenase (Enomoto 1991) and the cytochrome P450 enzyme 2E1 (Pirmohammed 1995). There are also possible genetic associations in individuals possessing the HLA haplotypes A2, B8, B13, DR2 and DR3 with alcoholic liver disease (Saunders 1982, Devor 1989); whilst the mechanism of these associations is unknown, they do suggest the possibility that genetic variation in immune mechanisms may influence the development of alcoholic liver disease.

Since the recognition and characterisation of increased expression and circulating levels of pro-inflammatory cytokines in alcoholic liver disease in the late 1980's and 1990's (McClain 1993), interest has developed into whether genetic variability in expression of cytokines may influence risk of developing the disease. Variations in expression of TNF- α in alcoholic liver disease are of particular interest since this cytokine plays a vital role in activating leucocyte effector functions and in upregulating expression of other cytokines (including chemokines) in this disease as discussed in the earlier chapters of this thesis. The TNF- α promoter gene lies on chromosome 6 near to the HLA histocompatibility gene complex and is a candidate gene for genetic variation in TNF- α expression. Two polymorphisms in the TNF- α promotor region have been described, including a substitution of guanine by adenine at base pair position -308 (-308_{G-A}), referred to as the TNF1/ TNF2 allele polymorphism (Wilson 1993), and a substitution of guanine by adenine at base pair position -238 (-238_{G-A}) (D'Alfonso 1994). The TNF2 allele is closely linked to the presence of the HLA A1, B8, DR3 haplotype, suggesting co-inheritance by linkage disequilibrium (Wilson 1993). Possession of this allele has been described as an independent risk factor for death from cerebral malaria (McGuire 1994) and severe sepsis

(Stuber 1996). Prior to the discovery of this polymorphism it was recognised that inducible TNF- α production was higher in subjects possessing the HLA DR3 and DR4 alleles compared to those possessing the DR2 allele (Jacob 1990), and this may now at least partly be explained by the discovery that the TNF2 allele leads to more avid transcription of TNF- α activity compared to the more common TNF1 allele (Wilson 1997), which also suggests that excessive TNF- α transcription may be a risk factor for more severe disease under some circumstances. However it remains unclear whether the association of certain HLA haplotypes (including HLA A1, B8, DR3) with inflammatory diseases occurs because of the co-existence of higher transcription of TNF- α activity, or whether the reverse association is true, i.e. the TNF2 allele being associated with higher mortality in some diseases because of its association with these HLA haplotypes. The TNF -238_{G-A} allele may also lead to higher transcription of TNF- α since it occurs within a putative regulation box of the TNF- α promoter region, similar to HLA class II promoter regions (D'Alfonso 1994). It has been shown that possession of this allele is a significant risk factor in susceptibility to pulmonary tuberculosis in Africans (Ruwende 1996).

It has been suggested that inheritance of TNF- α promoter polymorphisms may influence the risk of developing alcoholic liver disease. Allott (1996) found a weak but significant association between the TNF2 allele and clinically evident alcoholic cirrhosis. Grove (1997) failed to corroborate this association but found another weak but significant association between the TNF -238_{G-A} allele and the presence of hepatic steatohepatitis in biopsy-proven alcoholic liver disease.

We have examined the hypothesis that one or other of the 2 described polymorphisms in the TNF- α promoter region may be associated with a higher risk of alcoholic liver disease in our own patient population. If confirmed then any such association would strengthen the likelihood of other immunoregulatory genetic polymorphisms, including those for chemokine transcription, playing a role in the development of alcoholic liver disease. We also did haplotyping for the HLA alleles DR3 and DQ2 in order to (i) verify a previous study suggesting an association between HLA DR3 with alcoholic liver disease, (ii) extend this study to include the DQ2 allele, (iii) confirm and explore the reported independent associations between these TNF- α polymorphisms and HLA haplotypes and (iv) determine which provided the stronger association with alcoholic liver disease; TNF- α

polymorphisms or the HLA haplotypes studied. Our study was done only in patients with clinically severe liver disease and was done in conjunction with Dr. Munir Pirmohammed at the Department of Pharmacology and Therapeutics, Royal Liverpool University Hospital, as part of a larger collaborative study. The results presented here are only those of the patients from our own centre; the results from the patient group at Liverpool have been published previously (Allott 1996).

PATIENTS AND METHODS

Patients

We studied a total of 136 patients (male 90, female 46; median age 50 years, range 26-70). 128 patients were white caucasians and 8 were from the Indian subcontinent. Patients were only included in the analysis if they had clinically decompensated liver disease (i.e. presenting with any combination of ascites, variceal haemorrhage and jaundice with serum bilirubin $>100 \mu\text{M}$), and a history of prolonged alcohol abuse, and where other likely causes of liver disease had been excluded by blood tests (e.g. absent serum markers for viral hepatitis, autoimmune liver disease, haemochromatosis, α -1 antitrypsin deficiency and Wilson's disease), radiological imaging and, where possible, histological analysis of liver biopsy or explanted liver tissue. Liver disease was confirmed histologically (and other liver disease excluded) in 103/136 (76%) of cases (percutaneous biopsy; 34, transjugular biopsy; 43, explanted liver; 26), although in 17 cases the full histology reports were not available for critical review for this study as biopsies had been done at other centres. Of the 86 cases with histological review, 73 had cirrhosis and 13 were non-cirrhotic (but all with some degree of fibrosis), with 49/86 (57%) showing additional steatohepatitis (defined here as fatty change, ballooning degeneration and parenchymal leucocyte infiltration). Twenty six patients had undergone liver transplantation at the time of analysis and 15 patients had died from complications of liver disease before or in the absence of any consideration towards transplantation.

Control subjects (N=137) consisted of spouses of affected patients or members of the hospital workforce (male 63, female 74; median age 42 years, range 20-85). Of the control subjects, 126 were white caucasians and 11 were from the Indian subcontinent.

DNA extraction and analysis

The methods for extracting DNA are summarised in appendix 11; this was done in our own laboratories and samples were stored at -20°C prior to being sent to the Royal Liverpool University Hospital in batches. Analysis for the promoter polymorphisms was done by Dr. Pirmohammed's team by restriction fragment length polymorphism using the techniques described by Mansfield et al (1993) for the TNF1 / TNF2 (-308_{G-A}) polymorphisms; this technique involved PCR amplification of flanking regions and enzymatic digestion with the restriction enzyme *Nco*1, allowing identification of the polymorphic alleles by polyacrylamide gel electrophoresis and ethidium bromide staining. The techniques described by D'Alfonso et al (1994) were used to identify the TNF -238_{G-A} polymorphism; briefly this was determined using oligonucleotide probes designed to detect the appropriate genomic variation by dot blot analysis of amplified DNA material. Tissue typing for the HLA haplotypes DR3 and DQ2 was done at Dr. Pirmohammed's laboratory using a polymerase chain reaction-based technique with commercially available DNA restriction enzymes and primer sequences for the alleles in question (Olerup 1992).

Statistical analysis was done using Chi-squared test and Spearman's rank correlation where appropriate. A *p* value of <0.05 was used to indicate statistical significance. This study was done within the auspices of our 'leucocyte recruitment and alcoholic liver disease' project which was approved by the South Birmingham Health Authority Local Research Ethics Committee.

RESULTS

TNF- α polymorphisms

The frequency distribution of these polymorphisms amongst patients and controls is summarised in tables 1 and 2. The overall gene frequency for the TNF -308_{G-A} gene mutation was 19.5% in alcoholic liver disease subjects and 17.2% in healthy controls (difference not significant), whilst the gene frequency for the TNF -238_{G-A} gene mutation was 4.4 % in alcoholic liver disease subjects and 7.9% in healthy controls (difference not significant).

When both patients and control groups were combined, the overall sex-related frequencies were similar for both mutations (males 19% and females 17% for the TNF -308_{G-A} gene, and both males and females 6% for the TNF -238_{G-A} gene). Amongst Asian subjects (N=18), gene frequencies for the TNF-308_{G-A} and TNF-238_{G-A} genes were similar to the overall population at 17% and 11% respectively.

In patients with biopsy-proven alcoholic liver disease (N=103), the gene frequencies of the TNF-308_{G-A} and -238_{G-A} mutations were similar to the frequencies for the disease group as a whole, at 18.5% and 5.3% respectively. If patients were compared for presence (N=49) or absence (N=37) of steatohepatitis, then there was no excess in the gene frequency of the TNF-308_{G-A} mutation (20% in patients with steatohepatitis vs. 22% without) or the TNF-238_{G-A} mutation (1% with steatohepatitis vs. 8% without).

If patients who died (N=15) or were transplanted (N=26) for alcoholic liver disease were combined into one group (N=41), then gene frequencies of the TNF-308_{G-A} and -238_{G-A} mutations in this group were similar to the disease group as a whole, at 19.5% and 4.9% respectively.

DR3 and DQ2 haplotypes

The frequency of these haplotypes is shown in tables 3 and 4; in both cases there were non-significant trends towards higher frequencies of these haplotypes in alcoholic liver

disease subjects versus controls (DR3 positive, 34% vs. 27%, $0.1 < p < 0.5$; DQ2 positive, 54% vs. 44%, $0.05 < p < 0.1$, Chi-squared test).

When both patients and control groups were combined, the overall sex-related frequencies were similar for both haplotypes (males 29% and females 32% for DR3 positivity; males 51% and females 48% for DQ2 positivity). Amongst Asian subjects (N=18), frequencies of DR3 and DQ2 positivity were similar for the groups as a whole at 26% and 42% respectively.

In patients with biopsy-proven alcoholic liver disease (N=103), the frequencies of DR3 and DQ2 positivity were similar to the frequencies for the group as a whole, at 35% and 56% respectively. If patients were compared for presence (N=49) or absence (N=37) of steatohepatitis, then there was no excess in the frequency of DR3 positivity (36% in patients with steatohepatitis vs. 42% without) or DQ2 positivity (54% with steatohepatitis vs. 61% without).

If patients who died (N=15) or were transplanted (N=26) for alcoholic liver disease were combined into one group (N=41), then frequencies of DR3 and DQ2 positivity were similar to the disease group as a whole, at 29% and 51% respectively.

When all disease subjects and controls were analysed together, there were strongly positive correlations between DR3 and DQ2 haplotypes, and between these haplotypes and TNF- α polymorphisms as follows:

| <u>Combination</u> | <u>p value (Spearman rank correlation)</u> |
|----------------------|--|
| TNF2 (-308G-A) - DR3 | < 0.001 |
| TNF2 (-308G-A) - DQ2 | < 0.001 |
| TNF (-238G-A) - DR3 | < 0.05 |
| TNF (-238G-A) - DQ2 | 0.10 |
| DR3 - DQ2 | < 0.001 |
| TNF2 - TNFA | 0.33 |

(a) TNF- α -308_{G-A} polymorphism

TNF 1 = G at position -308
TNF 2 = A at position -308

| <u>Subjects</u> | <u>ALD</u> <u>(N=136)</u> N (%) | <u>Controls</u> <u>(N=137)</u> N (%) |
|-------------------------|---------------------------------------|--|
| TNF1 / TNF1 | 90 (66) | 92 (67) |
| TNF1 / TNF2 | 39 (29) | 43 (31) |
| TNF2 / TNF2 | 7 (5) | 2 (2) |
| (Gene frequency) | | |
| TNF1 | 81% | 83% |
| TNF2 | 19% | 17% |

(b) TNF- α -238_{A-G} polymorphism

TNF -238_G = G at position -238
TNF -238_A = A at position -238

| <u>Subjects</u> | <u>ALD</u> <u>(N=136)</u> N (%) | <u>Controls</u> <u>(N=137)</u> N (%) |
|-------------------------|---------------------------------------|--|
| TNF -238 _{G/G} | 125 (92) | 116 (85) |
| TNF -238 _{G/A} | 10 (7) | 21 (15) |
| TNF -238 _{A/A} | 1 (1) | 0 |
| (Gene frequency) | | |
| TNF -238 _G | 91% | 92% |
| TNF -238 _A | 9% | 8% |

(c) DR3 haplotype distribution

| <u>Subjects</u> | <u>ALD</u> <u>(N=136)</u> | <u>Controls</u> <u>(N=137)</u> |
|-----------------|------------------------------|-----------------------------------|
| DR3 + ve | 46 (34 %) | 37 (27 %) |

(d) DQ2 haplotype distribution

| <u>Subjects</u> | <u>ALD</u> <u>(N=136)</u> | <u>Controls</u> <u>(N=137)</u> |
|-----------------|------------------------------|-----------------------------------|
| DQ2 + ve | 74 (54 %) | 60 (44 %) |

Table 7.1 Distribution of TNF- α -308 G-A (7.1a) and -238 G-A (7.1b) polymorphisms, and DR3 (7.1c) and DQ2 (7.1d) haplotypes in alcoholic liver disease (ALD) subjects and controls. None of the differences in TNF- α polymorphisms or HLA haplotypes between ALD subjects and controls reached statistical significance.

DISCUSSION

This study does not support the hypothesis that TNF- α promoter polymorphisms at the -238 or -308 loci influence the risk of alcoholic liver disease. As with some of the previously published studies concerning genetic associations in this disease, our study could be criticised on methodological grounds. Owing to the nature of our practice, our patients were from diverse geographic backgrounds, whilst control subjects were predominantly local and not specifically matched to patients. Nevertheless, the gene frequency of the genetic mutations studied in our control subjects was similar to previously published series for both the -238 and -308 loci; Wilson et al (1993) and Allott et al (1996) found gene frequencies of 16% and 15% for the TNF2 (-308_{G-A}) mutation in controls, compared to our finding of 17%, whilst Grove et al (1997) found a gene frequency of 7% for the TNF -238_{G-A} mutation in controls, compared to our finding of 8%. Our alcoholic liver disease group was potentially flawed by design in that some patients did not have biopsy-proven disease; however the validity of this group was substantiated by having otherwise secure inclusion criteria and in our practice it is very unusual to find histological evidence of unexpected liver disease if a history of sustained alcohol abuse is obtained and other diseases have been excluded by careful clinical, serological and radiological analysis. Furthermore the inclusion criterion of clinically severe liver disease will by definition exclude those with otherwise milder or covert alcoholic liver disease and might therefore be expected to provide the most 'informative' group of patients for a study of this nature.

Whilst the possibility of the TNF2 (-308_{G-A}) allele as a risk factor for alcoholic liver disease as suggested by Allott (1996) was not excluded by our study, since there was a small trend towards this association, the most striking finding was the contrast with the findings of Grove (1997) in our failure to demonstrate any association between the TNF -238_{G-A} gene mutation and steatohepatitis. Those conclusions were based upon a relatively small number of patients with alcoholic liver disease carrying this mutation (only 13/ 150 patients were heterozygote carriers for the mutation, with no homozygotes being found, and 9 of the 13 heterozygote carriers had steatohepatitis). Those conclusions may therefore have been subject to a type 1 error (false-positive association). Furthermore, when stratifying patients for the presence of steatohepatitis it is important to consider that this

lesion is likely to diminish with sustained abstinence from alcohol (as evidenced by the rarity of steatohepatitis in explanted livers from patients undergoing transplantation for alcoholic liver disease at our centre), and this phenomenon will confound studies that attempt to identify genetic risk factors for steatohepatitis unless concurrent alcohol abuse is used as an inclusion criterion.

The increased incidence of the DR3 and DQ2 haplotypes in alcoholic liver disease subjects in our study group was slightly more convincing, although still not statistically significant. As with the TNF- α promoter polymorphisms, far greater numbers of patients with alcoholic liver disease may need to be studied in order to confirm or refute a significant association. Another approach using a different control population, which is considered more scientifically robust, is to use the transmission disequilibrium test (TDT) for genetic linkage whereby both parents of any affected individual are studied and used as the controls. Any excessive incidence of the target mutation in the patient group compared to the parental group can then be used as evidence for linkage (Spielman 1996). This test has been used to refute the possibility of mutations in the serotonin transporter gene (*HTT*), which had previously been proposed based upon population-based studies, acting as a risk factor for alcohol dependence (Edenberg 1998). In any event, in the case of the TNF- α promoter polymorphisms that we have studied, the lack of any definite conclusions from studies carried out so far suggest that these genetic factors are likely to be of limited importance in influencing the risk of alcoholic liver disease in any individual subject.

This study could suggest that the raised tissue and circulating levels of TNF- α occurring in alcoholic liver disease are therefore purely the result of 'upstream' activation occurring as a consequence of alcohol abuse, rather than a result of genetically determined excessive production. Alternatively, if the TNF2 (-308_{G-A}) mutation and/ or the HLA-DR3 and DQ2 haplotypes do have a true association with susceptibility to alcoholic liver disease, then since these genes are inherited in linkage disequilibrium it is possible that they are simply markers for another, more important, risk factor for disease that is also closely linked to the same area of the genome. The area of chromosome 6 where these genes are located is also interspersed with numerous promoter regions between the TNF- α promoter region and the HLA-D loci, including complement system components and cytochrome P450 enzymes. Thus the search should and will continue for other candidate immunological genes conferring susceptibility to alcohol liver disease.

Chapter 8

Conclusions and Future Directions

ALCOHOL, CHEMOKINES AND THE PATHOGENESIS OF ALCOHOLIC LIVER DISEASE

The studies illustrated in this thesis have shown that chemokine expression is an integral feature of alcoholic liver disease and these studies have important implications for the pathogenesis and therapy of this disease and for liver disease in general. Since the inception of the studies described in this thesis, the appearance of several prominent review articles has testified to a wider appreciation of the integral role played by chemokines in inflammatory disease (Adams 1997, Luster 1998, Baggiolini 1998).

Whilst we demonstrated that several liver cell subtypes express chemokines after appropriate stimulation, our studies suggest that alcohol itself is unlikely to act as a direct stimulant for chemokine secretion, and several important questions remain:

1. Are alcohol and its metabolites solely responsible for inducing the inflammatory and destructive lesions of alcoholic liver disease, or are other nutritional/ metabolic factors necessary for its pathogenesis ?
2. What is the role of lipopolysaccharide in the pathogenesis of alcoholic liver disease ?
3. Is the effect of cytokines and chemokines solely deleterious in alcoholic liver disease, or do these factors also play a role in liver regeneration and repair ?
4. What are the differences underlying the large variation in individual susceptibility to alcoholic liver disease ?

It will remain difficult to determine the answers to these questions by *in vitro* studies because of the difficulty in reproducing the complex interplay of inflammatory mechanisms associated with alcoholic liver disease *in vitro*. However, available evidence suggests that the pathogenesis involves an interplay of pro-inflammatory mediators that are released as a consequence of alcohol metabolism and possibly circulating lipopolysaccharide. Alcohol itself may (at least initially) inhibit these processes via several mechanisms which have been reviewed recently by Spitzer (1998). These include including altered signal transduction of protein kinases and transcription factors, altered G-protein function and altered membrane structure. There is now emerging evidence for a

'rebound' enhancement of inflammatory activity after alcohol is withdrawn or is fully metabolised which is likely to be of profound importance in disease pathogenesis (Szabo 1994, Enomoto 1998). In the former study, subjects admitted with acute alcohol intoxication had diminished monocyte production of TNF- α within 3 days of admission, but this was followed by a delayed increase up to 9 days thereafter, whilst in the latter study, acute administration of alcohol to rats led to an initial diminution of TNF- α release from Kupffer cells (i.e. a 50% reduction at 4 hours) which was followed by a delayed increase (100% increase at 24 hours) compared to baseline values.

In addition to the role of leucocyte activation that I have focussed upon in this thesis, a substantial part of the liver injury of alcoholic liver disease is likely to be induced by the toxicity of acetaldehyde and other reactive metabolites in interference with cellular protein trafficking and also peroxidation of lipids, DNA injury and possibly other regulatory processes. In addition to this direct toxicity, modification of hepatocellular proteins may also enhance leucocyte-derived destruction of hepatocytes via recognition of 'neoantigens'; this process would be expected to continue long after withdrawal of alcohol even though the generation of new reactive metabolites may have ceased.

A proposed model for the pathogenesis of alcoholic liver disease, with particular reference to the role of chemokine secretion, is summarised in figures 8.1 and 8.2.

Figure 8.1 emphasises the likely role of lipopolysaccharide in the pathogenesis of the disease. Circulating alcohol will increase intestinal permeability with a consequent increased absorption of lipopolysaccharide into the portal circulation. The presence of increased circulating lipopolysaccharide after alcohol ingestion and also in subjects with alcoholic liver disease suggests that lipopolysaccharide may either accelerate or facilitate the development of the disease, particularly when considering the known effects of lipopolysaccharide upon cytokine and chemokine secretion as suggested by studies described in and cited earlier in this thesis (Sylvester 1993, Enomoto 1998).

In *figure 8.2* the local effects of alcohol within the liver are illustrated. The presence of reactive oxygen species, along with increased levels of lipopolysaccharide from within the portal circulation, will trigger the synthesis and release of chemokines. The expression of acetaldehyde-modified proteins ('neoantigens') on the hepatocyte membrane may engage Kupffer cells and circulating monocytes which, in the presence of other co-stimulatory

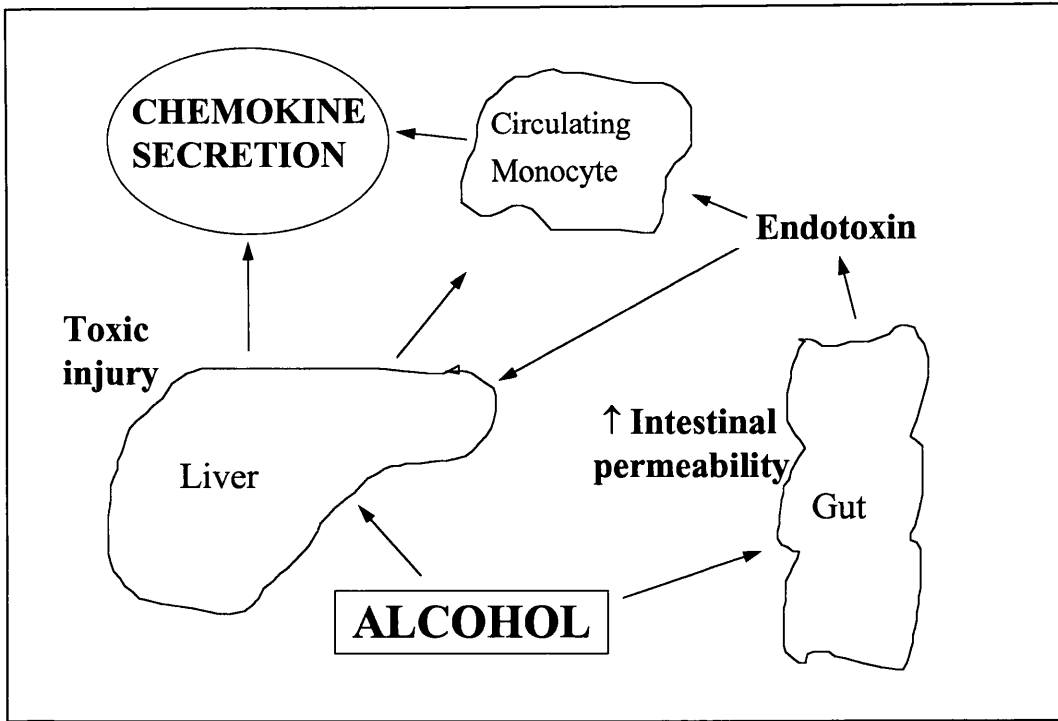


Figure 8.1

Schematic representation of role of alcohol and lipopolysaccharide in systemic chemokine expression

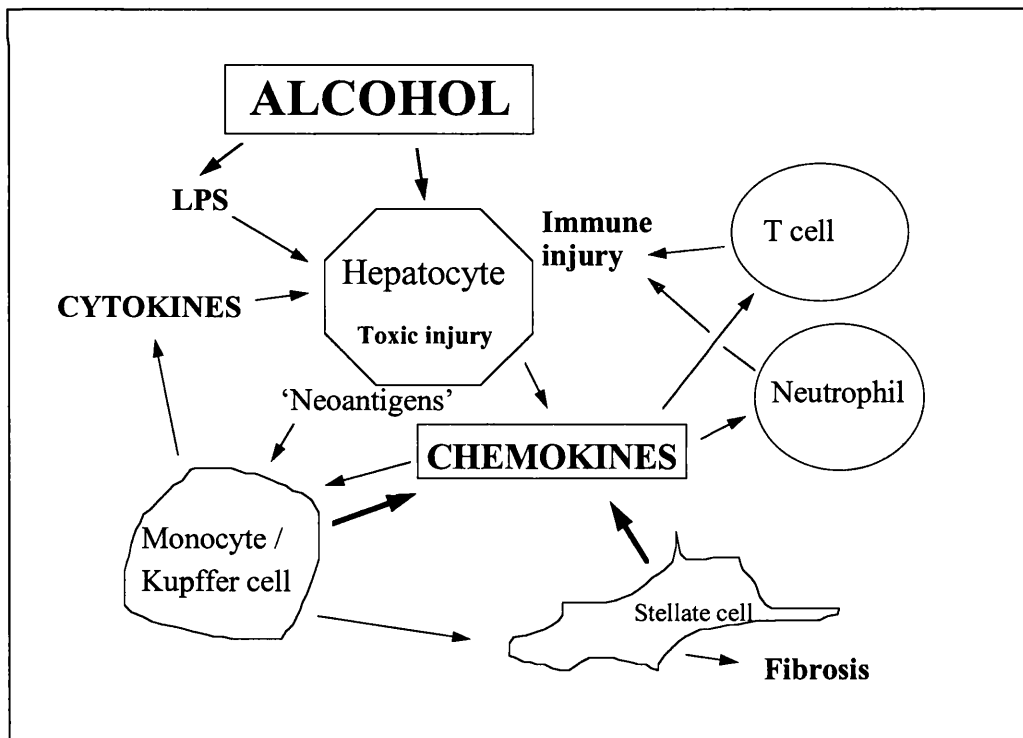


Figure 8.2

Schematic representation of alcohol and chemokine expression within liver

signals including circulating pro-inflammatory cytokines, will secrete (further) cytokines and chemokines (Le 1989, Taub 1996). Stellate cells may also become activated both by the presence of alcohol intermediaries and by soluble Kupffer cell-derived factors including TNF- α and TGF- β ; this will lead to further chemokine synthesis and induce transformation into a myofibroblastic phenotype with subsequent collagen release and fibrosis (Bachem 1992, Marra 1993). The presence of increased levels of local cytokine and chemokine expression may lead to a self-perpetuating 'paracrine' cycle and activation and recruitment of other circulating leucocytes which will amplify tissue injury (Bachem 1992, Marra 1997).

