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Role of Adhesion Molecules in Chronic Liver Allograft Rejection

by

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ABSTRACT

Chronic rejection after liver transplantation is characterized by accumulation of leukocytes into the graft. Like other inflammatory conditions, this white cell recruitment process is mediated by adhesion molecules expressed on the activated leukocytes and vascular endothelium. The present study investigated the roles of E-, P-, and L-selectins and ICAM-1 on leukocyte adhesion in sinusoids and post-sinusoidal venules during acute inflammation and chronic rejection of the liver using intravital microscopy. Mice deficient in selectins still exhibited leukocyte adhesion in the hepatic microvasculature in response to inflammatory stimuli, suggesting a minimal role for the selectins in the recruitment process within the inflamed liver. However, ICAM-1 was essential for stable adhesion. In a rat model of chronic hepatic allograft rejection, early leukocyte recruitment was demonstrated which could be blocked by ICAM-1 monoclonal antibody or ICAM-1 antisense oligonucleotides. Regression of cholestasis with prolonged graft survival in the ICAM-1 antisense-treated animals suggested a protective effect of anti-ICAM-1 therapy on the bile ducts.

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DEDICATION

To my beloved parents and Candy.

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LIST OF ABBREVIATIONS

ABC	avidin-biotin complex
DAB	diaminobenzidine
fMLP	formyl-methionyl-leucyl-phenylalanine
ICAM-1	intercellular adhesion molecule-1
LPS	lipopolysaccharide
μm	micrometer
μM	micromolar
mAb	monoclonal antibody
MPO	myeloperoxidase
P-sel KO	P-selectin knockout
E/P-sel KO	double mutant E-selectin/P-selectin knockout
P/ICAM-1 KO	double mutant P-selectin/ICAM-1 knockout

CHAPTER ONE: INTRODUCTION

1.1 The Liver

The liver is the largest and most metabolically complex organ of the human body, weighing 1.5 kg and receiving 1.5 L of blood per minute (McMinn, 1990; Johnson & Shields, 1991). It has a multitude of functions, including the maintenance of energy supply of the organism through metabolism of carbohydrate, fat and protein (Ganong, 1985). It synthesizes the majority of plasma proteins, including albumin and coagulation factors. It is the major site for storage of iron, copper, vitamins A and B_{12} (Johnson & Shields, 1991). The formation of bile is necessary for the efficient digestion and absorption of dietary fats. Bile also provides an excretory pathway for lipid waste products. The liver participates in heat production and some reticulo-endothelial activities. Moreover, it is a major control station of the endocrine system, contributing to the maintenance of peripheral hormonal levels by degrading most of them during a single passage. Furthermore, the liver has a rich enzyme system that allows the metabolism of many drugs, including alcohol. It also detoxifies noxious substances arriving from the gut preventing them from entering the systemic circulation and converts some lipophilic compounds into more water-soluble agents, which can then be excreted into the bile or urine. The liver also has a unique vasculature with dual blood supply (Johnson & Shields, 1991). Unlike other organs, the liver possesses a remarkable ability to replace lost tissue rapidly by compensatory cellular hypertrophy and hyperplasia.

1.1.1 The hepatic circulation

1.1.1.1 Anatomical considerations

The dual afferent blood supply, consisting of the hepatic artery and the portal vein is unique to the liver (Anderson, 1983). The hepatic artery brings oxygenated blood to the liver, while the portal vein carries venous blood rich in the products of digestion after being absorbed from the gastrointestinal tract. Together, the liver receives about 25% of cardiac output (Lautt & Greenway, 1987). The hepatic artery supplies 25-30% of the 1,500 ml blood that enters the liver every minute. By a delicate pressure arrangement with the portal venules, it determines the blood flow through the hepatic lobules. A hepatic arterial blood flow of 375 $ml.min^{-1}$ at an arterial pressure of 90 mmHg in a 70 kg man gives a hepatic arterial resistance of around 4 mmHg.ml.min.100g/ml (Richardson & Withrington, 1981). The hepatic artery is the second major branch of the celiac axis. It divides into the left and right branches in a Y-shaped manner at the porta hepatis to supply the corresponding lobes. There are aberrant hepatic arteries in about 50% of cases (Rappaport, 1982). Sometimes the common hepatic artery arises from the superior mesenteric artery or the aorta directly. The left and right hepatic branches may themselves arise from the left gastric or superior mesenteric arteries respectively (Johnson & Shields, 1991; McMinn, 1990; Rappaport, 1982).

The valveless portal vein is an afferent nutrient vessel of the liver, carrying 70-75% of the blood to the organ. This important vein is about 2 inches long, with a portal venous

pressure of 5-10 mmHg (Richardson & Withrington, 1981). It is formed by the union of superior mesenteric and splenic veins. It also divides into the left and right branches at the porta hepatis. The portal circulation begins as a capillary plexus in the organs it drains and ends by emptying into the sinusoids within the liver. The liver therefore receives venous outflow blood from the lower end of esophagus to halfway down the anal canal; from the pancreas, gallbladder and the spleen, together known as the "total portal tributary flow".

Within the liver, distributing branches of the hepatic artery and the portal vein run in parallel (McMinn, 1990). After repeated branching, terminal branches of these vessels (named hepatic arterioles and portal venules, respectively) supply blood to the hepatic sinusoids. Here, the arterial and portal venous blood become mixed. These sinusoids are the principal vessels involved in transvascular exchange between the blood and the parenchymal liver cells. The blood is then collected in small branches of hepatic veins (central venules or terminal hepatic venules) and is returned through three main hepatic veins to the inferior vena cava.

1.1.1.2 Hepatic microvascular system

The microcirculation is the most active part of the hepatic vasculature, because it regulates nutrition and function of the parenchyma and its supportive tissues. During the past century, the organization of the hepatic microvasculature has been the subject of some debate. Kiernan introduced the concept of "classic hepatic lobule" as the basic architecture in 1883 (Figure 1.1). He described a circumscribed pyramidal lobule bounded at its periphery by five to

six "portal triads", consisting of terminal branches of the hepatic artery and portal vein, with accompanying bile ducts, lymphatics and nerves. The axis of this classic hepatic lobule is the terminal hepatic venule (central venule), which is the origin of hepatic venous drainage (Kiernan, 1883). Columns of liver cells and blood-containing sinusoids extend between these two systems. However, in most mammals, the peripheral boundaries of these lobules are poorly defined, lacking a definite connective tissue border circumscribing each lobule. As a result, considerable sinusoidal anastomoses and mixing of blood occur between adjacent lobules. Thus, blood collected by each central venule may be supplied by several portal vessels. Due to these reasons and because of intralobular regional differences in oxygenation and metabolic functions, Rappaport proposed the "acinar concept" in 1973 to define the functional hepatic unit (Rappaport, 1973).

Although the hepatic acinus is the functional unit of the liver, it has no distinct morphological boundaries (McCuskey, 1994). Its axis is the portal tract containing a hepatic arteriole with a terminal portal venule. The peripheral boundary is circumscribed by an imaginary line joining the neighbouring terminal hepatic venules (central venules or post-sinusoidal venules). There are about 100,000 acini in the human liver, each about 2 mm in diameter (Lautt & Greenway, 1987). They are sometimes described as cluster of grapes on a vascular stalk comprising the terminal branches of portal vein, hepatic artery and the bile duct. An acinus is subdivided into three circulatory zones, each having different levels of oxygenation and metabolic functions (Junqueira *et al.*, 1986). Blood enters the acinus at the portal triad

(Zone 1 or periportal zone), flows through the sinusoids between plates of hepatocytes towards the terminal venules at the acinar periphery (Zone 3 or perivenous/centrilobular zone). Zone 2 is defined as the region between Zone 1 and Zone 3. Since hepatocytes in Zone 1 are situated close to the supplying vessels, they are bathed by blood of a composition similar to that in the afferent vessels. Therefore, they are well oxygenated and rich in nutrients, hormones and toxins. Flow in the adjacent sinusoids is concurrent. All entrances to the acinus occur in the periportal region while all exits occur at the periphery. Since Zone 3 lies on the outer limits of the acinus, it is perfused by the least oxygenated blood that has already undergone gaseous and metabolites exchange with the parenchymal cells of Zones 1 and 2 (Junqueira *et al.*, 1986).