These inflammatory mechanisms will be similar for both alcoholic hepatitis and cirrhosis but will presumably have to be more sustained in order for cirrhosis to develop. In practice patients frequently have a combination of both hepatitis and cirrhosis but occasional patients may have severe liver failure and death from a 'pure' alcoholic hepatitis whilst others may have a 'pure' cirrhosis despite active alcohol abuse up to the time of presentation. As shown in chapter 2 of this thesis, synthesis of both alpha and beta chemokines probably occurs from the same sources within the liver although with different intensities according to the severity of the stimulus, and so pure alcoholic hepatitis is likely to result from predominant secretion of alpha, and pure cirrhosis from beta, chemokines. The factors regulating expression of chemokine genes presumably allow for differential expression of the different chemokine families; pro-inflammatory cytokines such as TNF- α and transcription factors such as NF κ B are likely to be implicated (see below) but these factors are involved in regulation of both alpha and beta chemokines and it is not yet known how such differential expression would be regulated.

A further chemokine-related factor which may alter the phenotypic expression of alcoholic liver disease relates to the expression of specific chemokine receptors and development of a "T helper 1" (Th1) or a "T helper 2" (Th2) lymphocytic response; the former is associated with a more aggressive and pro-inflammatory tissue reaction whilst the latter is less aggressive and induces a predominantly antibody-mediated inflammatory response. It is now recognised that the chemokine receptors CXCR3 and CCR5 are preferentially expressed on Th1 cells whilst CCR3 and CCR4 are preferentially expressed on Th2 cells,

and CXCR4 and CCR2 are common to both types. Thus, for example, expression of MIP-1 α , MIP-1 β and RANTES would allow preferential recruitment of Th1 lymphocytes whilst expression of SDF-1 and MCP-1 would allow recruitment of both Th1 and Th2 lymphocytes (Sallusto 1998).

Finally in considering the role of chemokines in the pathogenesis of alcoholic liver disease, it is possible that they will play a role not just in facilitating inflammatory liver damage but also in promoting liver regeneration. Such a role has already been described for the 'proinflammatory' cytokine TNF- α (Brucoleri 1997). One way in particular in which chemokines may promote regeneration is via promotion of angiogenesis; this role has been demonstrated for CXC chemokines displaying the ELR motif (Strieter 1995).

The mechanisms of alcoholic liver disease that we have described will clearly only account for histologically evident liver disease; how these processes are regulated and inflammatory damage minimised or avoided with more modest or limited levels of alcohol consumption is not clear, but will presumably be dependent upon sufficient host antioxidant and hepatocyte regenerative capacity to limit the toxic effects of ethanol. It has long been believed that nutritional depletion is an important factor in precipitating alcoholic liver disease (Patek 1949), and supplementation with the natural antioxidant precursor S-adenosyl methionine and / or polyunsaturated lecithin (an essential fatty acid) has been shown to protect against development of alcoholic liver disease in non-human primates (Lieber 1994), whilst other studies have shown that depletion of natural antioxidant vitamins may occur in, and thus perhaps predispose to, alcoholic liver disease (Bjorneboe 1988).

FUTURE DIRECTIONS

Intracellular signalling and regulation of chemokine genes

Whilst it is known that proinflammatory cytokines and stimuli such as lipopolysaccharide and reactive oxygen species induce chemokine gene transcription, the signal transduction pathways involved remain a focus of much current research. Transcription factors such as nuclear factor-kappa B (NFκB) are probably involved; this agent is responsible for rapid induction of many cytokines (Barnes 1997) and is up-regulated in tandem with expression of chemokines in liver injury (Tsukamoto 1996); pharmacological blockade of NFκB has been shown to reduce chemokine gene expression in endothelial cells (Ebnet 1997). NFκB may in itself be upregulated by stress-activated protein kinases (SAPK's, also known as mitogen-activated protein kinases, MAPK's). This group of protein kinases includes extracellular-signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38^{MAPK}, and may be activated by engagement of proinflammatory cytokine receptors with their appropriate ligand or by oxidative stress. They have been implicated in the activation of hepatic stellate cells (Reeves 1998) and directly in stellate cell expression of MCP-1 (Marra 1997). Thus, a clarification of the signalling pathways involved in alcohol-mediated liver injury and chemokine expression would greatly enhance our understanding of the pathogenesis of alcoholic liver disease.

Genetic determinants of chemokine gene and chemokine receptor gene expression

The possibility of genetic polymorphisms in TNF-α expression influencing the risk of developing alcoholic liver disease was addressed in chapter 7. Whilst our data did not support a role for the polymorphisms studied, other genetic polymorphisms for pro-inflammatory cytokine gene expression are recognised and could be associated with an increased risk of liver disease. It will be interesting to see whether polymorphisms for chemokine promoter genes are determined since this could be another useful area for exploring genetic risk for alcoholic liver disease. At present there have not been any genetic polymorphisms for chemokine genes described; however there has been intense research into polymorphisms for chemokine receptor genes since it was realised that these influenced the risk of contracting HIV infection and developing AIDS in subjects exposed

to the virus. The first mutation determined to alter risk of HIV infection was a deletion ($\Delta 32$) in the CC-chemokine receptor 5 (CCR5) gene, which protects the individual from HIV infection since the virus uses the (wild type) CCR5 as a portal of entry into the leucocyte, and presence of the $\Delta 32$ mutation reduces the avidity of HIV-CCR5 binding (Samson 1996). Since this discovery was made, further mutations have been characterised including a polymorphism for the CCR5 promoter region, which also protects against HIV infection presumably from reduced transcription of the CCR5 gene (McDermott 1998). In addition to the role of chemokine receptor genes in HIV infection, the importance of the chemokine receptors in leucocyte recruitment has been determined in CCR2 knockout mice, which lack the receptor for MCP-1 and have severely impaired monocyte migration (Boring 1997). This animal model will allow the role of MCP-1 in fibrogenesis to be further investigated *in vivo*, and it will also be of great interest to determine whether polymorphisms in chemokine receptor genes or promoter regions play a role in determining susceptibility to other inflammatory diseases including alcoholic liver disease.

Therapy for alcoholic liver disease

Approaches to the pharmacological therapy of alcoholic liver disease may involve attempts to ameliorate the metabolic disturbances of the disease (including repletion of antioxidant vitamins and minerals), or to reduce the extent of immune injury directly with immunosuppressive or anti-inflammatory therapy. The latter approach could include non-specific immunosuppressive therapy with corticosteroids, which has been explored extensively, or therapy targeted at specific immune mediators, which has not. Corticosteroid therapy reduces expression of pro-inflammatory cytokines and chemokines (Xu 1996) and has been evaluated in numerous clinical studies in alcoholic hepatitis over the past 20 years. Whilst 2 of the most recent studies showed a benefit in outcome following 4 weeks' therapy (Carithers 1989, Ramond 1992), a recent meta-analysis of several published studies has suggested no overall benefit with this treatment (Christensen 1995). There are no published studies of therapy with other conventional immunosuppressive agents; however as discussed in chapter 6, a recent study suggests that pentoxifylline, which reduces expression of many pro-inflammatory cytokines and chemokines, may be beneficial in alcoholic hepatitis (Akriviadis 1997). Furthermore,

inhibition of pro-inflammatory cytokine and chemokine gene activation with proteasome inhibitors, which block the induction of NF κ B activity, reduces activation in cultured hepatic stellate cells and may be a useful therapy to explore *in vivo* (Hellerbrand 1998). However, as with other 'pro-inflammatory' cytokines, the widespread involvement of NF κ B in many vital cellular processes casts doubt on the potential benefit of such pharmacological blockade (Taub 1998). Nevertheless monoclonal antibodies to TNF- α have been shown to be beneficial in inflammatory conditions including rheumatoid arthritis and Crohn's disease; this approach may theoretically be beneficial in alcoholic liver disease and has been shown to reduce hepatic chemokine expression and inflammation in a rat model of the disease (Iimuro 1997).

Our own studies suggest that specific blockade of chemokine expression or function may be a useful approach in the therapy of alcoholic liver disease. Although not evaluated in inflammatory hepatic diseases, pharmacological blockade of the beta chemokines MCP-1 and RANTES with monoclonal antibodies has been shown to inhibit development of crescent formation and fibrosis in a murine model of nephritis (Lloyd 1997). However, an equally attractive and possibly preferable approach would be to block chemokine receptors since there is less redundancy amongst this family of receptors compared to the numerous chemokines described (Baggiolini 1997). Chemokine antagonists have been synthesised by truncation of the amino terminal of the chemokine molecule, resulting in a competitive agonist without biological effect (Clark-Lewis 1991, Gong 1995), and use of an inactive MCP-1 analogue synthesised this way has been shown to reduce disease severity in a mouse model of inflammatory arthritis (Gong 1997). Further research into the strategy of chemokine receptor blockade is likely to come from the AIDS field since such blockade may provide a potentially important means to inhibit viral replication (Baggiolini 1997).

We look forward enthusiastically to further exploration of the role of immune modulators in the management of alcoholic liver disease.

Reference List

- Abdullah, M.S., Wild, G., Jacob, V., Milford-Ward, A., Ryad, R., Zanaty, M., Ali, M.H. and el Nahas, A.M. (1997) Cytokines and the malnutrition of chronic renal failure. *Mineral and Energy Metabolism* **23**, 237-242.
- Adachi, Y., Bradford, B., Gao, W., Bojes, H.K. and Thurman, R.G. (1994) Inactivation of Kupffer Cells Prevents Early Alcohol-induced Liver Injury. *Hepatology* **20**, 453-460.
- Adams, D.H., Hubscher, S.G., Shaw, J., Rothlein, J. and Neuberger, J.M. (1989) Intercellular adhesion molecule-1 on liver allografts during rejection. *Lancet* **ii**, 1122-5.
- Adams, D.H., Hubscher, S.G., Shaw, J., Johnson, G.D., Babbs, C., Rothlein, R. and Neuberger, J.M. (1991) Increased expression of ICAM-1 on bile ducts in primary biliary cirrhosis and primary sclerosing cholangitis. *Hepatology* **14**, 426-432.
- Adams, D.H., Burra, P., Hubscher, S., Elias, E. and Newman, W. (1994a) Endothelial Activation and Circulating Vascular Adhesion Molecules in Alcoholic Liver Disease. *Hepatology* **19**, 594
- Adams, D.H., Harvath, L., Bottaro, D.P., Interrante, R., Catalano, G., Tanaka, Y., Strain, A., Hubscher, S.G. and Shaw, S. (1994b) Hepatocyte growth factor and macrophage inflammatory protein-1 β : structurally distinct cytokines that induce rapid cytoskeletal changes and subset-preferential migration in T-cells. *Proceedings of the National Academy of Science* **91**, 7144-7148.
- Adams, D.H., Afford, S.C., Fear, J., Shaw, S. and Hubscher, S.G. (1996) Hepatic expression of macrophage inflammatory protein-1b after liver transplantation. *Transplantation* **61**, 817-825.
- Adams, D.H. and Lloyd, A.R. (1997) Chemokines: leucocyte recruitment and activation cytokines (review article). *Lancet* **349**, 490-495.
- Akahoshi, T., Kobayashi, N., Hosaka, S., Sekiyama, N., Wada, C. and Kondo, H. (1995) In-vivo induction of monocyte chemotactic and activating factor in patients with chronic renal failure. *Nephrology, Dialysis and Transplantation* **10**, 2244-2249.
- Akriviadis, E., Botla, R., Briggs, W., Reynolds, T. and Shakil, O. (1997) Improved short-term survival with pentoxifylline treatment in severe acute alcoholic hepatitis. *Hepatology* **26**, 250A
- Allott, R.L., Quest, L.J., Pirmohammed, M., Kitteringham, N.R., Gilmore, I.T. and Park, B.K. (1996) Investigation of the role of the TNF-alpha gene polymorphism in alcoholic liver disease (abstract). *Hepatology* **24**, 443A
- Ambrus, J.L., Stadler, S. and Kulaylat, M. (1995) Hemorrhologic effects of metabolites of pentoxifylline (Trental). *Journal of Medicine* **26**, 65-75.
- Anderson, P., Cremona, A., Paton, A., Turner, C. and Wallace, P. (1993) The risk of alcohol. *Addiction* **88**, 1493-1508.
- Andus, T., Bauer, J. & Gerok, W. (1991) Effects of cytokines on the liver. *Hepatology* **13**, 364-75.
- Armendariz-Borunda, J., Seyer, J.M., Postlethwaite, A.E. and Kang, A.H. (1991) Kupffer cells from carbon tetrachloride-injured rat liver produce chemotactic factors for fibroblasts and monocytes: the role of tumour necrosis factor-alpha. *Hepatology* **14**, 895-900.

- Attal, M., Huguet, F., Rubie, H., Charlet, J.P., Schlaifer, D. and Huynh, A. (1993) Prevention of regimen-related toxicities after bone marrow transplantation by pentoxifylline: a prospective randomised trial. *Blood* **82**, 732-736.
- Aviado, D.M. and Porter, J.M. (1984) Pentoxifylline: a new drug for the treatment of intermittent claudication. Mechanism of action, pharmacokinetics, clinical efficacy and adverse effects (review). *Pharmacotherapy* **4**, 297-307.
- Bachem, M., Meyer, D., Melchior, R., Sell, K. and Gressner, A.M. (1992) Activation of rat liver lipocytes by transforming growth factors derived from myofibroblastlike cells. A potential mechanism of self-perpetuation in liver fibrogenesis. *Journal of Clinical Investigation* **89**, 19-27.
- Baggiolini, M., Dewald, D. and Moser, B. (1994) Interleukin-8 and related chemotactic cytokines: CXC and CC chemokines. *Advances in Immunology* **55**, 97-179.
- Baggiolini, M. and Moser, B. (1997) Blocking chemokine receptors (review). *Journal of Experimental Medicine* **186**, 1189-1191.
- Barnes, P.J. and Karin, M. (1997) Nuclear factor kappa B: a pivotal transcription factor in chronic inflammatory diseases. *New England Journal of Medicine* **336**, 1066-1071.
- Bauditz, J., Haemling, J., Ortner, M., Lochs, H., Raedler, A. and Schreiber, S. (1997) Treatment with tumour necrosis factor inhibitor oxpentifylline does not improve corticosteroid dependent chronic active Crohn's disease. *Gut* **40**, 470-474.
- Bautista, A.P. (1995) Chronic alcohol intoxication enhances the expression of CD18 adhesion molecules on rat neutrophils and release of a chemotactic factor by Kupffer cells. *Alcoholism, Clinical and Experimental Research* **19**, 285-290.
- Bautista, A.P. (1997) Chronic alcohol intoxication induces hepatic injury through enhanced macrophage inflammatory protein-2 production and intercellular adhesion molecule-1 expression in the liver. *Hepatology* **25**, 335-342.
- Bazan, J.F., Bacon, K.B., Hardiman, G. (1997) A new class of membrane-bound chemokine with a CX3C motif. *Nature* **385**, 640-644.
- Benbernou, N., Esnault, S., Potron, G. and Guenounou, M. (1995) Regulatory effects of pentoxifylline on T-helper cell-derived cytokine production in human blood cells. *Journal of Cardiovascular Pharmacology* **25**, S75-S79.
- Bessler, H., Gilgal, R., Djaldetti, M. and Zahavi, I. (1986) Effects of pentoxifylline on the phagocytic activity, cAMP levels, and superoxide anion production by monocytes and polymorphonuclear cells. *Journal of Leucocyte Biology* **40**, 747-757.
- Bianco, J.A., Appelbaum, F.R., Nemunaitis, J., Almgren, J., Andrews, F. and Kettner, P. (1991) Phase I-II trial of pentoxifylline for the prevention of transplant-related toxicities following bone marrow transplantation. *Blood* **78**, 1205-1211.
- Bienvenu, J., Doche, C., Gutowski, M., Lenoble, M., Lepape, A. and Perdrix, J.P. (1995) Production of proinflammatory cytokines and cytokines involved in the TH1/ TH2 balance is modulated by pentoxifylline. *Journal of Cardiovascular Pharmacology* **25**, S80-S84.

- Bird, G.L.A., Sheron, N., Goka, J., Alexander, G.J. and Williams, R.S. (1990) Increased plasma tumour necrosis factor in severe alcoholic hepatitis. *Annals of Internal Medicine* **112**, 917-920.
- Bjarnason, I., Peters, T.J. and Wise, R.J. (1984) The leaky gut of alcoholism: possible route of entry for toxic compounds. *Lancet* **1**, 179-182.
- Bjorneboe, G., Johnsen, J., Bjorneboe, A., Marklund, S.L., Morland, J. and Drevon, C.A. (1988) Some aspects of antioxidant status in blood from alcoholics. *Alcoholism, Clinical and Experimental Research* **12**, 806-810.
- Bode, C., Kugler, V. and Bode, J.C. (1987) Endotoxaemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. *Journal of Hepatology* **4**, 8-14.
- Boring, L., Gosling, J., Chensue, S., Kunkel, S., Farese, R., Broxmeyer, H. and Charo, I.F. (1997) Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *Journal of Clinical Investigation* **100**, 2552-2661.
- Bossink, A.W., Paemen, L., Jansen, P.M., Hack, C.E., Thijs, L.G. and Van Damme, J. (1995) Plasma Levels of the Chemokines Monocyte Chemoattractant Protein-1 and -2 are Elevated in Human Sepsis. *Blood* **86**, 3841-3847.
- Brett, J., Gerlach, H., Naworth, P., Steinberg, S., Godman, G. and Stern, D. (1989) Tumor necrosis factor/ cachectin increases permeability of endothelial cell monolayers by a mechanism using regulatory G proteins. *Journal of Experimental Medicine* **169**, 1977-1991.
- Bruccoleri, A., Gallucci, R., Germolec, D.R., Blacksheare, P., Simeonova, P., Thurman, R.G. and Luster, M.L. (1997) Induction of early-immediate genes by tumor necrosis factor contribute to liver repair following chemical-induced hepatotoxicity. *Hepatology* **25**, 133-141.
- Bryce, T.A., Chamberlain, J., Hillbeck, D. and Macdonald, C.M. (1989) Metabolism and pharmacokinetics of ¹⁴C-pentoxifylline in healthy volunteers. *Drug Research* **39**, 512-517.
- Burgmann, H., Stauffer, F., Hollenstein, U., Wiesinger, E., Graninger, W. and Breyer, S. (1995) Effect of pentoxifylline concentrations on macrophage inflammatory protein 1 alpha production (letter). *Antimicrobial Agents and Chemotherapy* **39**, 574-575.
- Burnstine, M.A., Elnor, S.G. and Elnor, V.M. (1998) Orbital fibroblast chemokine modulation: effects of dexamethasone and cyclosporine A. *British Journal of Ophthalmology* **82**, 318-322.
- Burra, P., Hubscher, S.G., Shaw, J., Elias, E. and Adams, D.H. (1992) Is the intercellular adhesion molecule-1/leukocyte function associated antigen 1 pathway of leukocyte adhesion involved in the tissue damage of alcoholic hepatitis? *Gut* **33**, 268-271.
- Butcher, E.C. (1991) Leucocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* **67**, 1033-1036.
- Camacho, K., Casey, C., Wiegert, R., Sorrell, M. and Tuma, D.J. (1993) Time course of ethanol-induced impairment in fluid-phase endocytosis in isolated rat hepatocytes. *Hepatology* **17**, 661-67.

- Carithers, R.L., Herlong, H.F., Diehl, A.M., Shaw, E.W., Combes, B., Fallon, H.J. and Maddrey, W.C. (1989) Methylprednisolone therapy in patients with severe alcoholic hepatitis. A randomised multicenter trial. *Annals of Internal Medicine* **110**, 685-690.
- Casey, C.A., Wiegert, R. and Tuma, D.J. (1995) Effect of hyperosmolarity on receptor-mediated and fluid-phase endocytosis in ethanol-fed animals. *Biochemical Pharmacology* **49**, 1117-1123.
- Chamba, A., Stockley, R.A. and Burnett, D. (1991) Effects of neutrophil adherence on the characteristics of receptors for tumor necrosis factor-alpha. *FEBS letters* **282**, 373-376.
- Chedid, A., Mendenhall, C.L., Gartside, P., French, S.W., Chen, T. and Rabin, L. (1991) Prognostic factors in alcoholic liver disease. *American Journal of Gastroenterology* **86**, 210-216.
- Christensen, E. and Gluud, C. (1995) Glucocorticoids are ineffective in alcoholic hepatitis: A meta-analysis adjusting for confounding variables. *Gut* **37**, 113-118.
- Ciuffetti, G., Mercuri, M., Ott, C., Lombardini, R., Paltriccia, R., Lupattelli, G., Santambrogio, L. and Mannarino, E. (1991) Use of pentoxifylline as an inhibitor of free radical generation in peripheral vascular disease. Results of a double-blind placebo-controlled study. *European Journal of Clinical Pharmacology* **41**, 511-515.
- Clark-Lewis, I., Schumacher, M., Baggiolini, M. and Moser, B. (1991) Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH₂-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities. *Journal of Biological Chemistry* **266**, 23128-34
- Clot, P., Bellomo, G., Tabone, M., Arico, S. and Albano, E. (1995) Detection of Antibodies against Proteins Modified by Hydroxyethyl Free Radicals in Patients with Alcoholic Cirrhosis. *Gastroenterology* **108**, 201-207.
- Clot, P., Tabone, M., Arico, S. and Albano, E. (1994) Monitoring oxidative damage in patients with liver cirrhosis and different daily alcohol intake. *Gut* **35**, 1637-1643.
- Cook, D.N., Beck, M.A. and Coffman, T.M. (1995) Requirement of MIP-1 α for an inflammatory response to viral infection. *Science* **269**, 1583-1585.
- Czaja, M.J., Geerts, A., Xu, J., Schmiedeberg, P. and Ju, Y. (1994) Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. *Journal of Leukocyte Biology* **55**, 120-126.
- D'Alfonso, S. and Momigliano Richiardi, P. (1994) A polymorphic variation in a putative regulation box of the TNF alpha promoter region. *Immunogenetics* **39**, 150-154.
- D'Souza, N.B., Bagby, G.J., Nelson, S., Lang, C.H. and Spitzer, J. (1989) Acute alcohol infusion suppresses endotoxin-induced serum tumour necrosis factor. *Alcoholism, Clinical & Experimental Research* **13**, 295-298.
- Dai, Y., Rashba-Step, J., Cederbaum, A. (1993) Stable expression of human cytochrome P450E1 in Hep G2 cells; characterization of catalytic activities and production of reactive oxygen intermediaries. *Biochemistry* **32**, 6928-6937