With the development of electron microscope, the hepatic microvascular structure and dimensions are studied in more detail (Weiss *et al.*, 1983). The **portal venule** is about 200 μm long with an inner diameter of 35 μm. Its wall is formed by an endothelial lining with a basement membrane. It has no smooth muscle cells and therefore the regulation of blood flow is not efficient here. However, the swelling and shrinking of endothelial cells may adjust the blood entering into the sinusoids (Weiss *et al.*, 1983). The flow in the portal vein and venules depends on the state of constriction or dilatation of mesenteric arteries. The portal venous pressure dropped by 40% when blood enters the sinusoids (Rappaport, 1973).

The **hepatic arterioles** have an average inner diameter of 25 μm. They have an elastica interna, a double layer of smooth muscle cells in the media, and a thin adventitia containing unmyelinated nerve fibres (Rappaport, 1973; Rappaport 1982). The **terminal**

arterioles have a single layer of smooth muscle cells richly supplied with unmyelinated nerve fibres. They split into capillaries that form the peribiliary plexus and join the sinusoids. Distribution of arterial flow to the various acini is regulated in an efficient way through the constriction and relaxation of the smooth muscle cells. The presence of unmyelinated nerve fibres that transmit nerve impulses to the smooth muscles of the vascular wall also plays a role in flow regulation.

Sinusoids are considered as the capillaries of the liver. They are the principal sites for transvascular exchange between the blood and liver cells (McCuskey & Reilly, 1993). The length of a human sinusoid varies from 150-500 μm, and is only 7-15 μm in width (Weiss *et al.*, 1983). Sinusoids in periportal areas are narrower and more tortuous than the wider and straighter perivenous ones (Wisse *et al.*,1983). In these narrow periportal sinusoids, moving blood cells will be forced against the wall, causing a massage of the space of Disse. This will influence the exchange of fluid and particles through the endothelial fenestrae, and will cause stirring of the fluid in the space of Disse. Ninety percent of the sinusoidal length is lined by a fenestrated endothelium. This porosity is of chief hemodynamic importance. Without these pores, the low hydrostatic pressure (2-3 mmHg) in the sinusoids is not efficient for quick exchange between blood plasma and hepatocytes. Zone 3 sinusoids have a higher degree of porosity, favouring transport and uptake processes in that region (Wisse *et al.*, 1983). It is proposed that Zone 1 periportal cells enjoy the privilege of priority in the uptake and metabolism of oxygen and nutrients arriving via the portal blood. The porosity in Zone 3

which favours uptake and exchange processes in this region may compensate partly for the periportal priority. The sinusoidal lining cells are composed of three populations: Kupffer cells, fat-storing Ito cells and endothelial cells (McCuskey & Reilly, 1993). These endothelial cells are structurally different from the vascular endothelium elsewhere in the body. They lack a basement membrane and contain numerous fenestrae that permit hepatocytes ready access to nutrients and macromolecules in the plasma.

Terminal hepatic venules (central venules or post-sinusoidal venules) have an average inner diameter of 47 μm in human liver. They are lined by endothelium with an incomplete basement membrane. These endothelial lining cells are joined by tight intercellular junctions. The venule is surrounded by a fine tissue space that is continuous with the space of Disse and contains fibroblasts and elastic fibres.

1.1.2 Regulation of hepatic blood flow

Due to its dual blood supply, total blood flow to the liver is determined by both the hepatic arterial vascular resistance and the portal venous blood flow, which represents the outflow from the intestines and spleen. It has been known for over 20 years that intrinsic (relatively weak) and extrinsic mechanisms tightly regulate the amount of flow to the liver.

1.1.2.1 Intrinsic regulation

One form of intrinsic regulation peculiar to the liver is the hydrodynamic interaction

between the hepatic artery and the portal vein. If the portal blood flow is reduced, the hepatic artery dilates; if portal flow is increased, the hepatic artery constricts (Lautt & Greenway, 1987). However, changes in the hepatic arterial perfusion do not alter portal venous flow. This has been termed the "Hepatic Arterial Buffer Response" by Lautt and Greenway (1987). The function of this intrinsic autoregulatory system is to maintain a constant total blood flow to the liver. This can be accounted by the "adenosine washout hypothesis" (Lautt & Greenway, 1987). This hypothesis states that adenosine is constantly released into the fluid space surrounding the portal venules and the hepatic arterioles. Local adenosine concentration is regulated by washout into these two channels. If portal blood flow is reduced, less adenosine is washed away and the elevated adenosine concentration leads to hepatic arterial dilatation, resulting in increase in hepatic arterial blood flow; and vice versa.

1.1.2.2 Extrinsic factors

Extrinsic vasodilators also influence the hepatic vascular bed (Richardson & Withrington, 1981). **Postprandial** increase in osmolality (both in the portal and systemic circulation) is associated with increase in hepatic arterial flow as well as portal flow as a result of augmented intestinal blood flow (Carr *et al.*, 1978). Intravenous administration of **bile salts** (sodium dehydrocholate) increases total liver blood flow largely due to an increased hepatic arterial supply (Mitchell & Torrance, 1966). The synthetic pentapeptide gastrin analog, **pentagastrin**, produces dose-dependent hepatic arterial dilatation in the dog (Post & Hanson,

1975; Richardson & Withrington, 1976). Intraarterial injections of **secretin** (Richardson & Withrington, 1976) and **cholecystokinin-pancreozymin (CCK)** (Richardson & Withrington, 1977) in dog also produces hepatic arterial vasodilation. The release of CCK during digestion also increases portal venous flow due to mesenteric vasodilation. The pancreatic hormone **glucagon** has a unique spectrum of actions on the hepatic arterial bed. Its combined effects of vasodilation and antagonism of vasoconstriction make it likely to have a pathophysiologic role during the stressed state in ensuring a high hepatic arterial blood flow which would be needed to supply the increased oxygen demands of the hepatocytes during glycogenolysis and gluconeogenesis.

The role of **neural** elements in regulating blood flow in the liver microcirculation is incompletely understood. Adrenergic neural stimulation and α-adrenergic agonists elicit constriction of portal venules, hepatic arterioles, sinusoids and terminal hepatic venules of rats (Reilly *et al.*, 1981; McCuskey & Reilly, 1993; McCuskey 1994;). β-receptors are present in the portal venules and sinusoids. Stimulation of these β-receptors causes dilatatory effects (McCuskey & Reilly, 1993; McCuskey, 1994). Cholinergic stimulation has been reported to elicit both vasoconstriction and vasodilation. Koo and Liang (1979) showed that vagal stimulation and intraportal infusion of acetylcholine and cholinergic agonists produced dilatation of the sinusoids. Reilly *et al.*(1982) demonstrated that cholinergic agonists elicited constriction of portal venules, sinusoids and terminal hepatic venules in the rat. These constrictive responses are thought to be partly due to release of mast cell constituents,

particularly serotonin by the cholinergic agonists, since a decrease in the number of visible mast cells was demonstrated. Vasoactive peptides have recently been co-localized with neurotransmitters in adrenergic and cholinergic nerves. Neuropeptide Y (NPY) is found in sympathetic nerves supplying the hepatic arterial, portal venous and the biliary systems, having a vasoconstrictive effect. Substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) are found to coexist in sensory nerves innervating branches of portal vein and hepatic artery (McCuskey & Reilly, 1993). Some of the NPY and CGRP fibres are also found distributed intralobularly adjacent to parenchymal cells suggesting that these peptides might affect not only the hepatic vasomotor tone but also the metabolism of liver cells.