- D'Souza, N.B., Nelson, S., Sumner, W.R. and Deaciuc, I.V. (1994) Expression of tumour necrosis factor-alpha and interleukin-6 cell-surface receptors of the alveolar macrophage in alcohol-treated rats. *Alcoholism, Clinical and Experimental Research* **18**, 1430-1435.
- Deaciuc, I.V., Alappat, J.M., McDonough, K.H. and D'Souza, N.B. (1996) Interleukin-6 tumour necrosis factor-alpha clearance and metabolism in vivo and by the isolated rat liver: effect of acute alcohol administration. *Alcoholism, Clinical and Experimental Research* **20**, 293-301.
- Derr, R.F., Porta, E.A., Larkin, E.C. and Ananda Rao, G. (1990) Is ethanol per se hepatotoxic? *Journal of Hepatology* **10**, 381-386.
- Devor, E. & Cloninger, C. (1989) Genetics of Alcoholism. *Annual Review of Genetics* **23**, 19-36.
- Dezube, B.J., Sherman, M.L., Fridovich-Keil, J.L., Allen-Ryan, J. and Pardee, J.B. (1993) Down-regulation of tumor necrosis factor expression by pentoxifylline in cancer patients: a pilot study. *Cancer Immunology and Immunotherapy* **36**, 57-60.
- Doherty, G.M., Jensen, J.C., Alexander, H.R., Buresh, C.M. and Norton, J.A. (1991) Pentoxifylline suppression of TNF gene transcription. *Surgery* **110**, 192-198.
- Ebnet, K., Kaldjian, E., Anderson, A. and Shaw, S. (1996) Orchestrated information-transfer underlying leucocyte-endothelial interactions. *Annual Reviews in Immunology* **14**, 155-177.
- Ebnet, K., Brown, K.D., Siebenlist, U.K., Simon, M.M. and Shaw, S. (1997) *Borrelia burgdorferi* activates nuclear factor-kappa B and is a potent inducer of chemokine and adhesion molecule gene expression in endothelial cells and fibroblasts. *Journal of Immunology* **158**, 3285-3292.
- Edenberg, H.J., Reynolds, J., Koller, D.L., Begleiter, H., Bucholz, K.K. and Foroud, T. (1998) A family-based analysis of whether promoter alleles of the serotonin transporter gene HTT affect risk for alcohol dependence. *Alcoholism, Clinical and Experimental Research* **22**, 1080-1085.
- Enomoto, N., Takase, S. and Takada, A. (1991) Alcoholic liver disease in heterozygotes of mutant and normal aldehyde dehydrogenase-2 genes. *Hepatology* **13**, 1071-1075.
- Enomoto, N., Ikejima, K., Bradford, B., Rivera, C., Kono, H., Brenner, D.A. and Thurman, R.G. (1998) Alcohol causes both tolerance and sensitization of rat Kupffer cells via mechanisms dependent on endotoxin. *Gastroenterology* **115**, 443-451.
- Fawcett, J., Hammond, B. and Smith, G.D. (1993) Acute effects of ethanol on hepatic endocytosis and processing of insulin in perfused rat liver. *American Journal of Physiology* **264**, E420-E427.
- Furie, M.B. and Randolph, G.J. (1995) Chemokines and tissue injury. *American Journal of Pathology* **146**, 1287-1301.
- Gimbrone, M.A., Obin, M.S., Brock, A.F., Luis, E.A., Hebert, C.A., Yip, Y.K., Leung, D.W. and Kohr, W.J. (1989) Endothelial interleukin-8: a novel inhibitor of leucocyte-endothelial interactions. *Science* **246**, 1601-3.
- Ganong, W.F. (1993) The General and Cellular Basis of Medical Physiology. In: Anonymous *Review of Medical Physiology*, 16th edn. Prentice Hall International]

- Goates, C.Y. and Knutson, K. (1994) Enhanced permeation of polar compounds through human epidermis. I. Permeability and membrane structural changes in the presence of short chain alcohols. *Biochimica et Biophysica Acta* **1195**, 169-179.
- Gong, J.H. and Clark-Lewis, I. (1995) Antagonists of monocyte chemoattractant protein 1 identified by modification of functionally critical NH₂-terminal residues. *Journal of Experimental Medicine* **181**, 631-640.
- Gong, J.H., Ratkay, L.B., Waterfield, J.D. and Clark-Lewis, I. (1997) An antagonist of monocyte chemoattractant protein-1 inhibits arthritis in the MRL-lpr mouse model. *Journal of Experimental Medicine* **186**, 131-137.
- Grove, J., Daly, A.K., Bassendine, M.F. and Day, C.P. (1997) Association of a tumor necrosis factor promoter polymorphism with susceptibility to alcoholic steatohepatitis. *Hepatology* **26**, 143-146.
- Hellerbrand, C., Jobin, C., Iimuro, Y., Licato, L., Balfour Sartor, R. and Brenner, D.A. (1998) Inhibition of NF κ B in activated rat hepatic stellate cells by proteasome inhibitors and an I κ B super-repressor. *Hepatology* **27**, 1285-1295.
- Hill, D.B., Marsano, L.S. and McClain, C.J. (1993) Increased plasma interleukin-8 concentrations in alcoholic hepatitis. *Hepatology* **18**, 576-580.
- Horuk, R., Chitnis, C.F. and Darbonne, W.C. (1993) A Receptor for the Malarial Parasite Plasmodium Vivax: The Erythrocyte Chemokine Receptor. *Science* **261**, 1182-1184.
- Huang, Y.S., Chan, C.Y., Wu, J.C., Pai, C.H., Chao, Y. and Lee, S.D. (1996) Serum levels of interleukin-8 in alcoholic liver disease: relationship with disease stage, biochemical parameters and survival. *Journal of Hepatology* **24**, 377-384.
- Iimuro, Y., Gallucci, R.M., Luster, M.I., Kono, H. and Thurman, R.G. (1997) Antibodies to tumor necrosis factor alpha attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat. *Hepatology* **26**, 1530-1537.
- International Group (1981) Alcoholic Liver Disease: Morphological Manifestations. *Lancet* (i), 707-711.
- Israel, Y., Orrego, H. and Niemela, O. (1988) Immune Responses to Alcohol Metabolites: Pathogenic and Diagnostic Implications. *Seminars in Liver Disease* **8**, 81-90.
- Jacob, C.O., Fronck, Z., Lewis, G.D., Koo, M., Hansen, J.A. and McDevitt, H.O. (1990) Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor alpha: Relevance to genetic predisposition to systemic lupus erythematosus. *Proceedings of the National Academy of Sciences* **87**, 1233-1237.
- Jaeschke, H. and Farhood, A. (1991) Neutrophil and Kupffer self-induced oxidant stress and ischaemia-reperfusion injury in rat liver. *American Journal of Physiology* **260**, G355-G362
- Jensen, K. and Gluud, C. (1994) The Mallory body: theories on development and pathological significance. *Hepatology* **20**, 1330-1342.

- Jiang, Y., Beller, D.I., Frenzl, G. and Graves, D.T. (1992) Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *Journal of Immunology* **148**, 2423-2428.
- Joplin, R., Hishida, T., Tsubouchi, H., Daikuhara, Y., Ayres, R., Neuberger, J.M. and Strain, A.J. (1992) Human intrahepatic biliary epithelial cells proliferate in vitro in response to human hepatocyte growth factor. *Journal of Clinical Investigation* **90**, 1284-9.
- Kelner, G.S., Kennedy, J., Bacon, K.B. (1994) Lymphotactin: a cytokine that represents a new class of chemokine. *Science* **266**, 1395-1399.
- Kelvin, D.J., Michiel, D.F., Johnston, J.A., Lloyd, A.R., Sprenger, H., Oppenheim, J.J. and Wang, J.M. (1993) Chemokines and serpentine: the molecular biology of chemokine receptors. *Journal of Leukocyte Biology* **54**, 604-612.
- Khoruts, A., Stahnke, L., McClain, C.J., Logan, G. and Allen, J.I. (1991) Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. *Hepatology* **13**, 267-276.
- Kingham, J.G.C. (1997) Alcoholic Liver Disease, Not Viral Hepatitis, Predominates in South Wales (letter). *Hepatology* **25**, 1297
- Kopenhagen, K. and Wenig, H.G. (1979) Action of pentoxifylline on hepatic circulation. Comparison study with ¹⁹⁸Au colloid scintigraphy in liver patients and healthy subjects. *Medicum Klinikum* **74**, 1254-1256.
- Kuijpers, T.W., Hakkert, B.C., Hart, M.H.L. and Roos, D. (1992) Neutrophil migration across monolayers of cytokine-prestimulated endothelial cells: a role for platelet-activating factor and IL-8. *Journal of Cell Biology* **117**, 565-572.
- Kunkel, S.L., Lukacs, N., Kasama, T. and Strieter, R.M. (1996) The role of chemokines in inflammatory joint disease. *Journal of Leukocyte Biology* **59**, 6-12.
- Landman, D., Sarai, A. and Sathe, S.S. (1994) Use of pentoxifylline therapy for patients with AIDS-related wasting- a pilot study. *Clinical Infectious Diseases* **18**, 97-99.
- Lasker, J.M., Raucy, J., Kubota, S., Bloswick, B.P., Black, M. Lieber, C.S. (1987) Purification and characterisation of human liver cytochrome P450-ALC. *Biochemical and Biophysical Research Communications* **148**, 232-238.
- Le, J, Vilcek, J. (1989) Interleukin-6: A multifunctional cytokine regulating immune reactions and the acute phase protein response. *Laboratory Investigation* **61**, 588-602.
- Lieber, C.S. and DeCarli, L.M. (1970) Quantitative relationship between amount of dietary fat and severity of alcoholic fatty liver. *American Journal of Clinical Nutrition* **23**, 474-78.
- Lieber, C.S., DeCarli, L.M. and Rubin, E. (1975) Sequential production of fatty liver, hepatitis, and cirrhosis in sub-human primates fed ethanol with adequate diets. *Proceedings of the National Academy of Science of the United States of America* **72**, 437-441.
- Lieber, C.S. (1994) Alcohol & the Liver: 1994 Update. *Gastroenterology* **106**, 1085-1105.

- Lloyd, C.M., Minto, A.W., Dorf, M.E., Proudfoot, A., Wells, T.N.C., Salant, D.J. and Gutierrez-Ramos, J.C. (1997) RANTES and MCP-1 play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *Journal of Experimental Medicine* **185**, 1371-1380.
- Lukacs, N.W., Strieter, R., Chensue, S. and Kunkel, S.L. (1996) Activation and regulation of chemokines in allergic airway inflammation. *Journal of Leukocyte Biology* **59**, 13-17.
- Lumeng, L. and Crabb, D.W. (1994) Genetic Aspects and Risk Factors in Alcoholism and Alcoholic Liver Disease. *Gastroenterology* **107**, 572-578.
- Luscinskas, F.W., Kiely, J.M., Ding, H., Obin, M.S., Hebert, C.A., Baker, J.B. and Gimbrone, M.A.J. (1992) In vitro inhibitory effect of IL-8 and other chemoattractants on neutrophil-endothelial adhesive interactions. *Journal of Immunology* **149**, 2163-2171.
- Luster, A.D. (1998) Chemokines - Chemotactic Cytokines that Mediate Inflammation. *New England Journal of Medicine* **338**, 436-445.
- Maddrey, W.C. (1990) Alcoholic Hepatitis: Pathogenesis and Approaches to Treatment. *Scandinavian Journal of Gastroenterology* **175**, 118-130.
- Maddrey, W.C., Boitnott, J.K., Bedine, M.S., Weber, F.L., Mezey, E. and White, R.I. (1978) Corticosteroid therapy of alcoholic hepatitis. *Gastroenterology* **75**, 193-199.
- Maher, J.J., Tzagarakis, C. and Gimenez, A. (1994a) Malondialdehyde stimulates collagen production by hepatic lipocytes only upon activation in primary culture. *Alcohol and Alcoholism* **29**, 605-610.
- Maher, J.J., Zia, S. and Tzagarakis, C. (1994b) Acetaldehyde-Induced Stimulation of Collagen Synthesis and Gene Expression Is Dependent on Conditions of Cell Culture: Studies with Rat Lipocytes and Fibroblasts. *Alcoholism: Clinical and Experimental Research* **18**, 403-409.
- Maher, J.J. (1995) Rat hepatocytes and Kupffer cells interact to produce interleukin-8 (CINC) in the setting of alcohol. *American Journal of Physiology* **269**, G518-G523.
- Maher, J.J., Scott, M., Saito, J. and Burton, M.C. (1996) Adenovirus-mediated expression of CINC/GRO (IL-8) in rat liver induces a neutrophilic hepatitis. *FASEB Journal* **10**, 22
- Mak, K.M. and Lieber, C.S. (1988) Lipocytes and transitional cells in alcoholic liver disease: a morphometric study. *Hepatology* **8**, 1027-1033.
- Maltby, J., Wright, S., Bird, G. and Sheron, N. (1996) Chemokine levels in human liver homogenates: association between GRO alpha and histopathological evidence of alcoholic hepatitis. *Hepatology* **24**, 1156-1160.
- Mandrekar, P., Catalano, D. and Szabo, G. (1997) Alcohol-induced regulation of nuclear regulatory factor-kappa beta in human monocytes. *Alcoholism, Clinical & Experimental Research* **21**, 988-994.
- Mansfield, J.C., Holden, H., Tarlow, J.K., Di Giovine, F.S., McDowell, T.L., Wilson, A.G., Holdsworth, C.D. and Duff, G.W. (1994) Novel genetic association between ulcerative colitis and the anti-inflammatory cytokine interleukin-1 receptor antagonist. *Gastroenterology* **106**, 637-642.

- Marcus, B.C., Wyble, C., Hynes, K. and Gewertz, B.L. (1996) Cytokine-induced increases in endothelial permeability after adhesion molecule expression. *Surgery* **120**, 411-416.
- Marra, F., Valente, A.J., Pinzani, M. and Abboud, H.E. (1993) Cultured human liver fat-storing cells produce monocyte chemotactic protein-1: Regulation by proinflammatory cytokines. *Journal of Clinical Investigation* **92**, 1674-1680.
- Marra, F., Pastacaldi, S., Lee, J.C., Pinzani, M., Laffi, G. and Gentilini, P. (1997) Activation of p38^{MAPK} is required for cytokine-stimulated expression of monocyte chemotactic protein-1 in stellate cells. *Hepatology* **26**, 184A
- Marra, F., DeFranco, R., Grappone, C., Milani, S., Pastacaldi, S., Pinzani, M., Romanelli, R.G., Laffi, G. and Gentilini, P. (1998) Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis - correlation with monocyte infiltration. *American Journal of Pathology* **152**, 423-430.
- Martinez, I., Sveinbjornsson, B., Vidal-Vanaclocha, F., Asumendi, A. and Smedsrod, B. (1995) Differential cytokine-mediated modulation of endocytosis in rat liver endothelial cells. *Biochemical and Biophysical Research Communications* **212**, 235-241.
- Marui, N., Offerman, M.K., Swerlick, R., Kunsch, C., Rosen, C.A., Ahmad, M., Alexander, R.W. and Medford, R.M. (1993) Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated via an antioxidant sensitive mechanism in human vascular endothelial cells. *Journal of Clinical Investigation* **92**, 1866-1874.
- Masumoto, T., Onji, M., Horiike, N. and Ohta, Y. (1993) Assay of serum interleukin-8 levels in patients with alcoholic hepatitis. *Alcohol and Alcoholism* **28**, 99-102.
- Matsuoka, M.M., Zhang, M.Y. and Tsukamoto, H. (1990) Sensitisation of hepatic lipocytes by high-fat diet to stimulatory effects of Kupffer cell-derived factors: implication in alcoholic liver fibrogenesis. *Hepatology* **11**, 173-185.
- McClain, C. and Cohen, D.A. (1989) Increased tumour necrosis factor production by monocytes in alcoholic hepatitis. *Hepatology* **9**, 349-351.
- McClain, C., Hill, D., Schmidt, J. and Diehl, A.M. (1993) Cytokines and alcoholic liver disease. *Seminars in Liver Disease* **13**, 170-182.
- McDermott, D.H., Zimmerman, P.A., Guignard, F., Kleeberger, C.A., Leitman, S.F. and Murphy, P.M. (1998) CCR5 promoter polymorphism and HIV-1 disease progression. *Lancet* **352**, 866-870.
- McNab, G., Afford, S.C., Morland, C., Strain, A.J. and Adams, D.H. (1996) Human hepatic sinusoidal endothelial cells upregulate adhesion molecule expression and chemokine secretion in response to proinflammatory cytokines in vitro. *Gut* **38**, A51
- Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Hub, E. and Rot, A. (1997) Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell* **91**, 385-395.
- Moore, K. (1997) The hepatorenal syndrome (review article). *Clinical Science* **92**, 433-43.

- Morland, C.M., Fear, J., McNab, G., Joplin, R. and Adams, D.H. (1997) Promotion of leukocyte transendothelial cell migration by chemokines derived from human biliary epithelial cells in vitro. *Proceedings of the Association of American Physicians* **109**, 1-11.
- Murakami, K., Abe, T., Miyazawa, M., Yamaguchi, M., Masuda, T., Matsuura, T., Nagamori, S., Takeuchi, K., Abe, K. and Kyogoku, M. (1995) Establishment of a New Human Cell Line, L190, Exhibiting Characteristics of Hepatic Ito (Fat-Storing) Cells. *Laboratory Investigation* **72**, 731-39.
- Nair, M.P.N., Schwartz, S.A., Kronfol, Z.A., Hill, E.M., Sweet, A.M. and Gregen, J.F. (1994) Suppression of tumour necrosis factor production by alcohol in lipopolysaccharide-stimulated culture. *Alcoholism - Clinical and Experimental Research* **18**, 602-607.
- Navarro, J., Punzon, M.C., Pizarro, A., Fernandez-Cruz, E., Fresno, M. and Munoz-Fernandez, M.A. (1996) Pentoxifylline inhibits acute HIV-1 replication in human T cells by a mechanism not involving inhibition of tumour necrosis factor synthesis or nuclear factor-kappa B activation. *AIDS* **10**, 469-475.
- Nelson, S., Bagby, G.J., Bainton, B.G. and Summer, W.R. (1989) The effects of acute and chronic alcoholism on tumor necrosis factor and the inflammatory response. *Journal of Infectious Diseases* **160**, 422-429.
- Neuner, P., Klosner, G., Schauer, E., Pourmojib, M., Macheiner, W., Grunwald, C., Knobler, R., Schwarz, A., Luger, T.A. and Schwarz, T. (1994) Pentoxifylline in vivo down-regulates the release of IL-1 beta, IL-6, IL-8 and tumour necrosis factor-alpha by human peripheral blood mononuclear cells. *Immunology* **83**, 262-267.
- Nordmann, R. (1994) Alcohol & antioxidant systems. *Alcohol and Alcoholism* **29**, 513-22.
- Ohkubo, K., Masuto, T., Hore, N. & Onji, M. (1998) Induction of CINC/interleukin-8 production in rat liver by nonparenchymal cells. *Journal of Gastroenterology & Hepatology* **13**, 696-702.
- Ohlinger, W., Dinges, H.P., Zatloukal, K., Mair, S., Gollowitsch, F. and Denk, H. (1993) Immunohistochemical detection of TNF- α , other cytokines and adhesion molecules in human livers with alcoholic hepatitis. *Virchows Archives* **423**, 169-176.
- Olerup O. and Zetterquist., H. (1992) HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* **39**, 225-235.
- Orrego, H., Blake, J.E., Blendis, L.M. and Medline, A. (1987) Prognosis of alcoholic cirrhosis in the presence and absence of alcoholic hepatitis. *Gastroenterology* **92**, 208-214.
- Paronetto, F. (1993) Immunological Reactions in Alcoholic Liver Disease. *Seminars in Liver Disease* **13**, 183-195.
- Patek, A.J. and Post, J. (1941) Treatment of cirrhosis of the liver by a nutritious diet and supplements rich in vitamin B complex. *Journal of Clinical Investigation* **20**, 481-505
- Pennington, H.L., Hall, P.M., Wilce, P.A. and Worrall, S. (1997) Ethanol feeding enhances inflammatory cytokine expression in lipopolysaccharide-induced hepatitis. *Journal of Gastroenterology and Hepatology* **12**, 305-313.

- Persson, J. (1991) Alcohol and the small intestine (review). *Scandinavian Journal of Gastroenterology* **26**, 3-15.
- Pirmohammed, M., Kitteringham, N.R., Quest, L.J., Allott, R.J., Green, V.J., Gilmore, I.T. and Park, B.K. (1995) Genetic polymorphism of cytochrome P4502E1 and risk of alcoholic liver disease in Caucasians. *Pharmacogenetics* **5**, 351-357.
- Powell, W.J. and Klatskin, G. (1968) Duration of survival in patients with Laennec's cirrhosis. *American Journal of Medicine* **44**, 406-420.
- Rames, A., Poirier, J.M., LeCoz, F., Midavaine, M., Lecocq, B. and Grange, J.D. (1990) Pharmacokinetics of intravenous and oral pentoxifylline in healthy volunteers and in cirrhotic patients. *Clinical Pharmacology and Therapeutics* **47**, 354-359.
- Ramond, M.J., Poynard, T., Rueff, B., Mathurin, P., Theodore, C. and Chaput, J.C. (1992) A randomised trial of prednisolone in patients with severe alcoholic hepatitis. *New England Journal of Medicine* **326**, 507-512.
- Reeves, H.L., Burt, A.D., Wood, S. and Day, C.P. (1996) Hepatic stellate cell activation occurs in the absence of hepatitis in alcoholic liver disease and correlates with the severity of steatosis. *Journal of Hepatology* **25**, 677-683.
- Reeves, H.L., Peak, M., Burt, A.D. and Day, C.P. (1998) Stress-activated protein kinases (SAPK's) are involved in the activation of hepatic stellate cells. *Gut* **43**, 156
- Reimund, J.M., Dumont, S., Muller, C.D., Kenney, J.S., Keding, M., Baumann, R., Poindron, P. and Duclos, B. (1997) In vitro effects of pentoxifylline on inflammatory cytokine release in patients with inflammatory bowel disease. *Gut* **40**, 475-480.
- Rieneck, K., Diamant, M., Haahr, P.M., Schonharting, M. and Bendtzen, K. (1993) In vitro immunomodulatory effects of pentoxifylline. *Immunology Letters* **37**, 131-138.
- Ross, R. (1993) The pathogenesis of atherosclerosis: 1990 update *Nature* **362**, 801-809.
- Rowell, D.L., Eckmann, L., Dwinell, M.B., Carpenter, S.P., Raucy, J.L., Yang, S.K. and Kagnoff, M.F. (1997) Human hepatocytes express an array of proinflammatory cytokines after agonist stimulation or bacterial invasion. *American Journal of Physiology* **273**, G322-332.
- Rubin, E. and Lieber, C.S. (1968) Alcohol-induced Hepatic Injury in Nonalcoholic Volunteers. *New England Journal of Medicine* **278**, 869-876.
- Rush, B.R., Gliksman, L. and Brook, R. (1986) Alcohol availability, alcohol consumption and related damage. I. The distribution of consumption model. *Journal of Studies on Alcohol* **47**, 1-10.
- Ruwende, C., McGuire, W., Coleman, E., Kwiatkowski, D., Corrah, T., Whittle, H. and Hill, A.V.S. (1996) Association of a tumour necrosis factor promoter polymorphism with susceptibility to pulmonary tuberculosis. *Clinical Science* **90**, 3P
- Sallusto, F., Lenig, D., Mackay, C.R. and Lanzavechia, A. (1998). Flexible programs of chemokine receptor expression on human polarised T helper 1 and 2 lymphocytes. *Journal of Experimental Medicine* **187**, 875-883.

Samson, M., Libert, F. and Doranz, B.J. (1996) Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**, 722-725.

Sanchez, S., Albornoz, L., Bandi, J.C., Gerona, S. and Mastai, R. (1997) Pentoxifylline, a drug with rheological effects, decreases portal pressure in an experimental model of cirrhosis. *European Journal of Gastroenterology and Hepatology* **9**, 27-31.

Saunders, J.B., Walters, J.R.F., Davies, P. and Paton, A. (1981) A 20-year prospective study of cirrhosis. *British Medical Journal* **282**, 263-266.

Saunders, J.B., Wodak, A.D., Haines, A., Powell-Jackson, P.R., Portmann, B., Davis, M. and Williams, R. (1982) Accelerated development of alcoholic cirrhosis in patients with HLA-B8. *Lancet* **1**, 1381-1384.

Scatchard, G. (1949) Analysis of binding affinity by competitive inhibition. *Annals of the New York Academy of Science* **51**, 660-672.

Schmidt, W. (1977) The epidemiology of cirrhosis of the liver: A statistical analysis of mortality data with special reference to Canada. In: Fisher, M.M. and Rankin, J.G. (Eds.) *Alcohol and the Liver*, pp. 1-26. New York: Plenum Press]

Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO Journal* **10**, 2247-2258.

Sekiyama, K.D., Yoshiba, M. and Thomson, A.W. (1994) Circulating proinflammatory cytokines (IL-1 β , TNF- α and IL-6) and IL-1 receptor antagonist (IL-1R α) in fulminant hepatic failure and acute hepatitis. *Clinical and Experimental Immunology* **98**, 71-77.

Sherman, D.I.N., Ward, R.J., Warren-Perry, M., Williams, R. and Peters, T.J. (1993) Association of restriction fragment length polymorphism in alcohol dehydrogenase 2 gene with alcohol induced liver damage. *British Medical Journal* **307**, 1388-1390.

Sheron, N., Bird, G., Koskinas, J., Portmann, B., Ceska, M., Lindley, I. and Williams, R. (1993) Circulating and tissue levels of the neutrophil chemotaxin interleukin-8 are elevated in severe acute alcoholic hepatitis, and correlate with neutrophil infiltration. *Hepatology* **18**, 41-46.

Shields, P.L., Morland, C.M., Salmon, M., Qin, S., Hubscher, S.G. and Adams, D.H. (1999) Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C infected liver. *Journal of Immunology* (in press).