Kupffer cells secrete cytokines, eicosanoids and reactive free radicals in response to a variety of stimuli, such as phagocytosis of particulates (McCuskey & Reilly, 1993). These substances include prostaglandins, leukotrienes, interleukins, interferons and tumor necrosis factors. Although limited data are available, they are all suspected to be involved in hepatic microvascular regulation during pathophysiological states.

1.2 Adhesion molecules and leukocyte recruitment

The hallmark of an acute inflammatory condition is the movement of leukocytes from the bloodstream to the injured tissue. Discrete microcirculatory events have been postulated to mediate this transvascular movement of white cells. Since Dutrochet first reported that white blood cells adhered to vessel wall and emigrated to tissues in 1824, the interactions between leukocytes and endothelium have attracted the interest of many investigators (Butcher, 1991; Ley *et al.*, 1993; Mayadas *et al.*, 1993). Visualization of leukocytes (mainly neutrophils) behaviour within inflamed vessels of translucent tissues such as the mesentery or cremaster has revealed that leukocytes tether to the endothelial lining and roll along the length of the venule before firmly adhering to the microvasculature (Kubes and Kanwar, 1994; Kubes *et al.*, 1995; Mayadas *et al.*, 1993; Ley *et al.*, 1995; Finger *et al.*, 1996). This leukocyte emigration process through the postcapillary venules is an active process, requiring an orchestrated interaction of a number of proteins with their respective ligands. Butcher presented a model of leukocyte-endothelial cell recognition process in 1991 involving three or more sequential events leading from the initial encounter to stable attachment (Figure 1.2). Rolling of leukocytes along the surface of vascular endothelium is the first step observed. This requires transient attachment to and detachment from the endothelium. Rolling is mediated by binding of P-selectin and/or E-selectin on the vascular endothelium to complex carbohydrates, such as sialyl Le^x on circulating leukocytes (Lawrence and Springer, 1991). Alternatively, L-selectin expressed on the

leukocytes also participates in the rolling process (von Andrian *et al.*, 1993). Under physiologic shear stress in most post-capillary venules, rolling on a selectin is a prerequisite for stable adhesion through integrins (Lindbom *et al.*, 1992).

The second step of the model involves leukocyte activation (Butcher, 1991). Neutrophils undergo an activation event in response to stimuli such as platelet-activating factor, interleukin-8, leukotriene-B_4 and other chemoattractants. On activation, dramatic and rapid changes in shape of the neutrophils can be observed with the cells becoming flattened and exhibiting pseudopodia. Furthermore, the avidity of the neutrophil proteins leukocyte function-associated antigen-1 (LFA-1) and Mac-1 for their ligand intercellular adhesion molecule-1 (ICAM-1) increases, resulting in stable binding to the endothelium (step 3) (Butcher, 1991; Dustin, 1990).

Once the marginated neutrophil forms a stationary adhesion with the endothelial cell, it is stimulated by chemotactic factors to downregulate the selectin-based adhesion and upregulate the adherence dependent on LFA-1 and Mac-1 (Smith, 1992). With these adhesion molecules interacting with ICAM-1 on the endothelial cell, the leukocyte rapidly emigrates into the surrounding tissue. This directed migration is termed chemotaxis. White cells can perceive a gradient of chemotactic factors resulting in an orientation of the cells toward the higher concentration of the stimulus (Zigmond, 1979).

Taylor *et al.* (1981), using an *in vitro* model with a chemotactic gradient established across an endothelial monolayer, demonstrated that human neutrophils would migrate into a

compartment containing the chemotactic tripeptide fMLP at a concentration of 10nM. On the other hand, without the chemotactic factor, very few neutrophils migrate.

In a different *in vitro* model of endothelial monolayers, Furie *et al.* (1984) also showed neutrophil transendothelial migration by a gradient of fMLP or leukotriene B_4. Using monolayers of human umbilical vein endothelial cells (HUVEC) on collagen gels described by Huber and Weiss (1989), Furie *et al.* further showed that neutrophil transmigration does not occur unless a chemotactic gradient is established across the monolayer; and approximately 40% of the neutrophils contacting the apical surface of the monolayer migrate past the endothelial cells into the collagen gel.

Other molecules that exhibit chemotactic activity for leukocyte include C5a, PAF (Kubes *et al.*, 1990) and IL-8 (Huber *et al.*, 1991). They stimulate leukocyte in a dose-dependent manner to initiate chemotactic response (Snyderman & Uhing, 1992). All apparently utilize a common molecular mechanism for the rapid stimulation of phosphatidylinositol metabolism and the release of calcium from intracellular stores. This results in rearrangement and complex interaction of cell cytoskeletal elements. The microtubules impart directionality and stabilize the cell while actin-myosin system provides contraction forces (Malech *et al.*, 1977; Stossel, 1992).

1.2.1 The selectin family

Selectins mediate the first contact between leukocytes and the activated endothelial

cells in most organs. They are cell surface glycoproteins in which the amino terminals share sequence homology with the carbohydrate-binding domain of animal lectins, hence their name derivation. Furthermore, they all have a domain homologous to epidermal growth factor (EGF) and variable numbers of cysteine-rich complement regulatory protein repeats (CR domains) (Lasky, 1992). In 1989, three members of the selectin gene family (E-, P-, and L-selectin) were identified using antibodies. All the three selectins were localized to the long arm of chromosome 1 in the following year (Watson *et al.*, 1990). All of them have been shown to mediate rolling both *in vivo* and *in vitro*, and each selectin can directly and independently mediate rolling.

The first selectin to be studied was **L-selectin**. It was recognized as an adhesion molecule by using a monoclonal antibody (MEL-14) raised against a murine lymphoma. This antibody effectively blocked lymphocyte adhesion to high endothelial venules of lymph nodes *in vitro* and blocked lymphocyte homing to lymph nodes *in vivo* (Gallatin *et al.*, 1983). L-selectin is therefore the best characterized of the lymphocyte homing receptors (Stoolman, 1989; Lasky, 1992). L-selectin is constitutively expressed on most leukocytes, including granulocytes, monocytes and most lymphocytes. It has been found on the tips of leukocyte microvilli, which are the first points of contact with the endothelium (Picker *et al.*, 1991). It is known that L-selectin binds to carbohydrate ligand(s) on the endothelium. In fact, von Andrian *et al.* (1993) showed that L-selectin initiates neutrophil adhesion to inflamed venular endothelium by two parallel mechanisms: carbohydrate ligand (sialyl Lewis^x) presentation to E-

and P-selectins and simultaneously, recognition mediated by direct binding of L-selectin to an endothelial cell counterpart.