Shimizu, Y., Newman, W. and Tanaka, Y. (1992) Lymphocyte interactions with endothelial cells. *Immunology Today* **176**, 245-253.

Shiratori, Y., Takada, H., Hikiba, Y., Nakata, R., Okano, K., Komatsu, Y., Niwa, Y., Matsumura, M., Shiina, S., Omata, M. and Kamii, K. (1993) Production of chemotactic factor, interleukin-8, from hepatocytes exposed to ethanol. *Hepatology* **18**, 1477-1482.

Smart, R.G., Mann, R.E. and Suurvali, H. (1998) Changes in Liver Cirrhosis Death Rates in Different Countries in Relation to Per Capita Alcohol Consumption and Alcoholics Anonymous Membership. *Journal of Studies on Alcohol* **59**, 245-249.

Smith, R.E., Strieter, R.M. and Zhang, K. (1995) A Role for CC Chemokines in Fibrotic Lung Disease. *Journal of Leucocyte Biology* **57**, 782-787.

Sozzani, S., Introna, M., Bernasconi, S., Polentarutti, N., Cinque, P., Poli, G., Sica, A. and Mantovani, A. (1997) MCP-1 and CCR2 in HIV infection; regulation of agonist and receptor expression. *Journal of Leukocyte Biology* **62**, 30-33.

Spielman, R.S. and Ewens, W.J. (1996) The TDT and other family-based tests for linkage disequilibrium and association. *American Journal of Human Genetics* **59**, 983-989.

Spitzer, J.J. and Bautista, A.P. (1998) Tolerance and sensitivity: Ethanol and Kupffer cells. *Gastroenterology* **115**, 494-496.

Sprenger, H., Kaufmann, A., Garn, H., Lahme, B., Gemsa, D. and Gressner, A. (1997) Induction of Neutrophil Attracting Chemokines in Transforming Rat Hepatic Stellate Cells. *Gastroenterology* **113**, 277-285.

Springer, T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-314.

Standiford, T.J. and Danforth, J.M. (1997) Ethanol feeding inhibits proinflammatory cytokine expression from murine alveolar macrophages ex vivo. *Alcoholism, Clinical & Experimental Research* **21**, 1212-1217.

Steinhoff, G., Behrend, M., Schrader, B., Duijvestijn, A.M. and Wonigeit, K. (1993) Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia - lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2 and LFA-3. *American Journal of Pathology* **142**, 481-488.

Strain, A.J., Ismail, T., Tsubouchi, H., Arakaki, N., Hishida, T., Kitamura, N., Daikuhara, Y. and McMaster, P. (1991) Native and recombinant human hepatocyte growth factors are potent promoters of DNA synthesis in both human and rat hepatocytes. *Journal of Clinical Investigation* **87**, 1853-1857.

Strieter, R.M., Poverini, P.J., Kunkel, S.L., Arenberg, D.A., Burdick, M.D., Kasper, J. and Shanafelt, A.B. (1995) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *Journal of Biological Chemistry* **270**, 27348-27357.

Strieter, R.M., Standiford, T.J., Huffnagle, G.B., Colleti, L.M., Lukacs, N.W. and Kunkel, S.L. (1996) The good, the bad and the ugly- the role of chemokines in models of human disease. *Journal of Immunology* **156**, 3583-3586.

Sylvester, I., Suffredini, A.F., Boujoukos, A.J., Martich, G.D., Danner, R.L., Yoshimura, T. and Leonard, E.J. (1993) Neutrophil attractant protein-1 and monocyte chemoattractant protein-1 in human serum. Effects of intravenous lipopolysaccharide on free attractants, IgG autoantibodies and immune complexes. *Journal of Immunology* **151**, 3292-3298.

Szabo, G., Mandrekar, P., Verma, B., Isaac, A. and Catalano, D. (1994) Acute ethanol consumption synergises with trauma to increase monocyte tumour necrosis factor alpha production late postinjury. *Journal of Clinical Immunology* **14**, 340-352.

- Szabo, G., Mandrekar, P., Girouard, L. and Catalano, D. (1996) Regulation of human monocyte functions by acute ethanol treatment: decreased tumour necrosis factor- α , interleukin-1 beta and elevated interleukin-10, and transforming growth-factor-beta production. *Alcoholism, Clinical and Experimental Research* **20**, 900-907.
- Tanaka, Y., Adams, D.H., Hubscher, S.G., Hirano, H., Siebenlist, U. and Shaw, S. (1993) T-cell adhesion induced proteoglycan-immobilised cytokine MIP-1 β . *Nature* **361**, 79-82.
- Taub, D.D., Conlon, K., Lloyd, A.R., Oppenheim, J.J. and Kelvin, D.J. (1993) Preferential migration of activated human CD4⁺ and CD8⁺ T cells in response to MIP-1a and MIP-1b. *Science* **260**, 355-358.
- Taub, D.D., Turcovski-Corrales, S.M., Key, M.L., Longo, D.L. and Murphy, W.J. (1996) Chemokines and T-lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte activation in-vitro. *Journal of Immunology* **156**, 2095-2103.
- Taub, R. (1998) Blocking NF κ B in the Liver: The Good and Bad News (Editorial). *Hepatology* **27**, 1445-1446
- Teli, M.R., Day, C.P., Burt, A.D., Bennett, M.K. and James, O.F.W. (1995) Determinants of Progression to Cirrhosis or Fibrosis in Pure Alcoholic Fatty Liver. *Lancet* **346**, 987-990.
- Thornton, A.J., Ham, J. and Kunkel, S.L. (1991) Kupffer cell-derived cytokines induce the synthesis of a leukocyte chemotactic peptide, interleukin-8, in human hepatoma and primary hepatocyte cultures. *Hepatology* **14**, 1112-1122.
- Tracey, K.J. (1995) TNF and Mae West or: death from too much of a good thing (editorial). *Lancet* **345**, 75.
- Tsukamoto, H., Wang, S.C. and Pham, T.V. (1996) NF- κ B activation and chemokine expression by hepatic stellate cells in cholestatic liver injury. *Hepatology* **24**, 331A
- Tuma, D.J., Casey, C.A. and Sorrell, M.F. (1991) Effects of alcohol on hepatic protein metabolism and trafficking (review). *Alcohol and Alcoholism* **1** (supplement), 297-303.
- Tuma, D.J., Todero, S.L., Barak-Bernhagen, M. and Sorrell, M.F. (1996) Effects of chronic ethanol administration on the endocytosis of cytokines by rat hepatocytes. *Alcoholism: Clinical and Experimental Research* **20**, 579-583.
- van Furth, A.M., Seijmonsbergen, E.M., Langermans, J.A., van der Meide, P.H. and van Furth, R. (1995) Effect of xanthine derivatives and dexamethasone on Streptococcus pneumoniae-stimulated production of tumour-necrosis factor alpha, interleukin-1 beta, and IL-10 by monocytes. *Clinical and Diagnostic Laboratory Immunology* **2**, 689-692.
- Van Zee, K.J., Fischer, E., Hawes, A.S., Hebert, C.A., Terrell, T.G., Baker, J.B., Lowry, S.F. and Moldawer, L. (1992) Effects of intravenous IL-8 administration in nonhuman primates. *Journal of Immunology* **148**, 1746-1752.
- Vane, J.R. and Botting, R. (1987) Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB Journal* **1**, 89

- Weissman, D., Rabin, R.L., Artos, J., Rubbert, A., Sworford, R., Venkatesan, S., Farber, J.M. and Fauci, A.S. (1997) Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature* **389**, 981-985.
- Wickramasinghe, S.N., Gardner, B. and Barden, G. (1987) Circulating cytotoxic protein generated after ethanol consumption: identification and mechanism of reaction with cells. *Lancet* **2**, 122-26.
- Wilhelmsen, K.C. (1997) Does tumor necrosis factor play a role in alcoholic steatohepatitis? The potential pitfalls of a case-controlled allelic association analysis (editorial). *Hepatology* **26**, 232-3.
- Willis, M.S., Klassen, L.W., Tuma, D.J. and Thiele, G.M. (1997) Different levels of protein adduction by alcohol metabolites induce antibody and T cell responses specific to the carrier protein in a dose response manner. *Hepatology* **26**, 254A
- Wilson, A.G., de Vries, N., Poctot, E., di Giovini, E., van der Putte, E. and Duff, G.W. (1993) An allelic polymorphism within the human tumour necrosis factor alpha promoter region is strongly associated with HLA A1, B8 and DR3 alleles. *Journal of Experimental Medicine* **177**, 557-560.
- Wilson, A.G., Symons, J.A., McDowell, T.L., McDevitt, H.O. and Duff, G.W. (1997) Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3195-3199.
- Witt, D.P. and Lander, A.D. (1994) Differential binding of chemokines to glycosaminoglycan populations. *Current Biology* **4**, 394-400.
- Worthington, B.S., Meserole, L. and Syrotuck, J.A. (1978) Effect of daily ethanol ingestion on intestinal permeability to macromolecules. *American Journal of Digestive Diseases* **23**, 23-32.
- Xu, Y., Rojkind, M. and Czaja, M.J. (1996) Regulation of monocyte chemoattractant protein-1 by cytokines and oxygen free radicals in rat hepatic fat-storing cells. *Gastroenterology* **110**, 1870-77.
- Zeni, F., Pain, P., Vindimian, M., Gay, J.P., Gery, P., Bertrand, M., Page, D., Vermesch, R. and Bertrand, J.C. (1996) Effects of pentoxifylline on circulating cytokine concentrations and hemodynamics in patients with septic shock: results from a double-blind, randomised, placebo-controlled study. *Critical Care Medicine* **24**, 207-214.
- Zhang, H. and Potter, B.J. (1992) The effect of ethanol metabolism on ferritin uptake by freshly isolated rat hepatocytes: Is acetaldehyde responsible for this alteration? *Alcoholism: Clinical and Experimental Research* **16**, 301-307.
- Zhang, Y.H., Broser, M. and Cohen, H. (1995) Enhanced interleukin-8 release and gene expression in macrophages after exposure to mycobacterium tuberculosis and its components. *Journal of Clinical Investigation* **95**, 586-592.
- Zickus, C., Kunkel, S.L., Simpson, K., Evanhoff, H., Glass, M., Strieter, R.M. and Lukacs, N.W. (1998) Differential regulation of CC chemokines during fibroblast-monocyte interactions: adhesion vs. inflammatory cytokine pathways. *Mediators of Inflammation* **7**, 269-274.

Appendices

Appendix 1: Antibodies used for immunohistochemical / flow cytometric analysis

1. Polyclonal (rabbit anti-human) antibodies

| <u>Antibody</u> | <u>Working Dilution</u> | <u>Source</u> |
|-----------------|-------------------------|--|
| MIP-1 α | 1:100 | Dr.U. Siebenlist, National Institutes of Health, USA |
| MIP-1 β | 1:100 | Dr.U. Siebenlist, NIH, USA |
| MCP-1 | 1:100 | Genzyme Diagnostics, Massachusetts USA |
| IL-8 | 1:50 | Dr SL Kunkel, University of Michigan, USA |

2. Monoclonal (mouse anti-human) antibodies

The following antibodies were used:

| <u>Antibody</u> | <u>Clone</u> | <u>Ig Isotype</u> | <u>Source</u> |
|-------------------------|--------------|-------------------|--|
| TNF- α | | | Serotec, UK |
| Fibroblast ¹ | | | Dianova, Hamburg, Germany |
| CXCR1 | 5A12 | IgG2b | Dr. Qin, Leukosite, Massachusetts, USA |
| CXCR2 | 6C6 | IgG1 | “ |
| CXCR3 | 1C6 | IgG1 | “ |
| CCR5 | 2D7 | IgG1 | “ |

Note 1. This antibody reacted with a 35kD epitope expressed exclusively on the cell membrane of human fibroblasts was used at a dilution of 1:200. This antibody was not found to show any positive staining with cytospin preparations of isolated hepatocytes, biliary epithelial cells, liver endothelial cells or leucocytes.

Appendix 2: Immunohistochemical methods

6µm cryostat sections were brought to room temperature for 30 minutes then fixed in absolute acetone for 10 minutes before incubation with the primary antibody. Binding of primary rabbit antibodies was detected using an alkaline phosphatase labelled swine anti-rabbit immunoglobulin secondary antibody at a dilution of 1:100 (Dako Ltd., High Wycombe, Bucks. UK) and fast red substrate (Sigma Diagnostics, Poole, Dorset, UK). Detection of mouse monoclonal primary antibodies was carried out using rabbit anti-mouse immunoglobulin at a dilution of 1:25 (Dako) followed by mouse alkaline-phosphatase/anti-alkaline phosphatase (APAAP) complexes at a dilution of 1:60 (Dako) and fast red substrate. All incubations were carried out for 45 minutes at room temperature and sections were washed for 5 minutes with 2 changes of Tris-buffered saline (TBS, pH 7.4) between incubations. In negative controls the primary antibody was substituted with non-immune serum.

Composition of reagents: all reagents were obtained from Sigma Chemical Co. Ltd., Poole, UK and made up as follows:

Tris-buffered saline (TBS, 0.05M), pH 7.4

| | |
|--|--------|
| Tris | 30.35g |
| NaCl | 40.50g |
| Distilled water | 5L |
| (pH adjusted to 7.4 with conc. HCl, approx. 16mls) | |

Tris-buffer, pH 8.2

| | |
|---|-------|
| Tris | 6.05g |
| Distilled water | 1L |
| (pH adjusted to 8.2 with conc. HCl, approx. 7mls) | |

Alkaline phosphatase substrate stock solution

| | |
|---|------|
| 1M levamisole | 50µl |
| TBS pH 8.2 | 49ml |
| Naphthol-phosphate AS-MX | 10mg |
| N,N,-dimethylformamide | 1ml |
| (The naphthol-phosphate AS-MX was dissolved in N,N,-dimethylformamide in a glass container before TBS pH 8.2 was added, followed by levamisole) | |

Alkaline phosphatase / Fast Red working solution

This was prepared freshly by dissolving 50mg of Fast Red TR in 50mls of the above stock solution, and filtering before usage.

Appendix 3: In situ hybridisation analysis

Tissue preparation: Tissue samples were fixed for 6-24 hours in freshly prepared 4% paraformaldehyde in RNAase-free phosphate-buffered saline (PBS), then fixative was exchanged for 15% sucrose/ 0.01% sodium azide in PBS for 24 hours. Sections were then mounted in Cryo-M-Bed medium, snap-frozen in liquid nitrogen, and stored at -70°C. Thereafter, 4 µm tissue sections were mounted onto poly-L-lysine coated slides and stored dessicated at -70°C until the in-situ hybridisation assay.

Probe preparation: ³⁵S uridine triphosphate-labelled antisense or sense cRNA probes were synthesised from chemokine specific cDNA sequences. The cDNAs for MIP-1α and β were 307 bp and 331 bp respectively ligated into an Eco R1/BamH1 restriction site of the plasmid vector Bluescript KS+/- . The cDNA for IL-8 (a gift from Dr.P.Watson University of Sheffield, Sheffield, UK) was a 300 bp PCR-generated cDNA sequence ligated into Pst 1 restriction site of the plasmid pCR II. In each case the ³⁵S cRNAs were characterised prior to use using polyacrylamide gel electrophoresis to ensure that both sense and antisense transcripts were comparable in size and specific activity.

Tissue hybridisation: Tissue was permeabilised with 1µg/ml proteinase K in tris/ EDTA buffer for 15-20 minutes. This reaction was stopped by washing in glycine/ PBS for 5 minutes then cells were refixed for 3 minutes in paraformaldehyde and washed again x2 in PBS. Sections were then acetylated in 0.25% acetic anhydride/ triethanolamine (pH 8.0) for 10 minutes and washed in diethylpyrocarbonate (DEPC)-treated water then air dried at 37°C. The diluted probe was then added to sections under siliconised coverslips and hybridised overnight in a sealed humidity chamber at 50°C. The next day coverslips were removed in 2x standard sodium citrate (SSC)/ 0.1% sodium dextran sulphate (SDS), washed x2 in 2x SSC/ 0.1% SDS with 10mM dithiothreitol (DTT) at room temperature and then washed x2 at 50°C. They were then washed x2 in 2xSSC, incubated for 15 mins at 37°C with 10µg/ml RNAase A, then washed x2 in 2xSSC and dehydrated through graded ethanol. Sections were then dipped in K5 photographic emulsion and exposed for 5-28 days then developed and counterstained with haemotoxylin.

Appendix 4: Chemokine mRNA measurement by RNase protection assays

Total RNA was prepared by guanidium thiocyanate extraction from freshly collected liver tissue that had been snap frozen in nitrogen and stored at -70°C . Approximately $20\mu\text{g}$ of total RNA was hybridised with ^{32}P CTP labelled cRNA probes generated from the templates described in appendix 3 (in situ hybridisation). To control for RNA loading, samples were simultaneously hybridised with a ^{32}P CTP labelled beta actin antisense cRNA generated from a commercially prepared template (pTRI- β -actin obtained from AMS Biotechnology UK Ltd., Witney, Oxon. UK). Control hybridisations substituting sample with yeast tRNA (Sigma Chemicals Poole, Dorset UK) were set up in parallel. Following hybridisation overnight at 45°C , samples were digested with $20\mu\text{g/ml}$ RNase A (Boehringer Mannheim, UK) and 250 U/ml RNase T1 (Gibco, UK) for 30 minutes at 37°C . Samples were then digested with $20\mu\text{L}$ of 10mg/ml proteinase K (Boehringer Mannheim, UK) for 20 minutes at 37°C prior to phenol chloroform extraction in the presence of carrier yeast tRNA ($1.5\mu\text{L}$ of 10mg/ml stock). Samples were then redissolved in buffer and denatured at 90°C for 5mins and subjected to electrophoresis on a 4% polyacrylamide/8M urea sequencing gel. Gels were autoradiographed, developed (approximately 1-7 days later) and assessed using laser densitometry.

Appendix 5: Isolation and culture of peripheral blood mononuclear cells (PBMC's) and neutrophils

PBMC's were isolated from citrated venous blood by density gradient centrifugation with ficoll/hypaque (Lymphoprep, Nycomed, Oslo, Norway). Under sterile conditions, blood was mixed with an equal volume of phosphate-buffered saline then 10mls of the resulting mixture was layered onto 10mls of Lymphoprep in a 20ml universal container then centrifuged for 30 minutes at 1600 rpm. The cell layer was then aspirated and washed twice in Hank's balanced salts solution (with re-centrifugation for 10 minutes at 1600 rpm inbetween washes) and counted by haemocytometry. Any visible erythrocyte contamination was lysed between steps with sterile red cell lysis buffer (comprising 0.144 M NH_4Cl and 1 mM NaHCO_3 in distilled water).

Cells were then cultured in 1ml. aliquots at a concentration of 10^6 cells/ ml. For most experiments, cells were cultured for 24 hrs at 37°C at 10^6 cells/ml in Rose Park Memorial Institute (RPMI) medium containing 10% foetal bovine serum, glutamine (2 mM/L), and antibiotics (penicillin (100 U/ml) and streptomycin (100 mg/ml) without additional stimulation; where additional agents were added to culture medium this is highlighted in the appropriate chapters.

Neutrophils were isolated from citrated venous blood by density gradient centrifugation using Percoll. Firstly, a two-tiered gradient of Percoll solutions in phosphate-buffered saline was created in 15ml conical tubes, with 2.5ml 54% Percoll overlying 2.5ml 77% Percoll. Under sterile conditions, blood was mixed with an equal volume of phosphate-buffered saline and 10mls of the resulting mixture was layered onto the Percoll gradient, then centrifuged for 30 minutes at 1250 rpm. Cells were then washed twice in phosphate-buffered saline and counted by haemocytometry prior to analysis.

Appendix 6: Measurement of MCP-1 and IL-8 levels using customised ELISA's

MCP-1: 96-microwell plates were coated using mouse monoclonal anti-human MCP-1 (500 ng/ml, R&D Systems, Oxon, UK) in carbonate/ bicarbonate buffer (pH 9.6) layered onto immunosorbent wells (Nunc) and incubated overnight at 4⁰C. Plates were then washed with phosphate buffered saline (PBS) + tween (100 µL/L), and non-specific binding blocked with 0.1% bovine serum albumin (BSA) in PBS tween. Test and reference samples were then added for 2 hrs at room temperature. Rabbit anti-human MCP-1 (2.5 µg/ml, Peprotech EC Ltd., London, UK) was added for 1 hour, followed by swine anti-rabbit antibody conjugated with horseradish peroxidase (1.3 µg/ml, Dako Ltd., Cambridge, UK) for a further hour, with washing inbetween each step. Bound antibody was detected by colorimetric analysis using tetramethylene blue (TMB) liquid substrate which was stopped with 1M sulphuric acid after 20 minutes.

IL-8: 96-microwell plates were coated using mouse anti-human IL-8 (1 µg/ml, Peprotech EC Ltd., London, UK) in carbonate/ bicarbonate buffer (pH 9.6) layered onto immunosorbent wells (Nunc) and incubated overnight at 4⁰C. The wells were then washed with PBS tween and non-specific binding was blocked with 1% BSA in PBS tween for 1 hour. Test and reference samples were then added for 2 hours after room temperature. After washing, rabbit anti-human IL-8 (kindly provided by Steve Kunkel, Michigan, USA) was added at a concentration of 50 ng/ml for 1 hour, followed by swine anti-rabbit antibody conjugated with horseradish peroxidase (1.3 mcg/ml) for a further hour, with washing after each step. Bound antibody was then detected by colorimetric analysis using TMB liquid substrate as above.

For both ELISA's, optical density was measured with an MRX electronic microwell plate reader (Dynatech Labs) reading at 450 nm, and values were analysed with Revell software. Standard curves were made using reference samples consisting of serial dilutions of recombinant human MCP-1 or IL-8 (Peprotech EC Ltd., London, UK).

The working ranges for these ELISA's were 0.125-6.667 ng/ml for MCP-1, and 2-20 ng/ml for IL-8. For the MCP-1 ELISA, intra- and inter-assay variability were measured at 5.1% and 10.3% respectively, and for the IL-8 ELISA, corresponding figures were 4.5% and 7.6% respectively.

Appendix 7: Preparation of primary adult human hepatocytes

This technique was described by Strain (1991). Normal human liver tissue was obtained from segments surgically removed from adult donor organs before reduced-graft transplant into paediatric recipients. Tissue was stored at 4°C for 12-24 hours prior to cell isolation. Hepatocytes were isolated by enzyme perfusion; two exposed vessels on the single cut surface of 100-200g segments were cannulated with 3mm internal diameter tubing. Other major vessels were sutured and the tissue was perfused at 50 ml/minute sequentially with 750 ml Ca²⁺/Mg²⁺-free Krebs ringer bicarbonate buffer containing 10mM Hepes (KRB), 1500 ml 0.5mM EGTA in KRB and 750 ml KRB. Perfusates were allowed to run to waste. Finally, 200ml enzyme solution (0.05% collagenase, 0.05% hyaluronidase, 0.1% dispase, 0.005% DNAase containing 5mM CaCl₂) was perfused with recirculation and enzymatic digestion continued for 30-45 minutes until the liver was judged to be substantially softened. The tissue was then minced with scissors in 500ml KRB, containing CaCl₂ and 10% foetal bovine serum (FBS), stirred for 10 minutes, and the cell suspension filtered through 60 µm nylon mesh. Cells were washed x3 in KRB/CaCl₂/10% FBS by centrifugation at 50g, and viability and yield assessed by trypan blue exclusion and haemocytometer counting. All solutions were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, were pregassed with 95% O₂/ 5% CO₂, and maintained at 37°C throughout.

Appendix 8: Culture of hepatocytes

Primary human hepatocytes

Cells were isolated as described in appendix 7 (Strain 1991) and were plated onto rat-tail tendon collagen-coated 35mm tissue-culture dishes in 2 mls of KRB/CaCl₂/10%FBS at a density of 10⁶ cells/ well. After 1 hour, attached cells were washed x2 in phosphate-buffered saline and then re-fed with culture medium consisting of Williams E medium supplemented with 5.5 µM (2µg/ml) hydrocortisone, 100 nM (0.124 IU/ml) insulin, 10 ng/ml hepatocyte growth factor, 2mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Thereafter, cells were washed with PBS and culture medium was replaced daily.

Hep G2 cells

These cells were obtained from the Centre for Applied Microbiology and Research, Salisbury, UK. Cells were cultured in uncoated 35mm tissue-culture dishes in RPMI-based medium as for PBMC's (appendix 5).

L190 cells

L190 cells are a human liver-derived neoplastic cell line showing considerable phenotypic similarities with activated stellate cells (Murakami 1995). These were kindly provided by Professor Matsuura, Jikei University, Tokyo, Japan). Cells were cultured in uncoated 35mm tissue-culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal bovine serum (FBS) and glutamine, penicillin and streptomycin as for PBMC's.

Splitting and reseeded of Hep G2 and L190 cells cultures: These cells have doubling times of approximately 24 hours and 72 hours respectively. When confluent, cells were incubated in trypsin/ EDTA solution (1ml per well) for approximately 5 minutes or until detached from plates. A solution of 50% FBS in PBS was then added (1ml per well) to neutralise trypsin and then cells were washed twice in PBS prior to reseeded at approximately 1:5 density.

Appendix 9: ¹²⁵Iodine-radiolabelling of MIP-1 α and IL-8

Recombinant chemokines were obtained from Peprotech EC Ltd., London, UK and ¹²⁵Iodine sodium (100 mCi/ml) was obtained from the University of Birmingham biochemistry department.

Chemokines (2 μ g of IL-8 or 9 μ g of MIP-1 α) were incubated with 3 μ l (300 μ Ci) of ¹²⁵Iodine sodium, 50 μ l of 0.1% chloramine T¹ and 50 μ l of 0.25% sodium metabisulphite² for 15 seconds. This mixture was then made up to a total of 260 μ l with a solution of phosphate-buffered saline / 3% bovine serum albumin / 5 mM potassium iodide. Iodinated chemokine protein was separated from free iodine on a sephadex column³ and stored at 4^oC.

For IL-8 and MIP-1 α this yielded specific activities of 5x 10⁶ dpm/ μ g and 6.3x 10⁶ dpm/ μ g respectively. Confirmation of radiolabelling was done with SDS polyacrylamide gel electrophoresis (PAGE) and autoradiography which confirmed pure signals with proteins of the expected molecular weights.

Notes

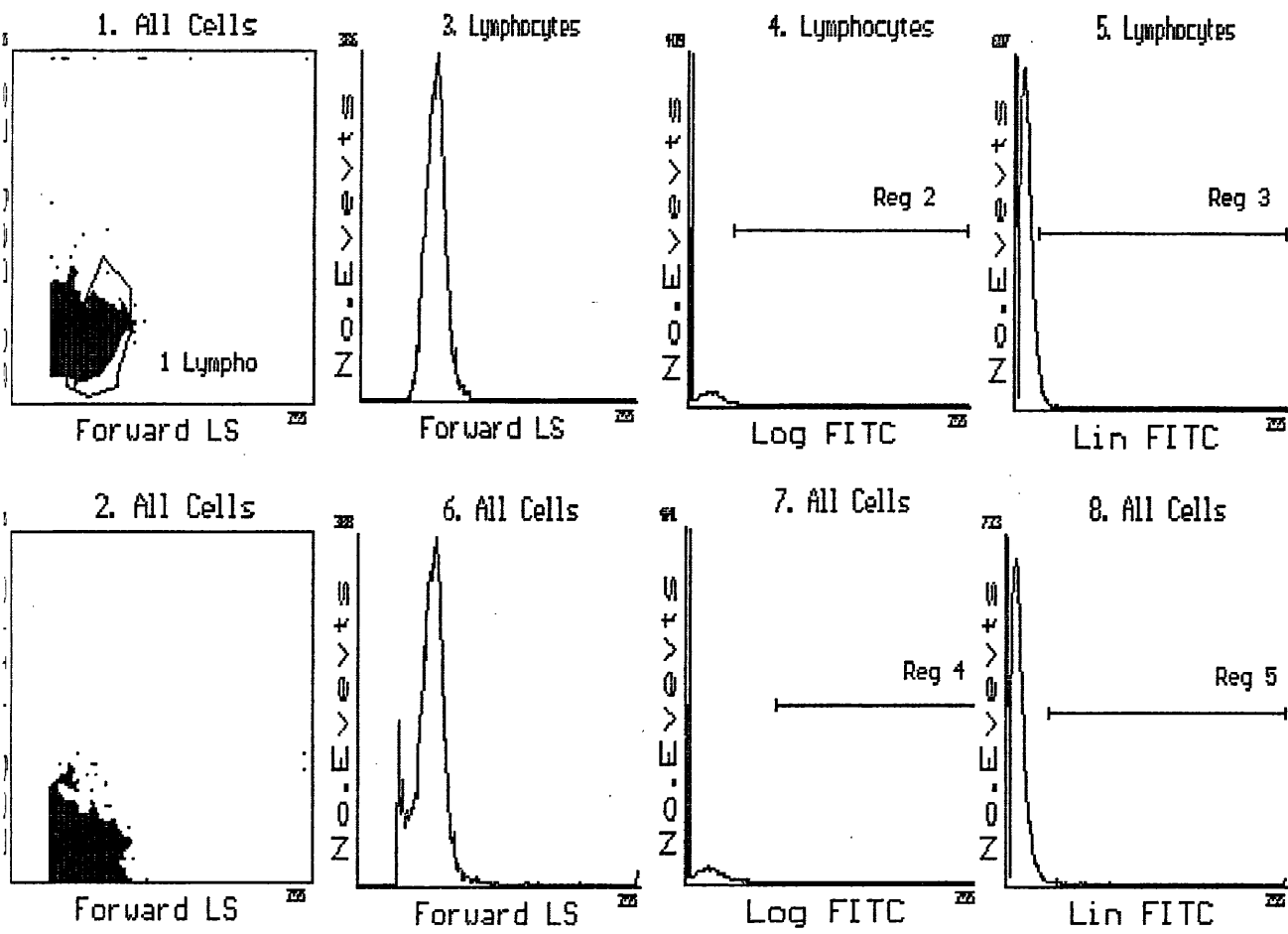
1. Chloramine T made up freshly in 0.05 M phosphate buffer (pH 7.5), containing 0.5 M sodium chloride.
2. Sodium metabisulphite in 0.5 M phosphate made up freshly in 0.05 M phosphate buffer (pH 7.5), containing 0.5 M sodium chloride.
3. Sephadex G10 column (approx. 10cm x 0.5cm) equilibrated in phosphate-buffered saline / bovine serum albumin (as above) and volume determined was voided with dextran blue.

Appendix 10: Labelling of cells for flow cytometry

Single cell suspensions were made with lymphocytes, neutrophils and hepatocytes obtained as described earlier. Cells were suspended in normal human immunoglobulin (to block Fc receptors) for 20 minutes at 4°C. Approximately 0.5×10^6 cells were then incubated with 5µl of the optimal concentration (between 10 and 50 µg/ml) of the primary antibody in 100 µl FACS medium (phosphate-buffered saline with 1% foetal bovine serum and 0.1% sodium azide) for 1 hour at 4°C. The cell suspensions were then centrifuged and washed in FACS medium (two cycles) and incubated with fluorescein isothiocyanate (FITC)-conjugated F(ab)₂ fragments of rabbit anti-mouse immunoglobulin (Dako, UK) at 1 in 20 dilution for a further 1 hour. Cells were then washed twice in FACS medium and incubated with normal mouse serum to saturate free binding sites on the FITC-conjugated F(ab)₂ fragments. After a further 2 washings, cells were fixed in 1% paraformaldehyde and stored in sealed opaque containers at 4°C prior to flow cytometric analysis.

Irrelevant mouse isotypes IgG1 and IgG2a were used as control antibodies. Flow cytometry analysis was kindly done by Roger Bird in the University of Birmingham Medical School with a FACS 440 analyser (Becton-Dickinson), with appropriate gating to exclude dead cells and debris. At least 10,000 cells were analysed in each sample.

Examples of flow cytometry histograms for CXCR2 antibody staining on PBMC's, neutrophils and hepatocytes are given on the following pages.



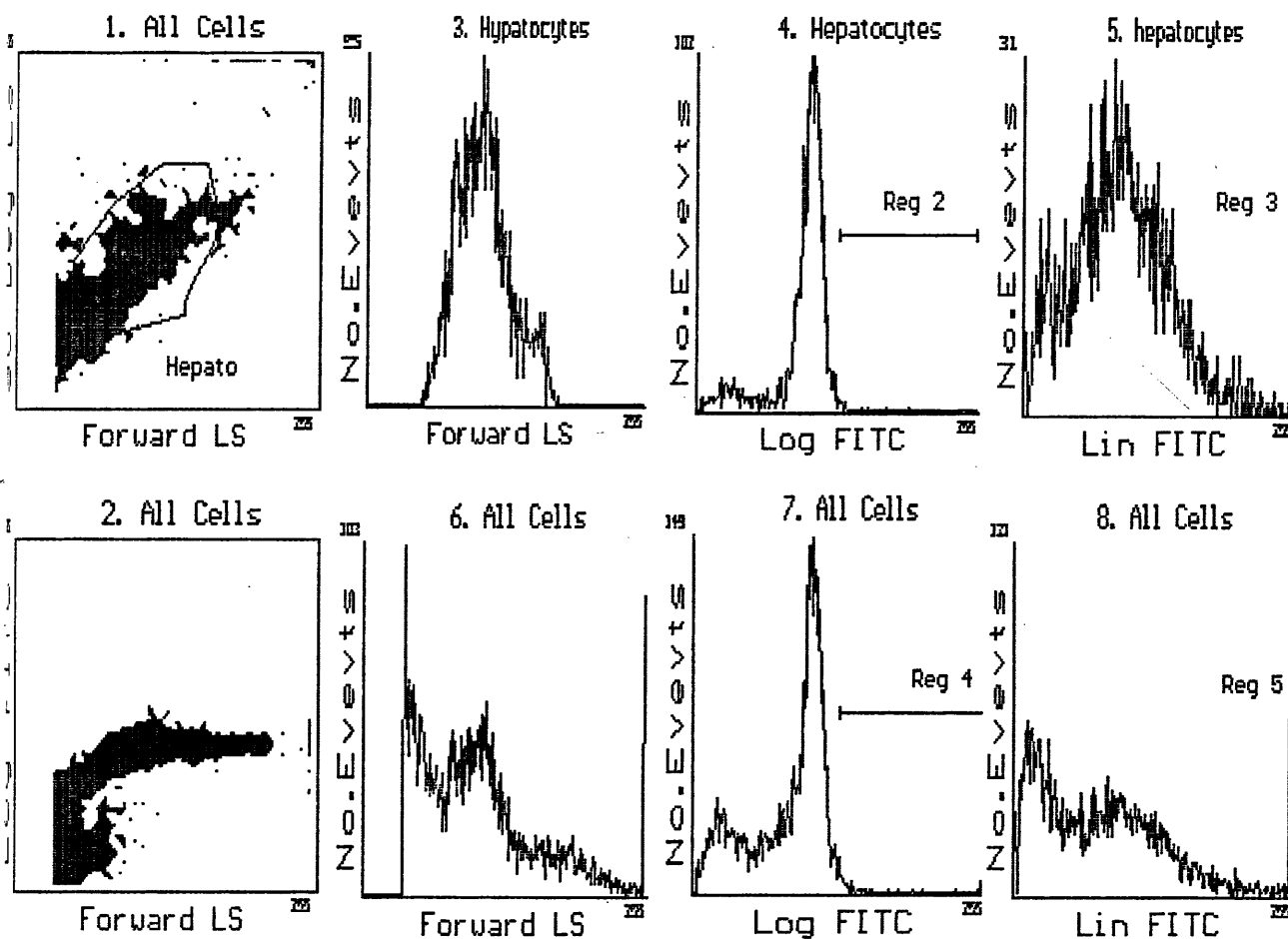
Histogram Statistics

| Gr\Pl | Title | File | Chan | Mean | SD | Med | Mode | Total |
|-------|------------|----------|------|-------|------|-----|------|-------|
| 3- 3 | Forward LS | FILEF006 | 255 | 67.90 | 0.72 | 67 | 69 | 8124 |
| 6- 4 | Forward LS | FILEF006 | 255 | 65.11 | 0.59 | 65 | 69 | 10001 |
| 4- 5 | Log FITC | FILEF006 | 255 | 10.70 | 0.10 | 0 | 1 | 8124 |
| 7- 6 | Log FITC | FILEF006 | 255 | 13.91 | 0.10 | 5 | 1 | 10001 |
| 5- 7 | Lin FITC | FILEF006 | 255 | 9.12 | 0.12 | 7 | 0 | 8124 |
| 8- 8 | Lin FITC | FILEF006 | 255 | 10.68 | 0.12 | 8 | 0 | 10001 |

Region Statistics

Linear Regions

| Region | L Ch | H Ch | Mean | SD | Med | Mode | Total (%) |
|------------------|--------------|------|-------|------|-----|------|-------------|
| Graph 4 - Plot 2 | 5 - Log FITC | | | | | | |
| 3 Reg 2 | 39 | 255 | 51.48 | 0.63 | 43 | 38 | 275 (3.39) |
| Graph 5 - Plot 3 | 7 - Lin FITC | | | | | | |
| 3 Reg 3 | 21 | 255 | 24.82 | 0.48 | 21 | 20 | 465 (5.72) |
| Graph 7 - Plot 4 | 6 - Log FITC | | | | | | |
| 4 Reg 4 | 78 | 255 | 96.99 | 1.25 | 86 | 77 | 139 (1.39) |
| Graph 8 - Plot 5 | 8 - Lin FITC | | | | | | |
| 5 Reg 5 | 39 | 255 | 81.28 | 1.12 | 60 | 255 | 154 (1.54) |



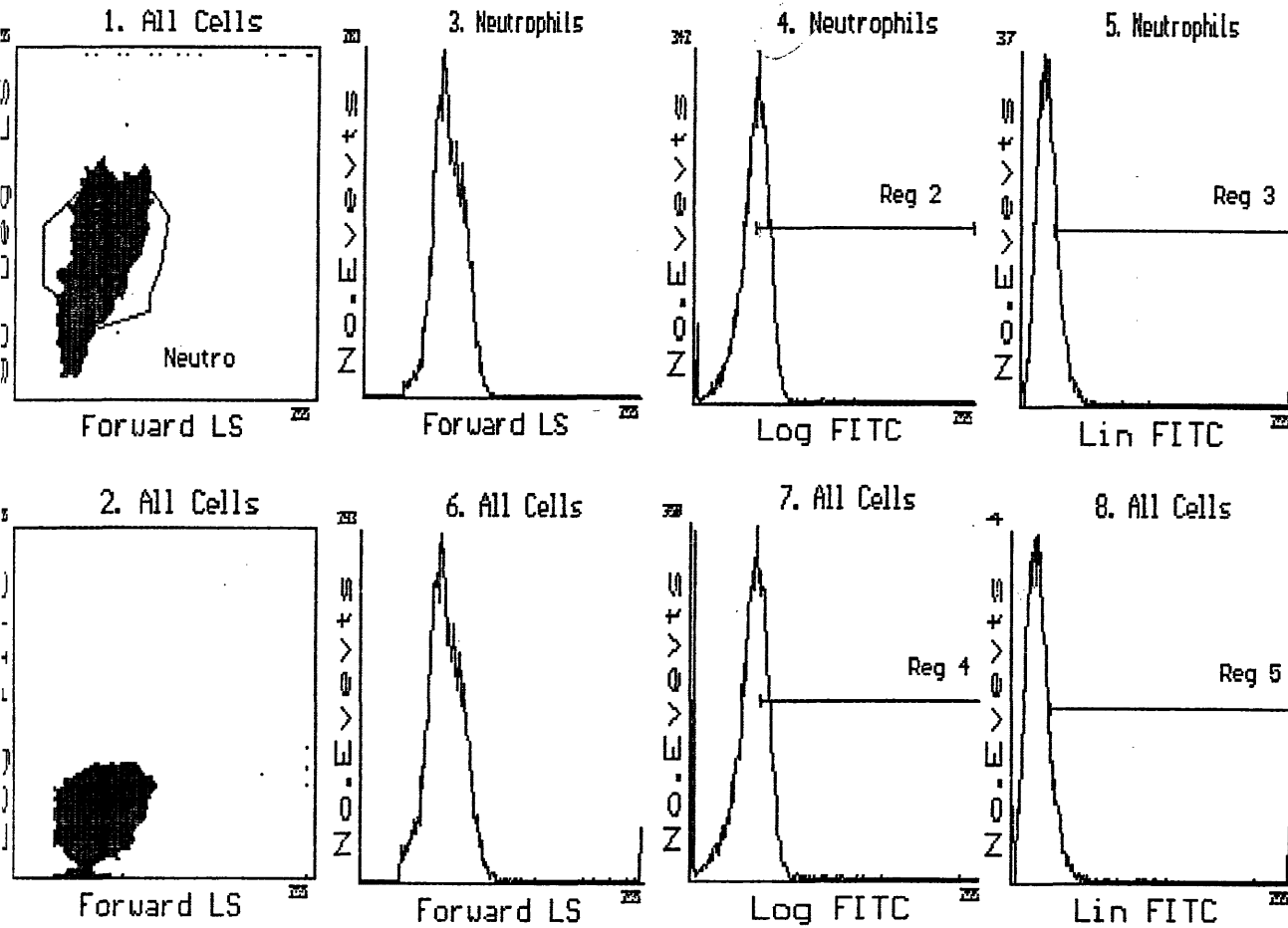
Histogram Statistics

| Gr\Pl | Title | File | Chan | Mean | SD | Med | Mode | Total |
|-------|------------|----------|------|--------|------|-----|------|-------|
| 3- 3 | Forward LS | FILEF018 | 255 | 105.31 | 0.58 | 103 | 105 | 2379 |
| 6- 4 | Forward LS | FILEF018 | 255 | 101.85 | 0.39 | 93 | 35 | 4820 |
| 4- 5 | Log FITC | FILEF018 | 255 | 94.96 | 0.94 | 100 | 103 | 2379 |
| 7- 6 | Log FITC | FILEF018 | 255 | 82.21 | 0.65 | 95 | 103 | 4820 |
| 5- 7 | Lin FITC | FILEF018 | 255 | 91.38 | 0.34 | 89 | 85 | 2379 |
| 8- 8 | Lin FITC | FILEF018 | 255 | 81.21 | 0.43 | 74 | 255 | 4820 |

Region Statistics

Linear Regions

| Region | L Ch | H Ch | Mean | SD | Med | Mode | Total (%) |
|-----------------------------|-----------------------|------|--------|------|-----|------|-------------|
| Graph 4 - Plot 5 - Log FITC | - FILEF018.LMD - HC6) | | | | | | |
| 2 Reg 2 | 129 | 255 | 141.71 | 3.99 | 133 | 134 | 14 (0.59) |
| Graph 5 - Plot 7 - Lin FITC | - FILEF018.LMD - HC6) | | | | | | |
| 3 Reg 3 | 255 | 255 | 0.00 | 0.00 | 0 | 255 | 14 (0.59) |
| Graph 7 - Plot 6 - Log FITC | - FILEF018.LMD - HC6) | | | | | | |
| 4 Reg 4 | 128 | 255 | 157.15 | 3.87 | 134 | 255 | 73 (1.51) |
| Graph 8 - Plot 8 - Lin FITC | - FILEF018.LMD - HC6) | | | | | | |
| 5 Reg 5 | 255 | 255 | 0.00 | 0.00 | 0 | 255 | 121 (2.51) |



Histogram Statistics

| Gr\Pl | Title | File | Chan | Mean | SD | Med | Mode | Total |
|-------|------------|----------|------|-------|------|-----|------|-------|
| 3- 3 | Forward LS | FILEF012 | 255 | 76.70 | 0.62 | 74 | 71 | 8708 |
| 6- 4 | Forward LS | FILEF012 | 255 | 77.10 | 0.57 | 74 | 71 | 10001 |
| 4- 5 | Log FITC | FILEF012 | 255 | 53.47 | 0.49 | 54 | 56 | 8708 |
| 7- 6 | Log FITC | FILEF012 | 255 | 51.91 | 0.46 | 53 | 56 | 10001 |
| 5- 7 | Lin FITC | FILEF012 | 255 | 24.00 | 0.23 | 21 | 19 | 8708 |
| 8- 8 | Lin FITC | FILEF012 | 255 | 25.06 | 0.23 | 21 | 23 | 10001 |

Region Statistics

Linear Regions

| Region | L Ch | H Ch | Mean | SD | Med | Mode | Total (%) |
|-----------------------------|----------------------|------|-------|------|-----|------|---------------|
| Graph 4 - Plot 5 - Log FITC | - FILEF012.LMD - N6) | | | | | | |
| 2 Reg 2 | 54 | 255 | 62.69 | 0.87 | 60 | 56 | 5035 (57.82) |
| Graph 5 - Plot 7 - Lin FITC | - FILEF012.LMD - N6) | | | | | | |
| 3 Reg 3 | 29 | 255 | 38.33 | 0.52 | 32 | 28 | 2637 (30.28) |
| Graph 7 - Plot 6 - Log FITC | - FILEF012.LMD - N6) | | | | | | |
| 4 Reg 4 | 61 | 255 | 68.43 | 1.10 | 64 | 60 | 3327 (33.27) |
| Graph 8 - Plot 8 - Lin FITC | - FILEF012.LMD - N6) | | | | | | |
| 5 Reg 5 | 34 | 255 | 51.73 | 0.79 | 38 | 34 | 1810 (18.10) |

Appendix 11: Preparation of purified DNA

10mls of ethylenediamine-tetra-acetic acid (EDTA)-preserved blood were obtained from each subject and transferred to universal containers then centrifuged at 2500 rpm for 10 minutes. The 'buffy coat' (white cell layer) was then aspirated, transferred to 15ml conical tubes and mixed with red cell lysis buffer for 10 minutes. White cell pellets were then purified by centrifugation and stored at -20°C prior to preparation of DNA which was done in batches of 12-24 samples. This was done by mixing the white cell pellet with 3 mls nuclear lysis buffer, resuspending the pellet several times then adding 200µL SDS and 125µL proteinase K solution (Boehringer Mannheim) to each tube. The tubes were incubated at 42°C in water baths overnight for 18 hours. The following day, DNA was separated by adding 1ml of 6M NaCl to each tube, agitating vigorously, adding 2mls chloroform and agitating again, then centrifuging for 30 minutes at 3000 rpm. The DNA layer was then aspirated and precipitated in 95% ethanol, then washed twice in 70% ethanol. Pellets were then decanted into Eppendorf tubes and centrifuged for 30 seconds at 14000 rpm, then drained, air-dried, and finally dissolved in 300µL sterile water and stored at -20°C.

Reagents

Red cell lysis buffer

1.44M NH₄Cl
10mM NaHCO₃
(prepared in distilled water)

Nuclear lysis buffer

10mM Tris-HCl, pH 8.2
0.4M NaCl
2mM Na₂EDTA, pH 8.0
(prepared in distilled water)

Proteinase K stock buffer

1% w/v sodium dextran sulphate (SDS)
2mM Na₂EDTA, pH 8.0
(prepared in distilled water)

Proteinase K working solution

2mg/ ml Proteinase K diluted in 10mls stock buffer

Publications

The following pages contain reprints of two articles which have been published in peer-reviewed journals following the work described in this thesis:

1. Afford, S.C., Fisher, N.C., Neil, D.A.H., Fear, J., Keogh, A., Brun, P., Hubscher, S.G. and Adams, D.H. (1998). Distinct patterns of chemokine expression are associated with leucocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis. *Journal of Pathology* **186**, 82-89.
2. Fisher, N.C., Neil, D.A.H., Williams, A. and Adams, D.H. (1999). Serum levels and peripheral secretion of the beta chemokines MCP-1 and MIP-1a in alcoholic liver disease. *Gut* **45**, 416-420.

DISTINCT PATTERNS OF CHEMOKINE EXPRESSION ARE ASSOCIATED WITH LEUKOCYTE RECRUITMENT IN ALCOHOLIC HEPATITIS AND ALCOHOLIC CIRRHOSIS

SIMON C. AFFORD^{1*}, NEIL C. FISHER¹, DESLEY A. H. NEIL¹, JANINE FEAR¹, PAOLA BRUN², STEFAN G. HUBSCHER³ AND DAVID H. ADAMS¹

¹*Liver Research Laboratories, Institute of Clinical Research, University of Birmingham, Edgbaston, Birmingham B15 2TH, U.K.*

²*Division of Gastroenterology and Histopathology, University of Padova, Italy*

³*Department of Pathology, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.*

SUMMARY

Alcoholic liver disease is associated with three histologically distinct processes: steatosis (parenchymal fat accumulation), alcoholic hepatitis (characterized by parenchymal infiltration by neutrophil polymorphs), and alcoholic cirrhosis (in which chronic inflammation and fibrosis dominate). Chemokines are cytokines that promote subset-specific leukocyte recruitment to tissues and could therefore play a crucial role in determining which leukocyte subsets are recruited to the liver in alcoholic liver disease. This paper reports that chemokine expression is increased in the liver of patients with alcoholic liver disease and, moreover, that distinct patterns of chemokine expression are associated with the different inflammatory responses to alcohol. Interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), and MIP-1 β were all detected in the parenchyma at sites of inflammation in alcoholic hepatitis, whereas in alcoholic cirrhosis, chemokines were restricted to inflammatory cells and endothelium in the fibrous septa and portal tracts. In alcoholic hepatitis, chemokine transcription was localized to sinusoidal cells, leukocytes, and fibroblasts in areas of parenchymal inflammation, but hepatocytes, despite staining strongly for chemokine protein, were negative. In alcoholic cirrhosis, chemokine mRNA was detected in portal tract endothelium, leukocytes, and fibroblasts. Thus, alcoholic hepatitis and alcoholic cirrhosis are associated with distinct patterns of chemokine expression that are likely to be important factors in determining whether a patient develops acute parenchymal inflammation and alcoholic hepatitis, or chronic septal inflammation and alcoholic cirrhosis. © 1998 John Wiley & Sons, Ltd.