The functions of L-selectin have been studied extensively in the recent years using antibody against it as well as genetic knockout mice deficient in this selectin. These studies showed that antibodies to L-selectin inhibit neutrophil rolling on venules of exteriorized mesenteric tissues, implicating L-selectin in neutrophil recruitment events during the earliest phase of tissue injury *in vivo* (Ley *et al.*, 1991; McEver, 1992; Lasky, 1992; Bevilacqua & Nelson, 1993), as well as in flow chambers *in vitro* (Smith *et al.*, 1991). Other experiments demonstrated a role for L-selectin in leukocyte recruitment during ischemia-reperfusion injury in several animal models (Kubes *et al.*, 1995; Ma *et al.*, 1993). Moreover, L-selectin antibody has been shown protective in a cobra venom model of neutrophil-mediated acute lung injury (Mulligan *et al.*, 1994). Furthermore, L-selectin deficient mice exhibited impaired inflammatory responses in models of chronic inflammation, including delayed type hypersensitivity (DTH) reactions and lipopolysaccharide-induced toxic shock (Tedder *et al.*, 1995).

P-selectin was first discovered by investigators interested in the biochemical events during platelet activation. Antibodies that bound to the surface of activated platelets were generated (Hsu-Lin *et al.*, 1984). Immunochemistry revealed a transmembrane glycoprotein associated with the α-granules in resting platelets and rapidly redistributed to the cell surface upon activation. Later, P-selectin was also found in storage granules of endothelial cells,

known as Weibel-Palade bodies (McEver *et al.*, 1989). Similar to those in platelets, endothelial P-selectin is synthesized constitutively, stored intracellularly, and mobilized to the cell surface rapidly following stimulation by thrombin, histamine, complement fragments and other mediators (Hattori *et al.*, 1989; McEver *et al.*, 1989). The expression of P-selectin at the cell surface is short lived, declining substantially within minutes, and returns to basal levels within 1 to 2 hours (Hattori *et al.*, 1989). The ligand for P-selectin has undergone extensive investigations. Leukocytes express sialyl Lewis^x and related glycans on many glycoproteins and glycolipids that are low-affinity ligands for P-selectin. The most extensively characterized ligand is P-selectin glycoprotein ligand-1 (PSGL-1), a disulfide-linked homodimer first identified by Moore *et al.* in 1992. It is consistent with the demonstrated requirements for sialic acid and fucose, and terminate in sLewis^x. PSGL-1 appears to be the principal ligand for P-selectin on all major classes of leukocytes (Kansas, 1996). Norman *et al.* (1995) showed that interaction of P-selectin with this high-affinity ligand is required for rolling of myeloid cells in mesenteric venules at physiologic shear stress *in vivo*.

The function of P-selectin is to mediate rolling during an inflammatory response. Dore *et al.* (1993) demonstrated that spontaneous rolling is blocked by antibody to P-selectin. In the same year, Mayadas *et al.* reported a total absence of leukocyte rolling in mesenteric venules and delayed recruitment of neutrophils to the inflamed peritoneal cavity of P-selectin deficient mice. P-selectin also contributes to chronic inflammation, selectively involved in accumulation of leukocytes in certain tissues. P-selectin is expressed by rheumatoid synovial endothelium

and supports the binding of monocytes to these vessels (Grober *et al.*, 1993). Furthermore, late monocyte entry into the inflamed peritoneum is impaired in P-selectin deficient mice (Johnson *et al.*, 1995). However, Mizgerd *et al.* (1996) showed that neutrophil margination in uninfected pulmonary capillaries does not require P-selectin. They further demonstrated that streptococcal pneumonia induces an E- and P-selectin-independent increase in white blood cell interaction with the noncapillary endothelium and that migration of neutrophils to alveoli can occur despite deficiency or inhibition of the selectins. The unique expression of P-selectin on activated platelets suggests that P-selectin may play a role on leukocyte-platelet interactions during wound healing and hemostasis. P-selectin mediates adhesion of activated platelets to white cells, bringing them to the sites of vascular injury (Larsen *et al.*, 1989). This induces expression of tissue factors, initiating the blood coagulation cascade.

A juxtacrine system for neutrophil activation and adhesion was shown by the coexpression of P-selectin and PAF by endothelium stimulated with histamine or thrombin (Lorant *et al.*, 1991; Zimmerman *et al.*, 1992). Lorant *et al.* demonstrated that when endothelial cells were stimulated, P-selectin would rapidly expressed on the endothelial cell surface, where it binds to a receptor on the neutrophil, tethering it to the endothelial cell. PAF is then rapidly synthesized and was coexpressed with P-selectin on the endothelial cell surface. PAF binds to a receptor on the neutrophil, serving as a signal for activation-dependent alteration in CD11/CD18 glycoproteins on the plasma membrane of neutrophil that makes them competent to bind to counterreceptors on the endothelial cells. In other words, the

tethering component mediated by P-selectin facilitates PAF interaction with its receptor on the neutrophil and enhances the PAF-stimulated adhesiveness (Lorant *et al.*, 1991). This defines a complex cell recognition system in which tethering of neutrophils by P-selectin facilitates juxtacrine activation of leukocyte by a signaling molecule, PAF (Zimmerman *et al.*, 1992).

E-selectin was also identified using monoclonal antibodies in 1989. The expression of E-selectin appears to be largely restricted to activated endothelial cells. In vitro, cultured endothelium will express E-selectin following stimulation by bacterial lipopolysaccharide, inflammatory cytokines interleukin-1 or tumor necrosis factor-α (Bevilacqua *et al.*, 1989). Induction of E-selectin requires *de novo* RNA and protein synthesis, with a maximal expression occurring 3 to 6 hours following cytokine treatment, returning to a basal level by 10 to 12 hours. Several lines of evidence suggest that E-selectin binds to oligosaccharides terminating with sialylated Lewis X antigen or sialylated Lewis a antigen.

Similar to the other two selectins, E-selectin also supports rolling of leukocytes at sites of inflammation and tissue injury *in vivo* (Olafsson *et al.*, 1994), and *in vitro* (Lawrence & Springer, 1993; Abbassi *et al.*, 1993). However, because of the requirement for *de novo* gene transcription for expression, E-selectin essentially plays no role in the earliest phase (first 2 hours) of leukocyte recruitment during acute inflammation. Because of concordant transcription of P-selectin in many tissues, E- and P-selectin are coexpressed in many inflamed tissues. As a result, inhibition of activity of either selectin often results in little or no effect on leukocyte recruitment. For example, in E-selectin knockout mice, no defect of leukocyte

recruitment to the inflamed peritoneum is evident unless P-selectin function is simultaneously blocked, illustrating the overlapping function of these endothelial selectins (Labow *et al.*, 1994). E-selectin is also expressed in sites of chronic inflammation, such as the rheumatoid synovium (Grober *et al.*, 1993) and in renal and cardiac graft rejection (Allen *et al.*, 1993; Brockmeyer *et al.*, 1993). Furthermore, E-selectin appears to serve as a tissue-specific homing receptor for memory T cells recruitment to the skin (Silber *et al.*, 1994).

Recent evidence supporting the importance of selectins in inflammation comes from a newly described disease called leukocyte adhesion deficiency (LAD) type 2. This rare disorder, reported by Etzioni *et al.* in 1992, is characterized by an inadequate inflammatory response to infection. Two unrelated patients with a congenital disorder of fucose metabolism do not express properly glycosylated ligands for selectins. They have recurrent bacterial infections, supporting the importance of selectins in the initial phase of inflammatory response.