KEY WORDS—alcoholic liver disease; leukocyte recruitment; chemokines; inflammation

INTRODUCTION

The development of alcohol-induced liver injury is determined, in part, by the immunological/inflammatory response that alcohol stimulates.^{1–3} Three histologically distinct processes may be observed: steatosis, characterized by parenchymal fat accumulation; alcoholic hepatitis, in which infiltration of the parenchyma by leukocytes, particularly neutrophils, is associated with hepatocyte damage; and cirrhosis, associated with chronic inflammation and fibrosis. All three manifestations may be present independently or in combination.^{4,5} Although alcoholic hepatitis is frequently associated with cirrhosis, it can present as a distinct clinical syndrome that, in its most extreme form, causes liver failure and has a high mortality.^{6,7} Although the reasons for these different responses to alcohol are not known, factors that regulate leukocyte recruitment to the liver will be crucial in determining whether patients develop acute inflammation, hepatocyte damage and alcoholic hepatitis, or a chronic response and cirrhosis.^{8,9}

Leukocyte recruitment from the circulation into tissues requires the presence of local chemotactic cytokines (chemokines) that are localized at sites of inflammation, where they activate subsets of leukocytes via specific cell surface receptors to undergo migration into tissue.^{10,11} Immunohistochemical studies have demonstrated chemokines on the luminal aspect of blood vessels in hepatic endothelium in allograft rejection,¹² but little is known about the distribution and expression of chemokines in alcoholic liver disease. There are over 30 known human chemokines that can be divided into three sub-families, based on their structure.^{13,14} These structural distinctions are important in determining the cell specificity of the chemokines;^{10,13} thus, many α -chemokines, of which interleukin-8 (IL-8) is the prototype, act on neutrophils, whereas β -chemokines, such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α and MIP-1 β , act on lymphocytes and monocytes.¹⁵ The composition, distribution, and duration of an inflammatory infiltrate in alcoholic liver disease will therefore depend on the combination of chemokines present in the local micro-environment.^{10,13,15,16}

It has been suggested that α -chemokines are important in the recruitment of neutrophils in alcoholic hepatitis, because circulating and tissue levels of IL-8 and GRO α are elevated in acute alcoholic hepatitis and

*Correspondence to: Dr S. C. Afford, Liver Research Laboratories, The Queen Elizabeth Hospital, Edgbaston, Birmingham B15 2TH, U.K.

Contract grant sponsors: Wellcome Trust; Endowment Fund of the Former Unit Birmingham Hospitals.

some of the histological features of alcoholic hepatitis can be reproduced in rats by transfecting hepatocytes *in vivo* with α -chemokines.^{17–20} These studies have concentrated on α -chemokines, but the expression of the β -chemokines may be equally important, as these are potent chemotactic factors for monocytes and lymphocytes, and likely to be involved in establishing the chronic inflammatory response to alcohol. Furthermore, little is known about either the local distribution of chemokines or the cells responsible for their synthesis in alcoholic liver disease. It is against this background that we investigated the expression of the four chemokines MCP-1, MIP-1 α , MIP-1 β , and IL-8 in patients with alcoholic liver disease, in order to determine whether particular patterns of chemokine expression are associated with the different manifestations of alcoholic liver disease.

MATERIALS AND METHODS

Sample collection and processing

Liver tissue was obtained from (a) patients with histological evidence of alcoholic hepatitis (transjugular liver biopsy specimens, $n=6$). All six patients had clinically severe alcoholic hepatitis (defined as liver failure and a discriminant function above 17 as described by Maddrey⁶ and Chendid *et al.*²¹). Four of the six patients had histological features of cirrhosis in addition to alcoholic hepatitis; (b) hepatectomy specimens from patients with end-stage alcoholic cirrhosis who had abstained from alcohol for 6 months ($n=10$). All hepatectomy specimens had characteristic histological features of end-stage alcoholic cirrhosis;⁵ (c) non-diseased liver ($n=6$) obtained from organ donors in whom the liver had been surgically reduced for paediatric transplantation. Liver disease was excluded biochemically and histologically. Tissue specimens were divided and either snap-frozen in liquid nitrogen for immunohistochemistry or fixed in fresh 4 per cent paraformaldehyde, and snap-frozen in Cryo-M-bed mounting medium to be used for *in situ* hybridization (ISH) studies.¹² All specimens were stored at -70°C until analysis.

Immunohistochemical studies

Immunohistochemistry was performed on snap-frozen tissues as described previously,^{22–25} with the following primary antibodies: (a) Polyclonal rabbit antibodies specific for MIP-1 α or MIP-1 β (a gift from Dr U. Siebenlist, NIH, U.S.A.) were used at a dilution of 1:100 for 1 h at room temperature.^{22,23} (b) Polyclonal rabbit antibodies to MCP-1 (Genzyme Diagnostics, Cambridge, MA, U.S.A.) were used at a dilution of 1:100. (c) Polyclonal rabbit anti-IL-8 and anti-MCP-1 (gifts from Dr S. L. Kunkel, University of Michigan, U.S.A.) were used at a dilution of 1:50.²⁶ (d) Anti-tumour necrosis factor- α (anti-TNF- α) was supplied by Serotec, U.K. (e) A monoclonal anti-human fibroblast antibody that reacted with a 35 kD epitope

expressed exclusively on the cell membrane of human fibroblasts was obtained from Dianova, Klein Fontenay 1, Hamburg, Germany and used at a dilution of 1:200. The latter antibody was found to be negative against cytospun preparations of isolated hepatocytes, biliary epithelial cells, liver endothelial cells, and leukocytes.

Binding of primary rabbit antibodies was detected using an alkaline phosphatase-labelled swine anti-rabbit immunoglobulin secondary antibody at a dilution of 1:100 (Dako Ltd, High Wycombe, Bucks, U.K.) and fast red substrate (Sigma Diagnostics, Poole, Dorset, U.K.). Detection of mouse monoclonal primary antibodies was carried out using rabbit anti-mouse at a dilution of 1:25 (Dako), followed by mouse alkaline-phosphatase/anti-alkaline phosphatase complexes at a dilution of 1:60 (Dako) and fast red substrate. In negative controls, the primary antibody was replaced by non-immune serum. In three patients with alcoholic hepatitis, two with cirrhosis, and two donor livers, dual staining was carried out with antibodies to TNF- α (Serotec, U.K.) as well as anti-chemokine antibodies.

Localization of chemokine mRNA by *in situ* hybridization

In situ mRNA hybridization was done using paraformaldehyde-fixed tissue as described previously.¹² Briefly, ³⁵S uridine triphosphate-labelled antisense or sense cRNA probes were synthesized from chemokine-specific cDNA sequences.²² The cDNAs for MIP-1 α and MIP-1 β were 307 and 331 bp respectively, ligated into an EcoRI/BamHI restriction site of the plasmid vector Bluescript KS+/- . The cDNA for IL-8 (a gift from Dr P. Watson, University of Sheffield, Sheffield, U.K.) was a 300 bp PCR-generated cDNA sequence ligated into the Pst I restriction site of the plasmid pCR II. In each case, the ³⁵S cRNAs were characterized prior to use, using polyacrylamide gel electrophoresis to ensure that both sense and antisense transcripts were comparable in size and specific activity. Sections were developed and examined for positivity after 7–14 days' autoradiography.

Histological assessment and scoring

Sections were assessed blind using a semi-quantitative system that we had previously validated by confocal microscopy.^{25,26} Staining was scored according to intensity and distribution on a five-point scale from negative to + + + +, where negative=no staining or positive hybridization relative to control sections, + = weak positivity, and + + + + = maximum positivity. The following structures were assessed: portal vessels (hepatic arterioles and portal venules); hepatic venules; sinusoidal cells (Kupffer cells and sinusoidal endothelium); bile ducts; hepatocytes; and infiltrating leukocytes.

Measurement of chemokine mRNA by RNase protection assays

Quantitative assessment of chemokine mRNA for MIP-1 α and MIP-1 β was carried out using an RNase

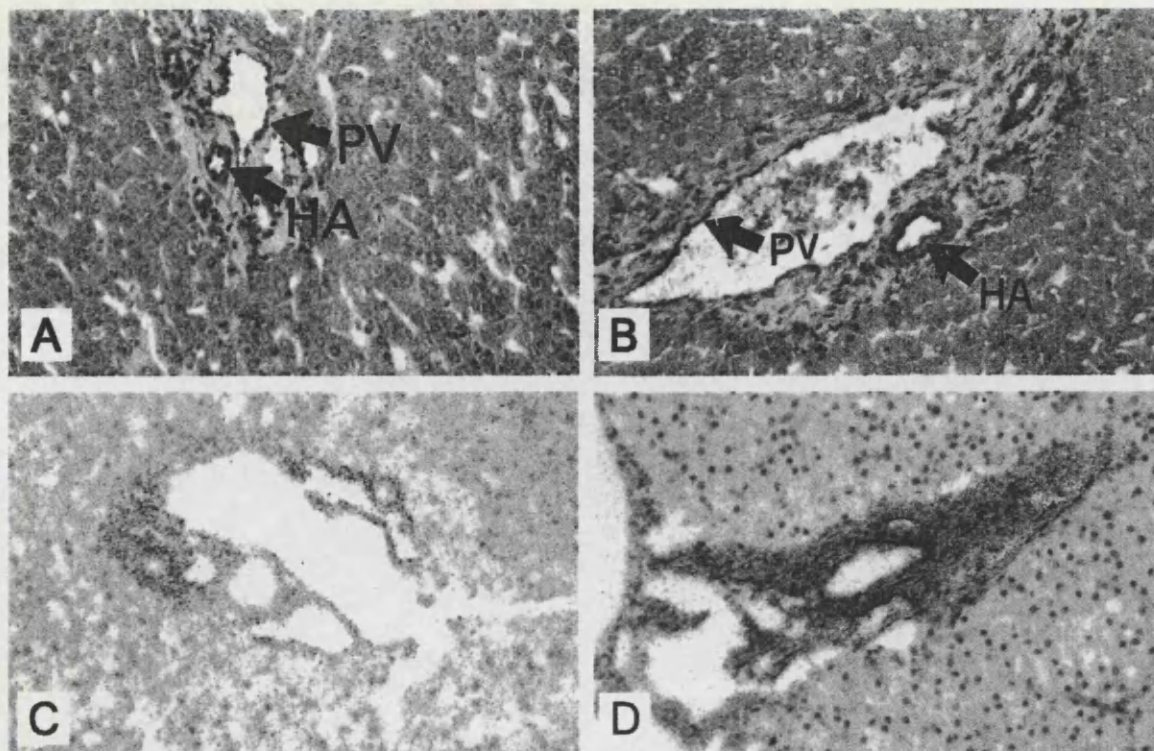


Fig. 1—Localization of chemokine protein and mRNA in non-inflamed liver tissue from organ donors. MCP-1 (panel A) and IL-8 (panel B) chemokine proteins were detected by immunohistochemistry in normal human liver tissue in portal veins (PV), hepatic arterioles (HA), and occasional inflammatory cells. Panels C and D show MCP-1 and IL-8 mRNA expression in normal tissue. Positive hybridization was confined to perivascular areas (portal tracts and central vein)

protection assay described in detail elsewhere.²⁷ Briefly, total RNA was prepared by guanidium thiocyanate extraction from freshly collected liver tissue that had been snap-frozen in nitrogen and stored at -70°C . Approximately $20\ \mu\text{g}$ of total RNA was hybridized with ^{32}P CTP-labelled cRNA probes generated from the templates described above (see *in situ* hybridization). Samples were simultaneously hybridized with a ^{32}P CTP-labelled β -actin antisense cRNA generated from a commercially prepared template (pTRI-b-actin obtained from AMS Biotechnology U.K. Ltd, Witney, Oxon, U.K.). Control hybridizations substituting yeast tRNA (Sigma Chemicals, Poole, Dorset U.K.) for sample were set up in parallel. Following hybridization and RNase digestion, samples were redissolved in buffer, denatured at 90°C for 5 min, and subjected to electrophoresis on a 4 per cent polyacrylamide/8 M urea sequencing gel. Gels were autoradiographed, developed (approximately 1–7 days later), and assessed using laser densitometry.

RESULTS

Chemokine expression in non-diseased liver

Immunohistochemistry demonstrated that MIP-1 α , MIP-1 β , MCP-1, and IL-8 protein could all be detected in non-diseased liver tissue. Staining was weak and confined to the vascular endothelium, sub-endothelial stroma, and occasional inflammatory cells (Figs 1A and 1B and Table I); hepatocytes were negative. *In situ* hybridization localized chemokine mRNA to the

occasional inflammatory cells and areas surrounding the vascular endothelium, indicating that low-level chemokine gene transcription was occurring even in the absence of active inflammation (Figs 1C and 1D and Table I).

Chemokine protein and mRNA expression in alcoholic liver disease

All four chemokine proteins were detected in ballooned hepatocytes at sites of active inflammation in severe alcoholic hepatitis. Variable staining ranging from moderate to intense was also observed in vascular and sinusoidal endothelium, intrahepatic bile ducts, and inflammatory cells (Fig. 2a and Table I). In patients with alcoholic hepatitis, ISH demonstrated greatly increased chemokine transcription in areas of inflammation, where positivity could be seen in inflammatory cells, vascular endothelium, sinusoidal lining cells, and particularly in areas of pericellular fibrosis (Fig. 2c and Table I). Intrahepatic bile ducts were negative. The cellular distribution of chemokine mRNA was similar for all chemokines and was associated with areas of active fibrosis as determined by immunohistochemical staining for fibroblasts (Figs 2e and 2f).

Chemokine proteins were detected in tissues obtained from patients with end-stage alcoholic cirrhosis without active alcoholic hepatitis. All chemokine proteins were detected in inflammatory cells, intrahepatic bile ducts, vascular endothelium, and, in particular, within fibrous septa. Unlike alcoholic hepatitis, no chemokine protein

Table I—Chemokine protein and mRNA expression in alcoholic liver disease. This table summarizes the expression of chemokine protein and mRNA in normal liver, alcoholic hepatitis, and alcoholic cirrhosis. Sections were scored according to standard criteria (see Materials and Methods)

| Chemokine | Distribution | Protein | | | mRNA | | |
|----------------|----------------------|---------|-----------|------------|--------|-----------|------------|
| | | Normal | Alc. hep. | Alc. cirr. | Normal | Alc. hep. | Alc. cirr. |
| MIP-1 α | Inflam. cells | + / + + | +++ | +++ | + | +++ | — |
| | Sinusoidal cells | — | +++ | — | — | +++ | — |
| | Biliary epithelium | — | +++ | +++ | — | — | — |
| | Vascular endothelium | + / + + | +++ | +++ | — | +++ | — |
| | Hepatocytes | — | +++ | — | — | + | — |
| | Fibrous septa | np | +++ | +++ | np | +++ | +++ |
| MIP-1 β | Inflam. cells | + / + + | +++ | +++ | + | +++ | — |
| | Sinusoidal cells | — | +++ | — | — | +++ | — |
| | Biliary epithelium | — | +++ | +++ | — | — | — |
| | Vascular endothelium | + / + + | +++ | +++ | — | +++ | — |
| | Hepatocytes | — | +++ | — | — | + | — |
| | Fibrous septa | np | +++ | +++ | np | +++ | +++ |
| MCP-1 | Inflam. cells | + / + + | +++ | +++ | ++ | +++ | — |
| | Sinusoidal cells | — | +++ | — | — | +++ | — |
| | Biliary epithelium | — | +++ | +++ | — | — | — |
| | Vascular endothelium | + / + + | +++ | +++ | ++ | +++ | +++ |
| | Hepatocytes | — | +++ | — | — | + | — |
| | Fibrous septa | np | +++ | +++ | np | +++ | +++ |
| IL-8 | Inflam. cells | + / — | +++ | ++ | ++ | +++ | ++ |
| | Sinusoidal cells | + | +++ | + / — | — | +++ | — |
| | Biliary epithelium | + / — | +++ | ++ | — | — | — |
| | Vascular endothelium | ++ | ++++ | +++ | ++ | +++ | +++ |
| | Hepatocytes | + / — | +++ | — | — | — | — |
| | Fibrous septa | np | +++ | + / — | np | +++ | +++ |

np=not present.

was detected in either hepatocytes or sinusoids (Fig. 2b and Table I). mRNAs for all chemokines were present throughout the fibrous septa, with MCP-1 and IL-8 transcripts also detected in vascular endothelium and vessel walls (Table I). mRNA expression in alcoholic cirrhosis was again strongly associated with the presence of fibroblasts, particularly at the interface of septa and adjacent liver (Fig. 2f).

Expression of chemokine and TNF- α in alcoholic liver disease

In normal liver, TNF- α protein was restricted to vascular endothelium and the occasional inflammatory cells and Kupffer cells; the staining was generally weak. In alcoholic cirrhosis, a similar pattern was observed, but the staining was stronger. In alcoholic hepatitis, there was strong staining in vascular endothelium, sinusoidal lining cells, and intense staining associated with inflammatory infiltrates in the parenchyma (Figs 3a and 3b). Double staining revealed co-localization of chemokines and TNF- α to areas of active inflammation (see Figs 3c and 3d).

Detection of chemokine mRNA by RNase protection assays

The RNase protection assay confirmed that chemokines were present in normal and diseased tissue.

Expression was low in normal, non-inflamed tissue and modestly increased in established cirrhosis without hepatitis. Chemokine mRNA was elevated substantially (two- to three-fold on average) in alcoholic hepatitis, confirming the observations in tissue sections. These data are summarized in Fig. 4.

DISCUSSION

Alcoholic liver disease is associated with two distinct inflammatory responses: alcoholic hepatitis, characterized by parenchymal infiltration by monocytes, lymphocytes, and large numbers of neutrophils; and alcoholic cirrhosis, where fibrosis and chronic inflammation with monocytes and lymphocytes are centred on portal tracts and fibrous septa. Our results suggest that locally secreted chemokines are important factors in determining the nature and distribution of the inflammatory response to alcohol. Chemokines expressed in the sinusoids in alcoholic hepatitis would promote the recruitment of neutrophils, monocytes, and lymphocytes to the parenchyma,^{5,21} whereas chemokines expressed in portal areas and fibrous septa during chronic inflammatory responses of alcoholic cirrhosis would promote recruitment and retention of inflammatory cells at that location. Our findings also demonstrate that chemokine expression alone is insufficient for leukocyte recruitment *in vivo*, because IL-8, which acts predominantly on

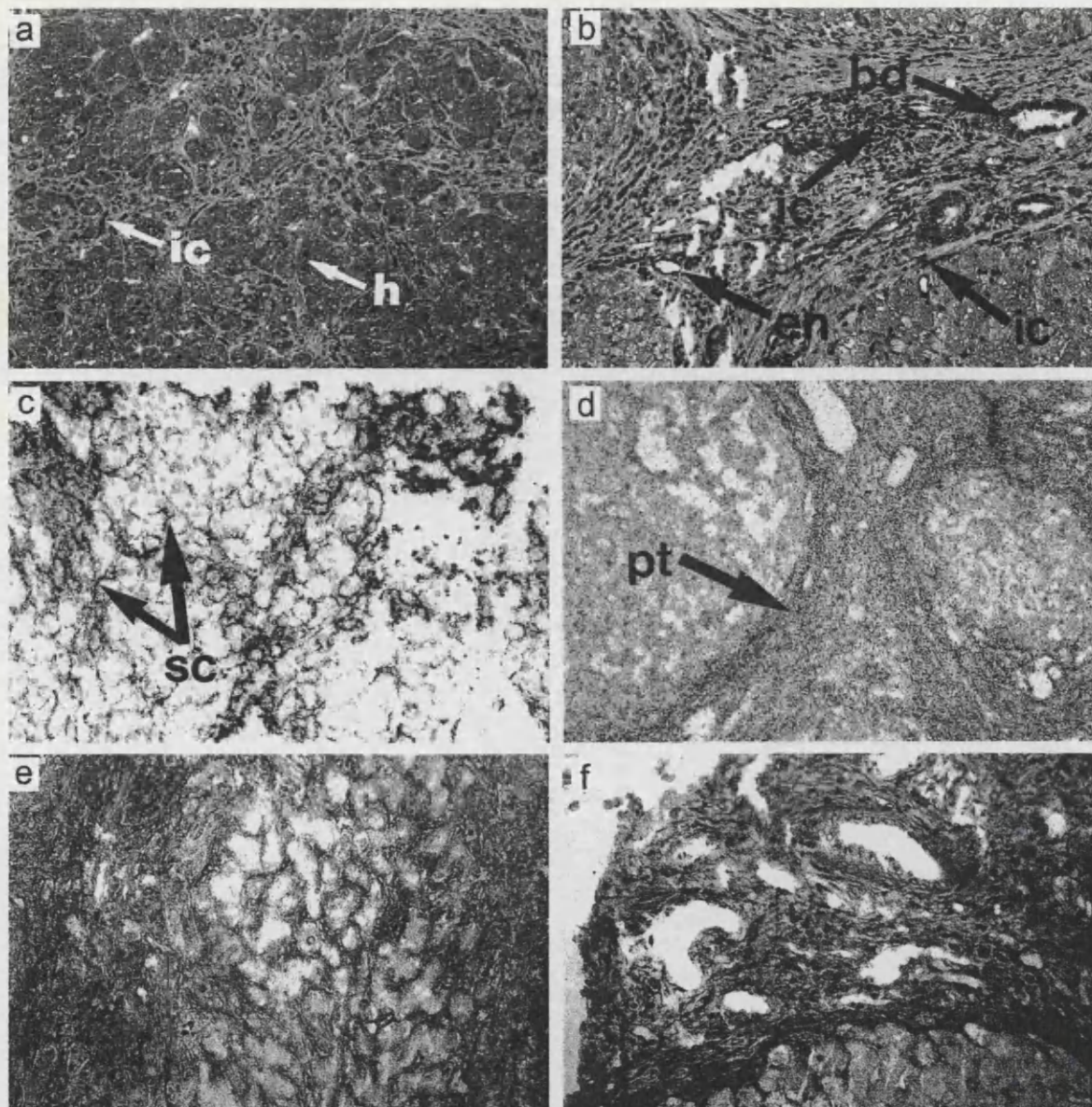


Fig. 2—Chemokine protein and mRNA expression in alcoholic hepatitis and alcoholic cirrhosis. The left-hand panels (a, c, e) show alcoholic hepatitis and the right-hand panels alcoholic cirrhosis for direct comparison. Panels a and b show immunohistochemical staining for IL-8 chemokine protein. In alcoholic hepatitis (a), there is strong staining in hepatocytes (h) and inflammatory cells (ic), whereas in alcoholic cirrhosis (b), staining is confined to portal tract endothelium (en), intrahepatic bile ducts (bd), and inflammatory cells (ic) in portal tracts and periseptal areas. Panels c and d show IL-8 and MIP-1 β mRNA localization (representative results; other chemokines were similar). In alcoholic hepatitis, mRNA is expressed in sinusoidal cells (sc) within the parenchyma, whereas in alcoholic cirrhosis, expression is confined to portal tracts (pt) and septal areas. Panels e and f show immunohistochemical staining for fibroblasts in patients with alcoholic hepatitis (e) and alcoholic cirrhosis (f), respectively

neutrophils, was detected in alcoholic cirrhosis in the absence of a neutrophil infiltrate; this suggests that other factors in addition to chemokines are required for leukocyte migration into tissues. In light of our previous reports of distinct patterns of endothelial activation in alcoholic hepatitis, these [factors] almost certainly include the presence of particular endothelial adhesion molecules.^{8,9}

Chemokine mRNA was expressed not only by infiltrating leukocytes, but also by liver endothelium and sinusoidal cells, implying that the liver itself is involved in the regulation of leukocyte recruitment in alcohol-induced liver injury. Many cell types have been shown to

produce MCP-1 and IL-8 when stimulated *in vitro*, including biliary epithelial cells, hepatocyte, and sinusoidal endothelium,^{28–30} and these cells may all contribute to the local levels of chemokines within the liver. In severe alcoholic hepatitis, but not cirrhosis, IL-8 mRNA was expressed strongly throughout the sinusoids and lobular regions in both inflammatory and sinusoidal cells. Overexpression of CINC (the rat homologue of IL-8) in rat hepatocytes *in vivo* results in a neutrophilic hepatitis and other studies have shown that hepatocytes secrete chemotactic factors in response to alcohol and its metabolites, implying a central role for hepatocyte-derived factors in generating the inflammatory response