1.2.2 The integrin family

The integrins, heterodimeric cell surface proteins, are composed of two noncovalently linked polypeptide chains, α and β, consisting of extracellular and transmembrane segments and cytoplasmic tails (Hynes, 1992). Integrin receptors exhibit an amazing diversity. At least 17 α and 8 β chains are currently known, some of which exists as various isoforms resulting from alternative splicing (Hynes, 1992). The integrins are expressed in most cells in the body, with many cells expressing several types of integrins. They interact with a wide variety of

proteins, including several extracellular matrix proteins such as fibronectin, laminin and collagen. Based on their complex characteristics of distribution, regulation and ligand-binding specificity, the integrins have been the subject of intensive investigation within a broad spectrum of biology. Several lines of evidence indicate that integrins are involved in tumor invasion and metastasis (Kishmimoto & Anderson, 1992). Malignant transformation is often associated with alterations of levels of expression or structural features of integrins which appear to play a role in cellular deposition of extracellular matrix by tumor cells (Kishimoto & Anderson, 1992). Certain tumors overexpress integrins. Osteosarcoma cells and melanoma cells show increase expression in β_1 and β_3 respectively. Integrin expression on malignant cells may also facilitate organ-specific dissemination of certain cancers via recognition of vascular cell adhesion molecules. Integrins associated with platelet adhesion are responsible for hemostasis (Kishimoto & Anderson, 1992). Platelet adhesion receptors mediate platelet localization and plug formation, providing a framework for fibrin deposition. A role for the integrins in tissue repair or wound healing has been increasingly recognized (Ruoslahti, 1991). Keratinocytes isolated from wound tissue express fibronectin receptors and attach to fibronectin substrates *in vitro*. The role of integrins in inflammatory functions is discussed in detail below.

The β_1-integrin family is the largest subfamily with 9 members. The β_1 family molecules have been designated VLA (very late activation) since two of them, VLA-1 and VLA-2, appear on lymphocytes 2 to 4 weeks after antigen stimulation *in vitro*. They mediate

the binding of different cells to the extracellular matrix and direct cell migration through tissues. VLA-4 binds to fibronectin and vascular cell adhesion molecule 1 (VCAM-1). The binding of lymphocytes to vascular endothelium is mediated by VLA-4.

The β_2-integrins are comprised of 3 members that share a common β_2-subunit (CD18) with distinct α-subunits (CD11a, CD11b, and CD11c). Each of them is constitutively present in the neutrophil plasma membrane. Monoclonal antibodies against each heterodimer inhibit adhesion of neutrophils to endothelial cells under the appropriate conditions (Furie *et al.*, 1991; Zimmerman *et al.*, 1992). Anti-CD18 antibody protects systemic circulation and prevents cardiovascular dysfunction in the lipopolysaccharide-induced septic shock model in rabbits (Thomas *et al.*, 1992).

Leukocyte function-associated antigen 1 (LFA-1) is comprised of an α-subunit (CD11a) and a β-subunit (CD18) and is expressed on all leukocytes. The known ligands for LFA-1 are ICAM-1, ICAM-2 and ICAM-3. LFA-1/ICAM-1 interaction may be responsible for changes in leukocyte shape and for the initial invasion of these cells into and through the endothelial layer. LFA-1 is also critical for adhesion and de-adhesion of lymphocytes (Dustin & Springer, 1989.) Furthermore, LFA-1 is required for cytotoxic T cells-antigen-dependent adhesion to, and killing of, some target cells.

Mac-1 (CD11b/CD18) is expressed on macrophages, granulocytes, monocytes, and large lymphocytes. Mac-1 binds complement fragment C3bi, fibrinogen, factor X and bacterial lipopolysaccharide, in addition to ICAM-1. Recent reports showed that Mac-1-dependent

adhesion to matrix proteins or endothelial cells contributes to a massive secretion of H_2O_2 by neutrophils activated by cytokines or chemotactic factors. The generation of reactive oxygen by neutrophils not only is essential for intracellular microbicidal activities of these cells, but also contributes significantly to tissue injury in a number of pathophysiologic conditions. The mechanism by which Mac-1-dependent adhesion potentiates respiratory burst by adherent stimulated neutrophils is unclear.

p150,95 (CD11c/CD18) exhibits the narrowest tissue distribution, expressed only on monocytes, macrophages and granulocytes. p150,95 plays a role in neutrophil adhesion to serum-coated surfaces, phagocytosis of latex particles, chemotaxis of peripheral blood monocytes and adherence of monocytes to endothelium.

Elucidation of the function of β_2-integrins was facilitated by the identification of a group of patients who exhibited a mutation in the β_2-subunit. Reports from the early 1980's revealed that these patients can be deficient in either the LFA-1 complex, Mac-1 complex or the p150,95 complex (Kishimoto & Anderson, 1992). Therefore, it was proposed that the primary defect in this disorder was β-subunit related. The disease was later found to be transmitted as an autosomal recessive mode and was named "Leukocyte Adhesion Deficiency" (LAD) in 1987. Features include widespread bacterial infections, defective neutrophil mobility and defects in neutrophil phagocytosis. Recurrent necrotic infections of soft tissues involving skin, mucous membranes and intestinal tract are clinical hallmarks of LAD. Staphylococcal or gram-negative organisms are often cultured from lesions despite long term antimicrobial

therapy. Cases of perirectal abscess, peritonitis, necrotizing enterocolitis, otitis media and gingivitis have been reported, mostly leading to death in infancy. Marked peripheral blood leukocytosis (4-24 times that of normal values) is a constant feature of LAD. The only treatment at present is bone marrow transplantation.

The β_3-integrins are expressed by the endothelial cells (vitronectin receptor, $\alpha_v\beta_3$), platelets (IIb/IIIa, $\alpha_{IIb}\beta_3$) and myeloid cells. They are involved in binding a variety of matrix and coagulation factors, including fibronectin, fibrinogen and von Willebrand factor.

1.2.3 The immunoglobulin supergene superfamily

This is a large family of proteins having one or more immunoglobulin domain(s). These proteins are composed of two antiparallel β-sheets, each made up of 3-4 short strands. The most prominent members of the immunoglobulin gene superfamily include ICAM-1, ICAM-2, ICAM-3, VCAM-1 and the T-cell receptor (TCR).

ICAM-1 is constitutively expressed on a wide variety of cell types including leukocytes, endothelial cells, fibroblasts, keratinocytes and some epithelial cells, occurring in only small amounts when resting but dramatically upregulated during inflammation or early antigen recognition (Osborn, 1990; Springer, 1990). The broad tissue and cellular distribution of this adhesion molecule suggests that it plays an extensive role in immune responses and leukocyte emigration. It is a 95-105 kDa glycosylated cell-surface protein with five immunoglobulin-like domains first identified in 1986 by the ability of a monoclonal antibody to

block phorbol ester-induced aggregation of a B-cell line (Rothlein *et al.*, 1986). Expression was markedly elevated in response to interleukin-1, tumor necrosis factor, interferon-γ and bacterial lipopolysaccharide (Dustin *et al.*, 1986; Rothlein *et al.*, 1988). ICAM-1 binds to two members of the β_2-integrin family, LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) (Marlin & Springer, 1987), both expressed on leukocytes. The LFA-1/ICAM-1-dependent cell-cell adhesion requires metabolic energy production, an intact cytoskeleton and the presence of Mg^{2+}, and is temperature dependent.