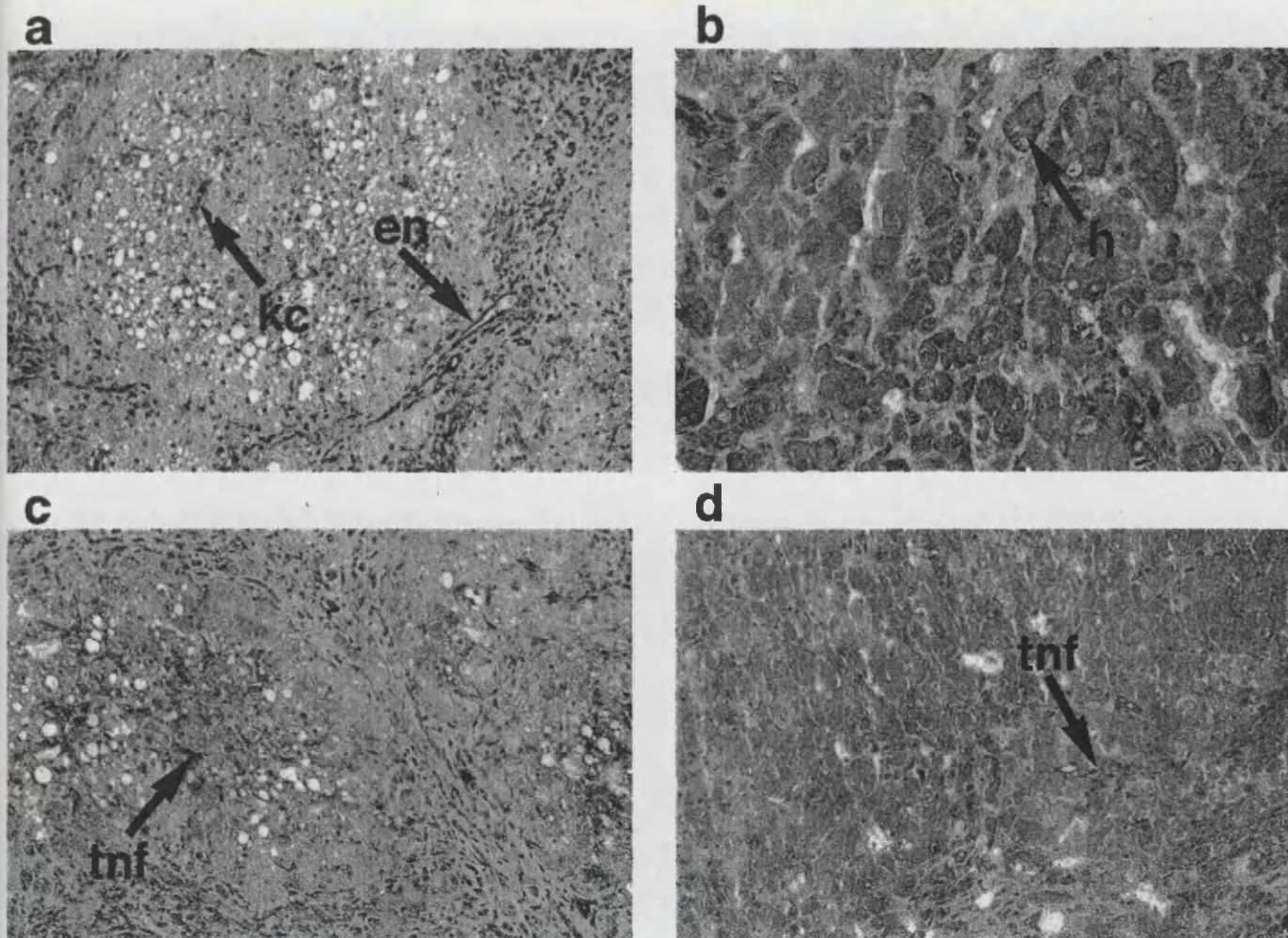


Fig. 3—Immunolocalization of TNF- α and chemokine protein in alcoholic hepatitis. Panel a shows immunolocalization of TNF- α using an alkaline phosphatase/fast red substrate in a patient with alcoholic hepatitis and cirrhosis demonstrating positive staining in vascular endothelium (en) and areas of inflammation in periseptal regions and within Kupffer cells in hepatic sinusoids in areas of steatohepatitis (kc). Panel b shows immunolocalization of the chemokine MIP-1 β using a horseradish peroxidase/DAB substrate in severe alcoholic hepatitis. There is strong expression of chemokine protein in ballooned hepatocytes (h). Panels c and d show dual immunostaining for TNF- α (red/pink stain) and MIP-1 α (widespread parenchymal brown stain) confirming that both cytokines are present in areas of inflammation and tissue damage

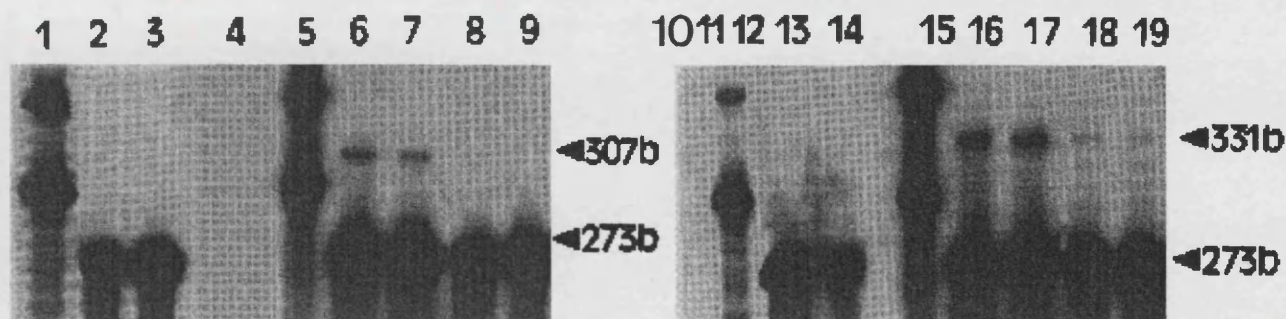


Fig. 4—RNase protection gel showing MIP-1 α and MIP-1 β mRNA expression in normal, alcoholic hepatitis, and alcoholic cirrhosis tissue extracts, respectively. Gel left to right tracks 1–9=MIP-1 α and β -actin (protected fragment size=307 and 273 bases, respectively); tracks 10–19=MIP-1 β and β -actin (protected fragment size 331 and 273 bases, respectively). 1=control non-hybridized MIP-1 α and β -actin probe mixture; 2, 3=probes hybridized with RNA from normal non-inflamed liver tissue; 4=control probe cocktail predigested with RNase; 5=control non-hybridized MIP-1 α and β -actin probe mixture; 6, 7=probes hybridized with RNA from severe alcoholic hepatitis; 8, 9=probes hybridized with RNA from end-stage alcoholic cirrhosis; 10=control MIP-1 β and β -actin probe cocktail predigested with RNase; 11=control non-hybridized MIP-1 β and β -actin probe mixture; 12, 13=probes hybridized with RNA from normal non-inflamed liver tissue; 14, 15=control non-hybridized MIP-1 β and β -actin probe mixture; 16, 17=probes hybridized with RNA from severe alcoholic hepatitis; 18, 19=probes hybridized with RNA from end-stage alcoholic cirrhosis

of alcoholic hepatitis.^{8,31} The contribution made by hepatocytes to chemokine synthesis in humans is not known, but our results suggest that they contribute relatively little because despite the presence of all four chemokine proteins in ballooned hepatocytes, no chemokine mRNA was detected. This finding suggests that hepatocytes take up or sequester chemokines synthesized and secreted by other cell types, a process that could increase the local concentration of chemokines in the parenchyma in alcoholic hepatitis.

Fibroblasts express chemokines *in vitro*³⁰ and activated fibroblasts may be particularly important contributors to chemokine secretion in alcoholic liver disease, because we found a striking co-localization of chemokine mRNA with the distribution of fibroblasts in both alcoholic cirrhosis and alcoholic hepatitis. Smith *et al.* have proposed a role for chemokines in fibrogenesis based on their observations that the administration of antibodies to MIP-1 α decreases cellular infiltrates and fibrosis in an animal model of interstitial lung fibrosis.^{32,33} Fibrogenesis involves complex interactions between leukocytes, fibroblasts, and sinusoidal cells, and chemokines are likely to play a central role in this process by recruiting and maintaining inflammatory cells at sites of active fibrosis.³⁴

In normal tissue, all four chemokine proteins were detected on endothelium and in inflammatory cells around blood vessels. These observations suggest that low-level chemokine secretion occurs in normal liver and could be important for the regulation of leukocyte recruitment during physiological immune surveillance. Alternatively, because the 'normal' liver came from organ donors, who were on intensive care units prior to organ removal, it is possible that the presence of endotoxin and cytokines in the portal blood accounts for this low-level expression. In normal liver, IL-8 and MCP-1 mRNA were detected in vascular but not sinusoidal endothelium. Previous studies have reported differences in adhesion molecules between sinusoidal and vascular endothelium³⁵ and these present observations provide further evidence that sinusoidal endothelium differs from portal and hepatic vascular endothelium. The endothelium can also sequester chemokines secreted by other cells. The ability of proteoglycans in the endothelial glycocalyx to bind glycosaminoglycan-binding sites on various chemokines allows chemokines to be retained at sites of inflammation *in vivo*.^{23,36-38} This mechanism could explain why we detected MIP-1 α and MIP-1 β chemokine protein on vascular endothelium in the absence of detectable mRNA.

Because *in situ* hybridization cannot quantify mRNA, we used RNase protection assays to provide a quantitative assessment of chemokine mRNA. Chemokine mRNA was markedly elevated in alcoholic hepatitis compared with cirrhosis, consistent with the increased inflammatory damage in alcoholic hepatitis.^{5,21} It has been proposed that increased production of TNF- α drives the inflammatory response in alcoholic hepatitis and TNF- α is a potent stimulus for chemokine release from several cell types *in vitro*, including Kupffer cells and human biliary epithelial cells.^{28,31} Although increased secretion of TNF- α by blood monocytes has

been reported in alcoholic hepatitis, the cellular origin of TNF- α within the liver is not known.^{18,39} We demonstrated co-localization of chemokine and TNF- α proteins in areas of inflammation in alcoholic hepatitis, suggesting the existence of a paracrine network in which TNF- α , produced by inflammatory cells, stimulates local secretion of chemokines in alcoholic hepatitis. Further evidence for the importance of TNF- α in alcoholic hepatitis comes from reports that patients who are genetically high TNF- α producers have an increased risk of alcoholic hepatitis.⁴⁰

The findings of our study imply that leukocyte recruitment to the parenchyma and portal tracts is controlled by different mechanisms. For instance, the expression of MCP-1 on portal vascular endothelium would promote the influx of monocytes and lymphocytes into portal tracts and in the presence of the appropriate stimulus activate fibrogenesis. In contrast, infiltration of the parenchyma, which is seen in alcoholic hepatitis, requires a more potent inflammatory stimulus to induce chemokine secretion from sinusoidal cells. This could be provided by high local levels of TNF- α . Therapeutic reduction of chemokine expression or function should thus reduce hepatic inflammation and prevent fibrogenesis in alcoholic liver disease. The recent development of chemokine receptor antagonists⁴¹ will provide reagents to allow us to test this hypothesis *in vivo*. If effective, these reagents will have broader applications in other types of inflammatory liver disease and cirrhosis. However, the widespread and diverse effects of chemokines mean that such approaches will need to take into account the potentially deleterious effects of inhibiting chemokine function *in vivo*.²⁹

ACKNOWLEDGEMENTS

We are grateful to Drs U. Siebenlist and J. Johnston (NIH, MD, U.S.A.), Dr P. Watson (University of Sheffield, U.K.) and Dr S. Kunkel (University of Michigan, Ann Arbor, U.S.A.) for providing reagents; Mrs A. Williams for technical assistance; our clinical colleagues in the Liver Unit for collection of biopsy specimens; and Dr James Neuberger for critical review of the manuscript. This work was supported in part by grants from the Wellcome Trust and the Endowment Fund of the Former Unit Birmingham Hospitals.

REFERENCES

1. Lieber CS. Alcohol and the liver: 1994 update. *Gastroenterology* 1994; **106**: 1085-1105.
2. Paronetto F. Immunologic reactions in alcoholic liver disease. *Semin Liver Dis* 1993; **13**: 183-195.
3. Adams DH. Leucocyte adhesion and alcoholic liver disease. *Alcohol Alcoholism* 1994; **29**: 249-260.
4. International Group. Alcoholic liver disease: morphological manifestations. *Lancet* 1981; **1**: 707-771.
5. French SW, Nash J, Shitabata P, Kachi K, Hara C, Chedid A, Mendenhall CL, the VA Cooperative Group. Pathology of alcoholic liver disease. *Semin Liver Dis* 1993; **13**: 154-169.
6. Maddrey WC. Alcoholic hepatitis: pathogenesis and approaches to treatment. *Scand J Gastroenterol* 1990; **175**: 118-130.
7. Sherlock S. Alcoholic hepatitis. *Alcohol Alcoholism* 1990; **25**: 189-196.
8. Fisher NC, Afford SC, Adams DH. Adhesion molecules and alcoholic liver disease. *Hepatogastroenterology* 1996; **43**: 1113-1116.

9. Adams DH, Burra P, Hubscher SG, Elias E, Newman W. Endothelial activation and circulating vascular adhesion molecules in alcoholic liver disease. *Hepatology* 1994; **19**: 588–594.
10. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994; **76**: 301–314.
11. Tanaka Y, Adams DH, Shaw S. Proteoglycan on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol Today* 1993; **14**: 111–114.
12. Adams DH, Fear J, Shaw S, Hubscher SG, Afford SC. Hepatic expression of macrophage inflammatory protein-1a and macrophage inflammatory protein-1b after liver transplantation. *Transplantation* 1996; **61**: 817–825.
13. Adams DH, Lloyd AR. Chemokines; biology and role in inflammatory disease. *Lancet* 1997; **349**: 490–495.
14. Kelner GS, Kennedy J, Bacon KB, et al. Lymphotactin—a cytokine that represents a new class of chemokine. *Science* 1994; **266**: 1395–1399.
15. Schall TJ, Bacon KB. Chemokines, leukocyte trafficking and inflammation. *Curr Opin Immunol* 1994; **6**: 865–873.
16. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science* 1996; **272**: 60–66.
17. Hill DB, Marsano LS, McClain CJ. Increased plasma interleukin-8 concentrations in alcoholic hepatitis. *Hepatology* 1993; **18**: 576–580.
18. Sheron N, Bird G, Koskinas J, et al. Circulating and tissue-levels of the neutrophil chemotaxin interleukin-8 are elevated in severe acute alcoholic hepatitis, and tissue-levels correlate with neutrophil infiltration. *Hepatology* 1993; **18**: 41–46.
19. Maltby J, Wright S, Bird G, Sheron N. Chemokine levels in human liver homogenates—associations between gro alpha and histopathological evidence of alcoholic hepatitis. *Hepatology* 1996; **24**: 1156–1160.
20. Maher JJ, Scott MK, Saito JM, Burton MC. Adenovirus-mediated expression of cinc/gro (il-8) in rat-liver induces a neutrophilic hepatitis. *FASEB J* 1996; **10**: 22.
21. Chedid A, Mendenhall CL, Garside P, French SW, Chen T, Rabin L, the VA Cooperative Group. Prognostic factors in alcoholic liver disease. *Am J Gastroenterol* 1991; **82**: 210–216.
22. Zipfel PF, Balke J, Irving SG, Kelly K, Siebenlist U. Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors. *J Immunol* 1989; **142**: 1582–1590.
23. Tanaka Y, Adams DH, Hubscher S, Hirano H, Siebenlist U, Shaw S. T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1b. *Nature* 1993; **361**: 79–82.
24. Adams DH, Hubscher SG, Shaw J, et al. Increased expression of ICAM-1 on bile ducts in primary biliary cirrhosis and primary sclerosing cholangitis. *Hepatology* 1991; **14**: 426–432.
25. Adams DH, Hubscher SG, Shaw J, Rothlein R, Neuberger JM. Intercellular adhesion molecule 1 on liver allografts during rejection. *Lancet* 1989; **2**: 1122–1125.
26. Goldstein LA, Streeter RM, Ivanov HL, Kunkel SL, Lukacs NW. TNF induced IL-8 and MCP-1 production in the eosinophilic cell line coll. *Mediators Inflammation* 1996; **5**: 218–223.
27. Belin D, Bost S, Vassalli JD, Strub K. A two step recognition signal of sequences determines the translocation efficiency of proteins. *EMBO J* 1996; **15**: 468–478.
28. Morland CM, Fear J, McNab G, Joplin R, Adams DH. Promotion of leukocyte transendothelial cell migration by chemokines derived from human biliary epithelial cells *in vitro*. *Proc Assoc Am Physicians* 1997; **109**: 1–11.
29. Strieter RM, Standiford TJ, Huffnagle GB, Colletti LM, Lukacs NW, Kunkel SL. The good, the bad, and the ugly—the role of chemokines in models of human-disease—commentary. *J Immunol* 1996; **156**: 3583–3586.
30. Baggolini M, Dewald D, Moser B. Interleukin-8 and related chemotactic cytokines: A and CC chemokines. *Adv Immunol* 1994; **55**: 97–179.
31. Maher JJ. Rat hepatocytes and Kupffer cells interact to produce interleukin-8 (cinc) in the setting of ethanol. *Am J Physiol—Gastrointestinal Liver Physiol* 1995; **32**: G518–523.
32. Smith RE, Strieter RM, Phan SH, et al. Production and function of murine macrophage inflammatory protein-1-alpha in belomycin-induced lung injury. *J Immunol* 1994; **153**: 4704–4712.
33. Smith RE, Strieter RM, Zhang K, et al. A role for c-c chemokines in fibrotic lung-disease. *J Leuk Biol* 1995; **57**: 782–787.
34. Friedman SL, Flier JS, Epstein F, Glickman R, Scheele G. Seminars in medicine of the Beth-Israel Hospital, Boston—the cellular basis of hepatic fibrosis—mechanisms and treatment strategies. *N Engl J Med* 1993; **328**: 1828–1835.
35. Steinhoff G, Behrend M, Schrader B, Duijvestijn AM, Wonigiet K. Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia—lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2 and LFA-3. *Am J Pathol* 1993; **142**: 481–488.
36. Adams DH, Harvath L, Bottaro DP, et al. Hepatocyte growth factor and macrophage inflammatory protein-1b: structurally distinct cytokines that induce rapid cytoskeletal changes and subset-preferential migration in T cells. *Proc Natl Acad Sci USA* 1994; **91**: 7144–7148.
37. Cockwell P, Adams DH, Savage COS. Glycosaminoglycans contribute to multiple functions of vascular endothelial cells. *Clin Exp Immunol* 1996; **104**: 113.
38. Witt DP, Lander AD. Differential binding of chemokines to glycosaminoglycan subpopulations. *Curr Biol* 1994; **4**: 394–400.
39. McClain C, Hill D, Schmidt J, Diehl AM. Cytokines and alcoholic liver disease. *Semin Liver Dis* 1993; **13**: 170–182.
40. Gove J, Daly AK, Bassendine MF, Day CP. Association of a tumour necrosis factor promoter polymorphism with susceptibility to alcoholic steatohepatitis. *Hepatology* 1996; **24**: 443A.
41. Xu LL, Kelvin DJ, Ye GQ, et al. Modulation of Il-8 receptor expression on purified human t-lymphocytes is associated with changed chemotactic responses to Il-8. *J Leuk Biol* 1995; **57**: 335–342.

Serum concentrations and peripheral secretion of the beta chemokines monocyte chemoattractant protein 1 and macrophage inflammatory protein 1 α in alcoholic liver disease

N C Fisher, D A H Neil, A Williams, D H Adams

Abstract

Background—Alcoholic liver disease is associated with increased hepatic expression of monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1 α (MIP-1 α).

Aims—To determine whether concentrations of chemokines in the peripheral circulation reflect disease activity, and whether chemokine secretion is restricted to the liver or is part of a systemic inflammatory response in alcoholic liver disease.

Patients—Fifty one patients with alcoholic liver disease and 12 healthy controls.

Methods—Peripheral vein (and hepatic vein in patients undergoing transjugular liver biopsy) chemokine concentrations were measured by ELISA. Chemokine secretion and transcription in isolated peripheral mononuclear cells were assessed using ELISA and *in situ* hybridisation in patients with severe alcoholic hepatitis.

Results—Serum MCP-1 concentrations were higher in alcoholic hepatitis compared with cirrhosis or healthy controls. MIP-1 α concentrations were below the assay sensitivity in most patients. Serum MCP-1 concentrations correlated significantly with serum aspartate aminotransferase and creatinine. In severe alcoholic hepatitis, MCP-1 concentrations were higher in hepatic compared with peripheral veins; in mild alcoholic hepatitis there was no difference. Mononuclear cell secretion of both MCP-1 and MIP-1 α was higher in severe alcoholic hepatitis compared with healthy controls, and chemokine mRNA was identified in monocytes.

Conclusions—Serum MCP-1 concentrations are raised in alcoholic liver disease and reflect severity of hepatic inflammation. Monocyte secretion of both MCP-1 and MIP-1 α is increased in severe alcoholic hepatitis. Both intrahepatic sources and peripheral mononuclear cells contribute to the raised serum MCP-1 concentrations.

(*Gut* 1999;45:416–420)

Keywords: alcoholic liver disease; chemokines; monocyte chemoattractant protein 1; macrophage inflammatory protein 1 α

variable degree of fibrosis.¹ While the key hepatotoxin underlying alcoholic liver disease (ALD) is self evident, the cascade of immunological events leading to inflammatory damage is complex and the variation in inflammatory response between individuals consuming similar amounts of alcohol is not understood.² We and others have previously shown that the expression of chemokines, a subgroup of cytokines with chemoattractant activity, correlates with clinical severity and inflammatory activity within the liver in alcoholic and other inflammatory liver diseases.^{3–7} The principal role of chemokines is in the recruitment of leucocytes to sites of inflammation and they play a vital role in a variety of infective and inflammatory diseases.^{8,9} Chemokines can be subdivided according to their structure into subgroups, of which the largest are the CXC, or alpha, and CC, or beta groups defined by the presence or absence respectively of an additional amino acid (“X”) between the first two cysteine residues in a conserved four cysteine motif.⁹ The alpha chemokines are further subdivided according to the presence or absence of a glutamine-leucine-arginine (ELR) amino acid sequence near the active terminal; those possessing this sequence are potent chemoattractants for neutrophils while those that do not are chemotactic for lymphocytes. Interleukin 8 (IL-8) possesses an ELR amino acid sequence and is the prototypic alpha chemokine, being exclusively chemotactic for neutrophils. The beta chemokines are exclusively chemotactic for mononuclear cells; the prototypes of this group are monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1 α (MIP-1 α), both of which are monocyte and lymphocyte chemoattractants.

Although the effects of chemokines are predominantly localised to sites of inflammation by binding to proteoglycans in the glycocalyx,¹⁰ they can also be detected in the circulation, and serum concentrations of the alpha chemokine IL-8 have been shown to correlate closely with clinical, histological, and laboratory indicators of severity in ALD.^{11–13} However, alcoholic hepatitis and active cirrhosis are also typically associated with a mononuclear

Liver Research
Laboratories, MRC
Centre for Immune
Regulation, Institute of
Clinical Science,
Queen Elizabeth
Medical Centre,
Birmingham B15 2TH,
UK

N C Fisher
D A H Neil
A Williams
D H Adams

Correspondence to:
Professor Adams.

Accepted for publication
18 March 1999

Alcoholic hepatitis is characterised by an intense leucocytic infiltration of the liver combined with hepatocyte damage and a

Abbreviations used in this paper: ALD, alcoholic liver disease; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PBMC, peripheral blood mononuclear cell; IL, interleukin; ELR, glutamine-leucine-arginine; TIPSS, transjugular intrahepatic portosystemic shunt; AST, aspartate aminotransferase.

Table 1 Summary characteristics of patients with severe alcoholic hepatitis (AH), mild to moderate AH, and quiescent cirrhosis, as defined histologically

| | Severe AH (n=24) | Mild/moderate AH (n=15) | Cirrhosis (n=12) |
|-------------------------|---------------------|----------------------------|---------------------|
| AST (U/l) | | | |
| Median | 64 | 50 | 36 |
| Range | 21-405 | 20-113 | 13-61 |
| Bilirubin (µmol/l) | | | |
| Median | 232 | 100 | 54 |
| Range | 45-1050 | 14-313 | 11-550 |
| PT (s) | | | |
| Median | 23 | 21 | 19 |
| Range | 17-43 | 14-39 | 15-30 |
| Discriminant function*† | | | |
| Median | 65.2 | 40.5 | 28.4 |
| Range | 29.6-151 | 5.6-138 | 9.8-111 |
| PSE | 13 | 4 | 2 |
| Transplanted‡ | 2 | 3 | 3 |
| Deaths‡ | 10 | 4 | 2 |

*After Maddrey,¹³ where discriminant function = [(prolongation in PT × 4.6) + (serum bilirubin/17)]. A discriminant function of >32 is indicative of a risk of mortality of at least 50%.

†Severe AH versus mild or moderate AH, p<0.05; severe AH versus quiescent cirrhosis, p<0.05; mild to moderate AH versus quiescent cirrhosis, p<0.05 (Mann-Whitney U test).

‡Occurring within three months after serum sampling. All deaths were directly related to liver disease.