The basic role of ICAM-1 is the induction of specific and reversible cell-cell adhesion, resulting in intercellular communication. Important functions of this adhesion molecule include localization of leukocytes to the area of inflammation, enhanced recognition of antigen-presenting cells to T lymphocytes, formation of lymphocyte germinal centers and enhancing natural killer cell response (Makgoba *et al.*, 1988; Boyd *et al.*, 1989; Springer, 1990; Furie *et al.*, 1991). ICAM-1 has also been shown to be associated with a variety of disorders characterized by local or generalized inflammation. As a receptor for rhinovirus, ICAM-1 is likely to play a role in upper respiratory tract infections (Greve *et al.*, 1991). It is also a receptor for *Plasmodium falciparum*-infected erythrocytes, suggesting a role in the pathophysiology of malaria (Ockenhouse *et al.*, 1992). In asthmatic patients, ICAM-1 is expressed by bronchial epithelial cells, and macrophages and eosinophils obtained from sputum or bronchoalveolar lavage (Vignola *et al.*, 1994). Elevated ICAM-1 expression has been observed in patients with autoimmune disorders such as rheumatoid arthritis (Aoki *et al.*, 1993)

and diabetes (Campbell *et al.*, 1989). Increased ICAM-1 expression on atherosclerotic plaques may contribute to fibrinogen deposition and monocyte attachment, followed by subendothelial migration, which represents a critical event in the development of atherosclerosis (Ross, 1993). Both increased and decreased ICAM-1 expression has been reported on malignant cells, possibly due to dedifferentiation or secondary to release of ICAM-1 regulatory factors. In general, reduced expression is associated with more aggressive malignant disease and a tendency to metastasize, presumably because these cancer cells can escape from immune surveillance (Kageshita *et al.*, 1993; Patros *et al.*, 1992). The role of ICAM-1 in ischemia/reperfusion injury and allogeneic organ transplantation is described in Section 1.2.4.

The second LFA-1 ligand, ICAM-2, is expressed on lymphoblastoid cell lines and thyroid follicular cells. Unlike many other endothelial adhesion molecules, ICAM-2 expression is not induced by cytokine treatment.

Vascular cell adhesion molecule-1 (VCAM-1) is another member of the immunoglobulin gene superfamily. It mediates adhesion between endothelial cells and activated lymphocytes, via its ligand VLA-4. VCAM-1 expression is induced on human umbilical vein endothelial cells (HUVEC) by IL-1, IL-4, TNF and LPS (Rice & Bevilacqua, 1989). Interestingly, VCAM-1 binds to most leukocytes but not the neutrophils. VCAM-1 are thought to play a role in mediating mononuclear leukocyte infiltration in vessels and interstitium in solid organ allograft rejection. Up-regulated expression of VCAM-1 within the liver and pancreas allografts at times of rejection was demonstrated by Bacchi *et al.* (1993).

1.2.4 Hepatic leukocyte recruitment and adhesion molecule expression in the liver

As in the case of other organs, neutrophils appear to contribute significantly to liver injury in several animal models. Jaecshke *et al.* (1990) showed that when rat livers were subjected to no-flow ischemia followed by reperfusion, two phases of liver injury were identified: an initial phase and a later progression phase. The later phase of reperfusion injury was mediated mainly by neutrophils, as shown in high power liver sections. Pretreatment with a monoclonal antibody against neutrophils, which caused consistent neutropenia, protected the liver from the reperfusion injury. Holman & Saba (1988) showed that hepatic parenchymal cell injury during intraperitoneal bacterial sepsis induced by cecal ligation with puncture was also mediated by neutrophils, as demonstrated by liver enzymes and hepatic morphology. Other published data (Jaeschke *et al.*, 1992) suggest an important role for adhesion molecules as immunoneutralization of CD11b with monoclonal antibodies significantly attenuated hepatic injury and reduced the number of polymorphonuclear cells in the post-ischemic liver. The mechanism of protection seems to involve not only the reduced accumulation of neutrophils in the liver, but also functional inactivation of these white cells, as demonstrated by their reduced spontaneous superoxide formation. Two studies were done in 1995 by different groups demonstrating the pivotal role of ICAM-1 in neutrophil-dependent ischemia-reperfusion injury in the rat liver. Vollmar *et al.* showed that lobar ischemia by clamping the hepatic artery and

portal vein branch for 60 minutes followed by reperfusion caused leukocyte adhesion in the hepatic sinusoids and post-sinusoidal venules. Monoclonal antibody against ICAM-1 effectively inhibited post-ischemic leukocyte adherence to the venular endothelial lining, with amelioration in bile flow and hepatocellular integrity. Using a very similar ischemia-reperfusion model, Farhood *et al.* showed an increase in ICAM-1 mRNA level in the affected lobes. ICAM-1 expression was enhanced in the sinusoids and hepatocytes after ischemia-reperfusion injury. Anti-ICAM-1 antibody also attenuated liver injury as indicated by liver enzymes and histology. A study by Essani *et al.* (1995) provided evidence that *Salmonella* endotoxin, through generation of tumor necrosis factor-alpha and interleukin-1, also induced expression of ICAM-1 mRNA in the liver. They further demonstrated that upregulation of both ICAM-1 and Mac-1 (CD11b/CD18) is necessary for a neutrophil-induced liver injury to occur. Blocking ICAM-1 by monoclonal antibody attenuated liver injury by 67-90% compared with the controls. In the same year, Essani *et al.* further showed that endotoxin induced a massive synthesis of E- and P- selectin mRNA in endothelial cells and Kupffer cells of the rat liver. In man, high circulating levels and tissue expression of E-selectin was observed in patients with alcoholic hepatitis (Adams *et al.*, 1993), and high levels of VCAM-1 in patients with alcoholic cirrhosis. Both diseases were associated with elevated levels of circulating ICAM-1.

Detailed clarification of adhesion molecule expression in specific regions and cell types of the liver further aid our understanding of the leukocyte recruitment process during inflammation of the organ. Using immunohistological analysis, Smith and Thomas (1990)

demonstrated a weak but localised ICAM-1 positive staining on hepatocytes in normal centrilobular areas around the central veins, in contrast with the absence of staining in the periportal cells. Steinhoff *et al.* (1993) further characterized the expression patterns of different adhesion molecules in the human liver. This is summerized in Table 1. E-selectin and P-selectin were basally expressed and inducible only on the portal venular and post-sinusoidal venular endothelia with acute and chronic liver inflammation. Sinusoidal endothelia, however, lacked this mechanism, even with severe inflammation. Expression and induction of ICAM-1 and LFA-3 showed a similar pattern, and very different from the selectins. In normal liver tissues, ICAM-1 and LFA-3 were expressed weakly on sinusoidal endothelia. An induction of ICAM-1 and LFA-3 on sinusoidal endothelia was demonstrated in all biopsies with hepatic inflammation. With chronic rejection, ICAM-1 and LFA-3 were strongly expressed on endothelia of portal venules and post-sinusoidal venules. There was a strong basal expression of ICAM-2 on the portal arterioles, but this expression displayed no major changes during rejection or inflammation. VCAM-1 was present in normal liver basally expressed on some Kupffer cells. Inflammation-induced (cholangitis and viral hepatitis) expression of this molecule was found mainly on portal venules and post-sinusoidal venules. Sinusoidal endothelium was also positive for VCAM-1 during chronic rejection. LFA-1 (CD11a) was expressed on adherent leukocytes in the normal sinusoids. The number of LFA-1 positive cells in sinusoids was greatly increased shortly after transplant reperfusion, acute rejection and post-operative infection. VLA-1, a cell matrix receptor for collagen and laminin, was expressed in

normal liver endothelial cells, portal interstitial cells, Kupffer cells and hepatocyte. Induced expression of these cell types was found in rejection. VLA-2 was present in normal bile duct epithelium. VLA-3 and VLA-5 were expressed on portal artery and portal vein endothelial cells. VLA-4 was mainly expressed on Kupffer cells and lymphocytes, whereas VLA-6 was only found in portal endothelium.