AST, aspartate aminotransferase; PT, prothrombin time; PSE, portosystemic encephalopathy (defined where clinically obvious).

cell infiltrate including monocytes and lymphocytes, and the hepatic expression of the chemokines MCP-1 and MIP-1α correlates with intensity of infiltration with these mononuclear cells in ALD.⁶ Serum concentrations of beta chemokines have not been studied in inflammatory liver diseases, although MCP-1 concentrations are elevated in human sepsis.¹⁴ For these reasons we studied circulating concentrations and peripheral secretion of MCP-1 and MIP-1α in ALD in order to determine: (a) whether concentrations of chemokines in the peripheral circulation reflect hepatic disease activity; and (b) whether chemokine secretion is restricted to the liver or is part of a systemic inflammatory response.

Patients and methods

PATIENTS

We studied 51 patients with biopsy proved ALD. All patients had consumed at least 80 g alcohol daily for more than 10 years and other potential causes of liver disease had been carefully excluded by comprehensive clinical, radiological, and serological analysis. Patients were managed with nutritional supplementation but not corticosteroids or other immunosuppressants. Patients with active sepsis or recent gastrointestinal haemorrhage were excluded from the study. Patients were subdivided by histological criteria¹ into those with severe alcoholic hepatitis (ballooning hepatocyte degeneration, Mallory's hyaline, and dense leucocyte infiltration including neutrophils; n=24), mild to moderate alcoholic hepatitis (with ballooning hepatocyte degeneration, Mallory's hyaline, and absent or light leucocyte infiltration without neutrophils; n=15), and inactive alcoholic cirrhosis (without histological stigmata of alcoholic hepatitis; n=12). The majority of patients with alcoholic hepatitis (18/24 with severe and 11/15 with mild to moderate alcoholic hepatitis) also had cirrhosis, and all remaining patients had fibrosis of at least mild to moderate severity.

In addition to histological features of disease we recorded routine clinical and laboratory

laboratory data for each patient (table 1). For comparison we also studied 12 healthy volunteers.

SERUM SAMPLING

Serum samples obtained from peripheral venous blood were analysed for all subjects. Some of the patients with coagulopathy underwent transjugular biopsy for clinical indications and in these patients we took the opportunity to sample blood from the hepatic vein. In a further five patients, transjugular intrahepatic portosystemic shunts (TIPSS) were inserted radiologically for control of ascites or because of previous problematic variceal haemorrhage; in these patients we took blood samples from peripheral, hepatic, and portal veins. Blood was collected into tubes containing clot activator; serum was separated within 30 minutes by centrifugation and stored at -70°C prior to analysis.

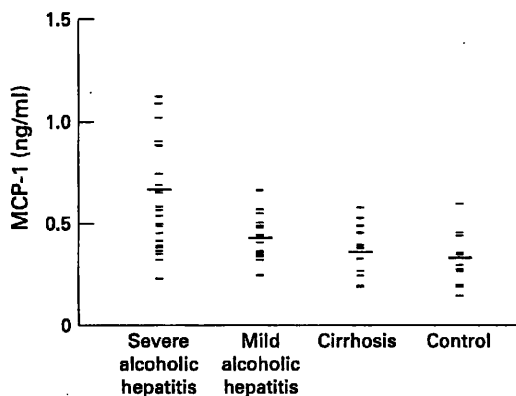
PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) CHEMOKINE SECRETION

MCP-1 and MIP-1α secretion from cultured PBMCs was measured in selected patients with severe alcoholic hepatitis (n=12) and healthy controls (n=12). PBMCs were isolated from citrated venous blood by Ficoll/Hypaque density gradient centrifugation for 30 minutes at 1600 rpm; cells were then washed twice in Hank's balanced salt solution, counted by haemocytometry, and cultured in 1 ml aliquots at a concentration of 10⁶ cells/ml. Cells were cultured for 24 hours at 37°C at 10⁶ cells/ml in RPMI containing 10% fetal bovine serum, glutamine (2 mM/l), and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) without additional stimulation. Supernatants were collected by centrifugation and stored at -70°C prior to analysis.

CHEMOKINE MEASUREMENT

MCP-1 and MIP-1α concentrations were measured using commercially available ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The lower limits of sensitivity for these assays were 31 and 47 pg/ml respectively. For measurement of PBMC secretion of MCP-1 we developed a customised ELISA. Ninety six microwell plates were coated with mouse monoclonal antihuman MCP-1 (500 ng/ml; R&D Systems, Oxon, UK) overnight at 4°C. Plates were then washed and non-specific binding blocked with 0.1% bovine serum albumin before adding the test sample for two hours at room temperature. Rabbit antihuman MCP-1 (2.5 µg/ml; Peprotech EC Ltd, London, UK) was added for one hour, followed by swine antirabbit antibody conjugated with horseradish peroxidase (1.3 µg/ml, Dako Ltd, Cambridge, UK) for a further hour, with washing between each step. Bound antibody was then detected using tetramethylene blue liquid substrate and colorimetric analysis. Reference samples consisted of serial dilutions of recombinant human MCP-1 (Peprotech EC Ltd, London, UK). This ELISA gave reproducible results with values within 10% of the commercial ELISA, with both intra-assay and interassay variability

Figure 1 Serum monocyte chemoattractant protein 1 (MCP-1) concentrations in patients with severe or mild to moderate alcoholic hepatitis, inactive alcoholic cirrhosis, and controls. Thick horizontal bars represent mean values. Concentrations in severe alcoholic cirrhosis were significantly higher than in cirrhotics or controls ($p < 0.01$, Mann-Whitney U test).



of less than 5% and with a lower limit of sensitivity of 250 pg/ml.

ANALYSIS FOR PBMC CHEMOKINE mRNA

Chemokine mRNA in peripheral blood leucocytes was determined by *in situ* hybridisation of cytospin preparations of PBMCs isolated as described above. Freshly isolated PBMCs were resuspended and mounted onto coated slides, fixed in 1% paraformaldehyde, and stored at -70°C prior to analysis. Chemokine mRNA hybridisation was done with a probe for MIP-1 α using techniques described elsewhere.⁴ Briefly, ^{35}S uridine triphosphate labelled antisense or sense cRNA probes were synthesised from a cDNA sequence specific for MIP-1 α , which was ligated into an Eco R1/Bam H1 restriction site of the plasmid vector Bluescript KS+/- . The ^{35}S cRNAs were characterised prior to use by polyacrylamide gel electrophoresis to ensure comparable size and specific activity. These were then hybridised with cytospin preparations and developed after 7–14 days of autoradiography.

STATISTICAL ANALYSIS

Tests for non-parametric data (Mann-Whitney U test, Wilcoxon rank sum, and multivariate linear regression) were done using SPSS statistical software. A p value of less than 0.05 was considered to indicate statistical significance. This study was approved by the Research Ethics Committee of the University Hospital Birmingham NHS Trust and patients gave informed consent before participation in the study.

Results

SERUM CHEMOKINE CONCENTRATIONS

Peripheral vein MCP-1 concentrations were higher in alcoholic hepatitis (severe, 640 (SD 285) pg/ml; mild, 435 (110) pg/ml) compared

Table 2 Correlation of laboratory indexes of disease severity with serum monocyte chemoattractant protein (MCP) 1 concentrations in patients with alcoholic liver disease, using Spearman's rank test

| Laboratory index | r Value | p Value |
|----------------------------------|-----------|-----------|
| Serum aspartate aminotransferase | +0.47 | <0.01 |
| Creatinine* | +0.36 | <0.01 |
| Bilirubin* | +0.35 | <0.01 |
| Albumin | -0.33 | <0.05 |
| Prothrombin time increase | +0.15 | NS |
| Peripheral leucocyte count | +0.18 | NS |

*Values transformed logarithmically for statistical analysis. Linear regression analysis identified serum aspartate aminotransferase ($p < 0.01$) and creatinine ($p < 0.05$) as significant independent variables.

with cirrhosis (355 (105) pg/ml) or healthy controls (325 (120) pg/ml) (severe alcoholic hepatitis versus healthy controls, $p < 0.01$; severe alcoholic hepatitis versus inactive cirrhosis, $p < 0.01$; mild alcoholic hepatitis versus healthy controls, NS; Mann-Whitney U test; fig 1). MCP-1 concentrations showed significant positive correlations with serum aspartate aminotransferase (AST) ($p < 0.01$), serum creatinine ($p < 0.01$), and serum bilirubin ($p < 0.01$), and a significant negative correlation with serum albumin ($p < 0.05$) (table 2). Linear regression analysis showed that serum AST ($p < 0.01$) and creatinine ($p < 0.05$) retained independent associations with MCP-1 concentrations. There was a non-significant trend towards higher MCP-1 concentrations in non-survivors compared with survivors (mean concentrations 763 and 565 ng/ml respectively, $p = 0.12$). In the alcoholic hepatitis groups, there was no difference in MCP-1 concentrations between those with and those without cirrhosis (mean concentrations 654 and 678 pg/ml respectively, NS). Peripheral vein MIP-1 α concentrations were below the lower limit of detection of the assay (that is, less than 46 pg/ml) in all healthy controls and in all ALD patients tested ($n = 32$) except one.

PERIPHERAL AND HEPATIC VEIN CHEMOKINE CONCENTRATIONS

In patients with severe alcoholic hepatitis in whom paired hepatic and peripheral vein samples were available, MCP-1 concentrations were higher in hepatic compared with peripheral venous blood (mean values 647 and 600 pg/ml respectively, $p < 0.01$, Wilcoxon rank sum), while in patients with mild to moderate alcoholic hepatitis or inactive cirrhosis the hepatic and peripheral vein MCP-1 concentrations were similar (mean values 293 and 301 pg/ml respectively, NS; fig 2). In the five patients undergoing TIPSS insertion, the highest MCP-1 concentrations were found in the hepatic veins, with the lowest concentrations occurring in the portal veins (mean concentrations in the hepatic, peripheral, and portal veins were 437, 421, and 402 pg/ml respectively; hepatic vein versus portal vein, $p = 0.07$, Wilcoxon rank sum). Hepatic vein MIP-1 α concentrations were below the lower limit of

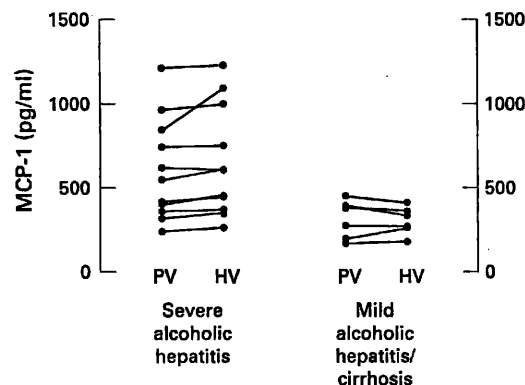
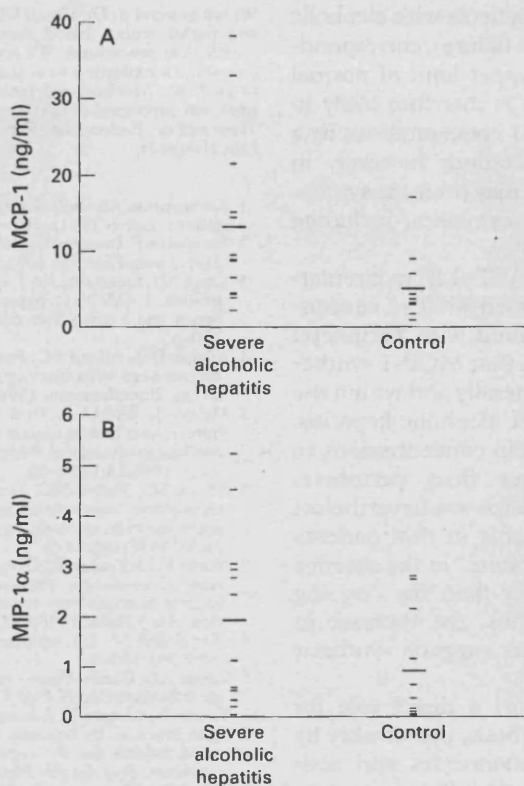


Figure 2 Paired hepatic and peripheral vein (HV and PV) monocyte chemoattractant protein 1 (MCP-1) concentrations in patients with severe alcoholic hepatitis compared with mild to moderate hepatitis or cirrhosis.

Figure 3 Spontaneous peripheral blood mononuclear cell secretion of (A) monocyte chemoattractant protein 1 (MCP-1) and (B) macrophage inflammatory protein 1 α (MIP-1 α) in patients with severe alcoholic hepatitis and healthy controls. Thick horizontal bars represent mean values.



detection of the assay (as for peripheral vein concentrations) in all patients with ALD.

PBMC CHEMOKINE SECRETION

Spontaneous secretion of MCP-1 and MIP-1 α from cultured PBMCs was higher in patients with severe alcoholic hepatitis compared with healthy controls (fig 3). In alcoholic hepatitis MCP-1 secretion was 13.13 (11.34) ng/ml and for controls 5.27 (4.77) ng/ml ($p=0.015$, Mann-Whitney U test); MIP-1 α secretion was 1.98 (1.78) ng/ml in alcoholic hepatitis and 0.84 (1.10) ng/ml in controls ($p=0.04$, Mann-Whitney U test). For patients with alcoholic hepatitis there was a significant positive correlation between PBMC secretion of and serum concentrations of MCP-1 ($r=0.68$, $p<0.05$, Spearman's rank test), and between PBMC secretion of MCP-1 and serum AST ($r=0.60$, $p<0.05$). However, there was no significant correlation between PBMC secretion of MIP-1 α with MCP-1 secretion or serum AST.

IDENTIFICATION OF CHEMOKINE mRNA

In situ hybridisation of cytospin preparations using the probe for MIP-1 α mRNA showed positive staining (mainly localised to monocytes) in patients with alcoholic hepatitis (fig 4).

Discussion

Our results suggest that secretion of MCP-1 is associated with disease activity in ALD. These findings complement previous observations of chemokine upregulation in the liver in ALD and suggest a role for chemokines in recruiting mononuclear cells to the liver following alcohol induced toxic injury. Previous reports of circulating IL-8 in alcoholic hepatitis have highlighted the importance of neutrophils in the pathogenesis of alcoholic hepatitis¹¹⁻¹³; however, while a neutrophilic infiltrate is the char-

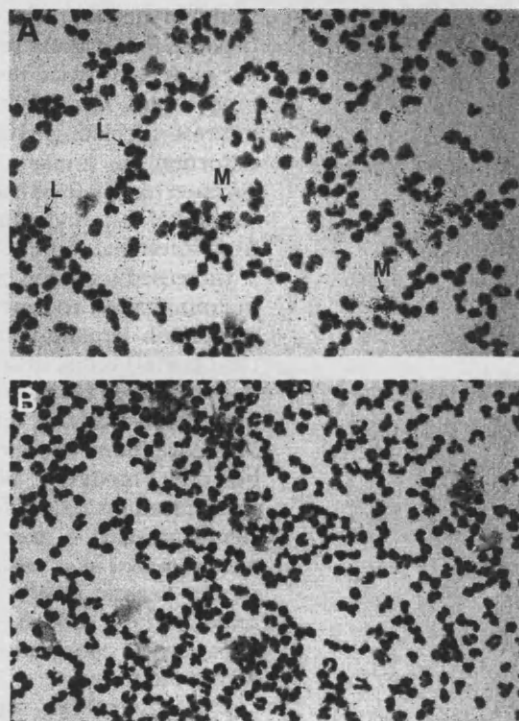


Figure 4 PBMC cytospin preparation hybridised with cDNA for macrophage inflammatory protein 1 α (MIP-1 α) chemokine mRNA (A, antisense probe; B, sense probe). Positive hybridisation is represented by dark granules, which are seen mostly in association with monocytes (M); lymphocytes (L) are mostly negative.

acteristic hallmark of alcoholic hepatitis, the inflammatory infiltrate includes mononuclear cells which play a vital role in inflammation and fibrogenesis.¹⁷ Thus beta chemokines such as MCP-1 are likely to play a central role in mediating these processes in ALD. We were unable to show increased serum concentrations of the beta chemokine MIP-1 α in ALD despite showing enhanced PBMC secretion; it is possible that circulating concentrations were increased but below the range detectable by the assay.

The significance of raised circulating concentrations of MCP-1 remains unclear and while this may simply reflect increased tissue synthesis of MCP-1, circulating MCP-1 may also have immunomodulatory effects, including increased expression of adhesion molecules on circulating monocytes and promotion of proinflammatory cytokine secretion, thus amplifying the inflammatory cascade.¹⁶ Furthermore, circulating IL-8 has been shown to enhance neutrophil sequestration into the liver and MCP-1 may play a similar role in enhancing hepatic uptake of mononuclear cells.¹⁷ However, the dynamic effects of a sustained increase in circulating chemokines are not clear and it is possible that persistent ligand occupancy of leucocyte chemokine receptors from circulating chemokines may inhibit their transendothelial migration.¹⁸

Our finding of a positive correlation between MCP-1 concentrations and serum creatinine probably reflects in part the renal excretion of chemokines; by virtue of its relatively small protein size, some excretion of MCP-1 is likely to occur from the kidneys. Measurable amounts are present in the urine of most healthy subjects¹⁹ and following this study we found high concentrations (1.34 and 1.36 ng/ml respec-

tively) in the urine of two patients with alcoholic hepatitis (but without renal failure), corresponding to at least twice the upper limit of normal renal values. Renal failure is therefore likely to increase circulating MCP-1 concentrations by a reduction in urinary excretion; however, in addition renal failure itself may promote synthesis of MCP-1 and other cytokines, including from intrarenal sources.^{20 21}

Increased secretion of MCP-1 from circulating monocytes, and increased MCP-1 concentrations in hepatic compared with peripheral (and portal) veins, suggest that MCP-1 synthesis is increased both peripherally and within the liver, respectively, in severe alcoholic hepatitis. While we found hepatic vein concentrations to be only modestly higher than peripheral concentrations, this difference was nevertheless significant and is remarkable in that patients were sampled in a "steady state" in the absence of any hepatic insult other than the ongoing inflammatory process. Thus, the increase in hepatic vein concentrations suggests synthesis within the liver.

Our observations support a direct role for MCP-1 in disease pathogenesis, presumably by recruiting and retaining monocytes and activated lymphocytes within the liver in severe alcoholic hepatitis. The fact that circulating monocytes showed increased spontaneous secretion of MCP-1 in alcoholic hepatitis suggests that recruitment of these cells into the liver would further elevate local concentrations and act as an amplifying signal to enhance liver inflammation. The circulating monocytes might have been activated within the hepatic circulation during transit through the hepatic sinusoids or directly by soluble circulating factors.

The stimulus for chemokine production in ALD is not known but is likely to involve several signals. Proinflammatory cytokines (such as tumour necrosis factor α (TNF- α) and IL-1) and lipopolysaccharide are potent inducers of MCP-1; all these factors have been reported to be increased in the circulation in alcoholic hepatitis.^{22 23} The stimulus within the liver might be local TNF- α , the expression of which is increased in severe alcoholic hepatitis,^{6 24} or the direct effects of alcohol metabolites or alcohol induced reactive oxygen mediators. Synthesis of the chemokines is likely to occur predominantly from Kupffer and stellate cells within the liver.^{3 25} Alcohol itself is unlikely to act as a direct stimulus as this normally decreases cytokine or chemokine secretion²⁶; however, toxic alcohol metabolites may induce a "rebound" increase in cytokine synthesis following withdrawal of alcohol.^{12 27}

The further evidence we provide of a role for chemokines in the pathogenesis of ALD has therapeutic implications, particularly in light of observations that mortality in severe alcoholic hepatitis remains high even after withdrawal of alcohol.²⁸ If, as we propose, MCP-1 plays a central role in this process then treatment aimed at preventing MCP-1 secretion or inhibiting its effects might present an opportunity to alter the clinical course of an otherwise devastating disease.

We are grateful to Dr Simon Olliff for help in obtaining hepatic and portal venous blood samples from patients undergoing radiological procedures. We are also grateful to our physician colleagues for allowing us to study patients under their care and to Dr Clare Morland and Janine Fear for practical help. This work was supported in part by grants from the Sir Jules Thorn Trust and the Endowment Fund of the former United Birmingham Hospitals.

- 1 Anonymous. Alcoholic liver disease: morphological manifestations. *Lancet* 1981;ii:707-11.
- 2 Paronetto F. Immunological reactions in alcoholic liver disease. *Semin Liver Dis* 1993;13:183-95.
- 3 Czaja MJ, Geerts A, Xu J, et al. Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. *J Leukocyte Biol* 1994;55:120-6.
- 4 Adams DH, Afford SC, Fear J, et al. Hepatic expression of macrophage inflammatory protein-1 β after liver transplantation. *Transplantation* 1996;61:817-25.
- 5 Maltby J, Wright S, Bird G, et al. Chemokine levels in human liver homogenates: association between GRO alpha and histopathological evidence of alcoholic hepatitis. *Hepatology* 1996;24:1156-60.
- 6 Afford SC, Fisher NC, Neil D, et al. Distinct patterns of chemokine expression are associated with leucocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis. *J Pathol* 1998;186:82-9.
- 7 Marra F, DeFranco R, Grappone C, et al. Increased expression of monocyte chemoattractant protein-1 during active hepatic fibrogenesis—correlation with monocyte infiltration. *Am J Pathol* 1998;152:423-30.
- 8 Baggiolini M. Chemokines and leukocyte traffic. *Nature* 1998;392:565-8.
- 9 Luster AD. Chemokines—chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998;338:436-45.
- 10 Tanaka Y, Kimata K, Adams DH, et al. Modulation of cytokine function by heparan sulfate proteoglycans: sophisticated models for the regulation of cellular responses to cytokines. *Proc Ass Am Phys* 1998;110:118-25.
- 11 Hill DB, Marsano LS, McClain CJ. Increased plasma interleukin-8 concentrations in alcoholic hepatitis. *Hepatology* 1993;18:576-80.
- 12 Masumoto T, Onji M, Horiike N, et al. Assay of serum interleukin-8 levels in patients with alcoholic hepatitis. *Alcohol Alcohol* 1993;28:99-102.
- 13 Sheron N, Bird G, Koskinas J, et al. Circulating and tissue levels of the neutrophil chemotaxis interleukin-8 are elevated in severe acute alcoholic hepatitis, and tissue levels correlate with neutrophil infiltration. *Hepatology* 1993;18:41-6.
- 14 Bossink AW, Paemen L, Jansen PM, et al. Plasma levels of the chemokines monocyte chemoattractant proteins-1 and -2 are elevated in human sepsis. *Blood* 1995;86:3841-7.
- 15 Maddrey WC. Alcoholic hepatitis: pathogenesis and approaches to treatment. *Scand J Gastroenterol* 1990;175:118-30.
- 16 Jiang Y, Beller DI, Frenzl G, et al. Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *J Immunol* 1992;148:2423-8.
- 17 Van Zee KJ, Fischer E, Hawes AS, et al. Effects of intravenous IL-8 administration in nonhuman primates. *J Immunol* 1992;148:1746-52.
- 18 Lusinskas FW, Kiely JM, Ding H, et al. In vitro inhibitory effect of IL-8 and other chemoattractants on neutrophil-endothelial adhesive interactions. *J Immunol* 1992;149:2163-71.
- 19 R&D Quantikine (Human MCP-1 immunoassay) handbook. Catalogue no. DCP00. Oxon, UK: R&D Systems Europe.
- 20 Abdullah MS, Wild G, Jacob V, et al. Cytokines and the malnutrition of chronic renal failure. *Miner Energy Metab* 1997;23:237-42.
- 21 Akahoshi T, Kobayashi N, Hosaka S, et al. In-vivo induction of monocyte chemoattractant and activating factor in patients with chronic renal failure. *Nephrol Dial Transplant* 1995;10:2244-9.
- 22 Khoruts A, Stahnke L, McClain CJ, et al. Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. *Hepatology* 1991;13:267-76.
- 23 Bode C, Kugler V, Bode JC. Endotoxaemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. *J Hepatol* 1987;4:8-14.
- 24 McClain CH, Schmidt J, Diehl AM. Cytokines and alcoholic liver disease. *Semin Liver Dis* 1993;13:170-82.
- 25 Armendariz-Borunda J, Seyer JM, Postlethwaite AE, et al. Kupffer cells from carbon tetrachloride-injured rat liver produce chemotactic factors for fibroblasts and monocytes: the role of tumour necrosis factor-alpha. *Hepatology* 1991;14:895-900.
- 26 Maher JJ. Rat hepatocytes and Kupffer cells interact to produce interleukin-8 (CINC) in the setting of alcohol. *Am J Physiol* 1995;269:G518-23.
- 27 Enomoto N, Ikejima K, Bradford B, et al. Alcohol causes both tolerance and sensitization of rat Kupffer cells by mechanisms dependent on endotoxin. *Gastroenterology* 1998;115:443-51.
- 28 Orrego H, Blake JE, Blendis LM, et al. Prognosis of alcoholic cirrhosis in the presence and absence of alcoholic hepatitis. *Gastroenterology* 1987;92:208-14.