1.3 Liver transplantation and graft rejection

1.3.1 Background

The first report of a technique that would enable experimental transplantation of a new liver was performed in dogs by Welch (Welch, 1955). Starzl and his group in Denver, Colorado, reported the first successful human hepatic transplant in a 3-year-old boy suffering from biliary atresia in 1963 (Starzl *et al.*, 1963). Thereafter, the number of transplants escalated. More than 3,000 patients are now transplanted in 130 centres in the United States every year. In Canada, over 200 patients receive a new liver annually. Active centres are located in Halifax, Montreal, Toronto, London, Edmonton and Vancouver (Altraif & Levy, 1994). Possible candidates are those suffering from irreversible liver failure such as cirrhosis, cholestatic liver diseases, primary metabolic diseases or hepatic malignancy not responsive to conventional therapy. With increased experience of liver transplantation and improvement in surgical skills, graft rejection remains the only major problem. There are two broad categories of rejection, conventionally termed "acute" and "chronic". Acute rejection usually becomes clinically apparent in the first post-operative week, but chronic rejection may also have an early onset. Some hepatologists favour a division into "reversible" and "irreversible" rejection. Others prefer a morphological definition, known as the "vanishing bile duct syndrome". The short term results of acute rejection have significantly improved since the early 1980's, with the introduction of cyclosporine and other immunosuppressive agents. However, chronic rejection

is still the leading cause of late graft failure (Hayry *et al.*, 1993). Chronic hepatic rejection is irreversible, characterized by progressive loss of bile ducts, increasing cholestasis and an obliterative vasculopathy affecting the medium and large sized arteries (Lowes *et al.*, 1993). The incidence reported by different centres ranges from 2.4% to 16.8% of all transplanted cases. To date, the mechanisms underlying chronic hepatic rejection remain poorly understood, and conservative treatment is ineffective. The only hope for these patients is re-transplantation.

1.3.2 Definitions

An **allogeneic graft** (or **allograft**) is a graft transplanted between two genetically different individuals of the same species. **Rejection** refers to response of the recipient's immune system to the transplanted graft. **Chronic rejection** of the liver is a combined clinical-morphologic designation for long-lasting ductopenic rejection, characterized by loss of interlobular bile ducts, without any reference to onset or duration. The condition is considered irreversible in most of all instances. It is also a synonym for "vanishing bile duct syndrome" (Ludwig, 1992).

1.3.3 Clinical presentations

The onset of chronic rejection can vary from six weeks to nine months post-transplant. The typical course is the development of progressive cholestasis. The patient may present

with malaise, fever, right upper quadrant discomfort and jaundice. In most cases, the symptoms are slowly progressive, with increasing icterus and declining hepatic synthetic function manifested as coagulopathy and falling serum albumin levels. Chronic rejection is not reversed by immunosuppressive agents; re-transplantation remains the only effective treatment (Hayry *et al.*, 1993; Lowes *et al.*, 1993; Sherlock, 1993).

1.3.4 Diagnosis

Serum bilirubin, transaminases and prothrombin time increase in patients with chronic liver rejection. Alterations in echogenicity of the transplanted liver may be seen on ultrasound examination. Hepatic angiography may show narrowed hepatic arteries with no peripheral filling and often with occlusions of branch vessels. Bile duct stricturing may be observed on a cholangiogram (Sherlock, 1993), but all these are not specific to chronic rejection.

The definite diagnosis is confirmed by liver biopsy. Pathological changes are seen in the portal tracts, the hepatic parenchyma and occasionally the vascular supply (Robbins & Kumar, 1987). There is usually a portal tract infiltrate consisting of a mixture of lymphocytes, macrophages, plasma cells and neutrophils (Lowes *et al.*, 1993). T-lymphocytes and neutrophils surround the basement membrane of bile ducts leading to an immunologic attack, and eventually ductal necrosis. Parenchymal changes include hepatocyte damage with cholestasis (Lowes *et al.*, 1993). Foam cells of macrophage origin may be seen in the sinusoids. Vascular changes can be seen in both the arterial side and the venular side. The

lumen of medium and large arteries is narrowed with foam cells in the intimal layer. These cells contain cholesterol and may eventually lead to progressive obliteration of arterial lumen, a condition known as "obliterative vasculopathy" (Oguma *et al.*, 1989). On the venular side, endothelial inflammatory changes may be seen. Under high magnification, inflammatory cells are seen adhering to the venular endothelium, infiltrating into the liver parenchyma.

1.3.5 Mechanisms

The precise pathophysiology of chronic liver rejection leading to graft destruction remains uncertain. A variety of factors have been investigated.

1.3.5.1 Cellular response

Before a host can mount an immunological reaction against an allograft, it must first recognize the graft as being "foreign". This recognition process depends on a number of histocompatibility loci, each encoding an antigenic dterminant capable of causing rejection of a graft. The major histocompatibility complex (MHC) antigens, termed human leukocyte antigens (HLA) in man, are encoded for by closely linked genes situated on the short arm of chromosome 6. Human liver HLA class I (HLA-A, -B, -C) and class II (HLA-DR, -DP, -DQ) are found on sinusoidal lining cells (Hubscher *et al.*, 1990). Usually only class I is found on vascular endothelium, and class I and class II antigens on biliary epithelium. CD8+ lymphocytes recognize foreign class I MHC molecules; whereas CD4+ lymphocytes recognize

class II antigens (Hansen *et al.*, 1993). Rejection of a graft by a recipient is mainly determined by incompatibility of these histocompatibility antigens.

"Antigen presentation" is the process in which transplant antigens are presented to the recipient immune system to evoke a rejection response. The donor antigen may be processed by the host antigen-presenting cells (APCs) and presented in conjunction with host class II HLA; or the donor antigen can be presented directly to alloantigen-specific host cells by donor APCs without the need for processing by the host. Cells of macrophage lineage (Kupffer cells of the liver) are usually associated with antigen presentation. The APCs present transplant antigens to recipient CD4+ T-helper cells. These T lymphocytes expressing cell surface molecule CD4 preferentially recognize antigens of the MHC class II complexes (Hansen *et al.*, 1993). The interaction of the T4+ cells with class II antigens leads to the release of interleukin-1 (IL-1) from the antigen presenting cells. IL-1 promotes the proliferation of T4+ lymphocytes and the release of interleukin-2 (IL-2) from the cells. IL-2 further augments the proliferation of T4+ cells and also provides helper signals for the differentiation of class I-specific T8+ cytotoxic cells (Robbins & Kumar, 1987).

The other loci detectable (by early skin grafting experiments) are generally referred to as minor histocompatibility loci. At least 11 histocompatibility systems have been identified in the mouse (Sell 1987), and these apparently weak systems are also present in humans. If the organ donor and recipient are matched for the MHC but differ in one of these minor systems, rejection may not be as rapid or as severe. Mismatch in these systems can be more readily

overcome by immunosuppressive therapy (Sell, 1987). Hence, in clinical liver transplantation, if one matches donor and recipient at the major locus (such as within families), survival times are extremely good (Hansen et al., 1993).

Activated lymphocytes secrete cytokines (interferon, neutrophil-activating factors, interleukin-4) capable of recruiting and activating other inflammatory cells, such as neutrophils, eosinophils and macrophages. Inflammatory mediators including platelet-activating factor, platelet derived growth factor, thromboxane, tumor necrosis factor and leukotrienes are found to be released from damaged endothelium and activated platelets (Hoffmann *et al.*, 1993; Lowes *et al.*, 1993). The generalized inflammatory response results in several effector mechanisms leading to graft destruction, including the release of proteolytic enzymes and superoxide radicals.

Lymphocytes being the major cell type causing graft destruction in allograft rejection share many similarities with the neutrophils. Peripheral blood lymphocytes readily emigrate from the bloodstream to extravascular sites of inflammation. The recirculation of lymphocytes is a dynamic process that allows for constant surveillance of extravascular tissue by these immunocompetent cells (Harlan, 1985). T cell adhesion receptors include LFA-1, which binds to its counter-receptors ICAM-1 and ICAM-2; and CD2, which binds to its counter-receptor LFA-3. Makgoba *et al.* provided strong evidence supporting ICAM-1 as a ligand for LFA-1-dependent adhesion in T lymphocyte-mediated cytotoxicity. ICAM-1 monoclonal antibody blocks the LFA-1 pathway but not the CD2/LFA-3 pathway of adhesion.

When endothelial cells lining the vessels of a transplanted organ have been activated by ischemia, surgical manipulation, reperfusion injury or transplant incompatibility, they may produce a gradient of cytokines, such as tumor necrosis factor, or up-regulate adhesion molecules, particularly the selectins (Heemann *et al.*, 1994). As a consequence, circulating white cells in close proximity to the vascular endothelium will begin to slow down and tether via up-regulated selectin receptors on their surfaces. Leukocytes are pushed aside by the force of the flow and interact with the next selectin on the same or adjacent endothelial cell. These serial interactions result in the rolling effect where cells literally roll along the vessel walls, slowing progressively, and eventually coming to a complete stop. The presumed function of this phenomenon is to establish a physically close relationship between the leukocyte and endothelium, thereby forcing the leukocyte to remain in areas of higher cytokine concentrations and simultaneously up-regulating other surface adhesion molecules. The mechanism has been discussed in the previous sections.

The presence of cytokines and the binding of selectins result in a conformational shift of integrin receptors on the white cells from low to high affinity. The up-regulated β_2-integrin (particularly LFA-1) then adhere to other molecules appearing on the endothelial cells, such as ICAM-1, leading to permanent binding. This crucial adhesion allows the T cells to make a "lethal hit" to the target. The cytotoxic T cells then secrete (exocytose) the contents of some of their cytoplasmic granules in the areas of contact with their target cells (Kupfer & Singer, 1989). A pore-forming protein, present as a monomer in the granule, comes in contact with

extracellular concentrations of 1-2 mM calcium and undergoes polymerization in the lipid bilayer of the target cells. The polymerized form of the pore-forming protein can now act as an ion-permeable channel in the target cell plasma membrane, leading to osmotic swelling and lysis of the target (Podack *et al.*, 1991). Other components of the granules, i.e. the serine esterases, the cell toxins, and the proteoglycans may also cause nonspecific injury to the target cells (Young *et al.*, 1988). The third mechanism of lysis involves activating enzymes within the target cell to digest its own DNA (Abbas *et al.*, 1991). Once the DNA is fragmented, the target cell nuclei will also undergo fragmentation, a process known as apoptosis. The signal that induces DNA degradation have not been identified. The cytotoxic T lymphocytes are not injured during the lysis of the target cells. Moreover, each individual lymphocyte is capable of sequentially killing multiple target cells.

1.3.5.2 Humoral response

Humoral response has also been thought responsible for chronic rejection in the liver. Demetris *et al.* (1987) showed a deposition of immunoglobulin M (IgM) and complement C1q in the arterial walls of chronically rejected livers using immunoperoxidase technique. Furthermore, diffuse cytoplasmic immunoperoxidase staining for IgM and IgG of bile ductular epithelium was seen. The bile within the ductular and canaliculi lumen was also stained positive for IgA and IgM. The reactive antibodies with the donor organ are capable of inducing damage of the graft. They may be preformed in the recipient serum prior to

transplantation, or develop after engraftment with the assistance of T lymphocytes. Binding of complement-fixing antibodies to the endothelium of the graft may result in activation of the complement cascade, leading to endothelial damage, platelet activation, and loss of normal antithrombogenic milieu. This results in vasospasm, microvascular thrombosis and increased vascular permeability (Demetris, *et al.*, 1992). It is interesting to note that the liver parenchyma is more resistant to antibody mediated injury than the biliary tree. The large liver mass, dual afferent vasculature and the presence of Kupffer cells to neutralize immune complexes may account for this difference (Nakamura, *et al.*, 1993).

1.3.5.3 Ischemia

It is known that the hepatic artery is the only source of blood supply to the intra-hepatic biliary tree. Ischemia of the bile ducts due to chronic obliterative arteriopathy has been suggested to be one of the nonimmune mechanisms leading to ductopenic rejection. It has been shown that the severity of ductopenia parallels the degree of obliterative arteriopathy as measured by histometric analysis (Oguma *et al.*, 1989). The medium size arteries are affected most severely. The intima contains subendothelial foam cells and smooth muscle cell proliferation is apparent. The result is gradual arterial occlusion and diminished blood flow to the dependent bile ducts, eventually leading to ductal necrosis. The demonstration of hepatic vascular endothelial damage with increased circulating hyaluronic acid levels provides further evidence supporting vascular injury as a contributing factor in chronic liver rejection (Adams *et*

al., 1989).

1.3.5.4 Cytomegalovirus infection

Cytomegalovirus (CMV) infection is another possible contributing factor that deserves emphasis. CMV is one of the major infectious agents complicating the postoperative course of liver transplant patients (Maddrey & van Thiel, 1988). O'Grady and his group (1988) reported an increased incidence of chronic liver rejection to occur in association with serologic evidence of CMV infection. However, the mechanism underlying this association is not fully understood.

1.3.5.5 Pre-existing diseases

Pre-existing liver diseases, such as **primary sclerosing cholangitis** and **primary biliary cirrhosis** have also been reported to be associated with increased risk of developing chronic rejection (Klintmalm *et al.*,1989; Demetris *et al.*, 1988; Maddrey & van Thiel, 1988). A trend has been noted toward an increased incidence of allograft loss because of chronic rejection in patients suffering from primary biliary cirrhosis as compared to those who did not have this disorder before the transplant. A highly speculative conjecture for this correlation may be that these patients' immune system is sensitized against biliary antigens. Thus, the unique immunologic milieu of these patients may be a contributory factor in the postoperative course.

1.3.6 ICAM-1 expression and liver graft rejection

In the past few years, experiments have shown that adhesion molecules play an integral role in infiltration, activation, and binding of effector cells (T lymphocytes) to target tissues (bile duct epithelium and endothelial cells of the liver). This interaction plays a significant role in alloantigen recognition and effector cytolysis.

Special attention has been drawn to the expression of ICAM-1 on rejecting liver grafts. Adams *et al.* (1989) studied the expression of this particular adhesion molecule on liver tissues after transplantation, using standard immunohistology with peroxidase staining method. Expression of ICAM-1 on bile ducts, endothelium and perivenular hepatocytes (structures affected by the rejection process) was greater in patients with acute rejection than in those with stable transplants. This expression was even more significant in patients in whom there was progression to chronic rejection, suggesting that ICAM-1 expression might be useful in monitoring the progression of this disorder. On the other hand, in patients with resolving acute rejection, ICAM-1 expression was greatly reduced after high dose corticosteroid treatment.

Steinhoff and Behrend (1990, 1991, 1993) showed that in normal liver tissues obtained at hepatic resections, a weak expression of ICAM-1 was found on sinusoidal endothelia and Kupffer cells and not detectable on hepatocytes and bile ducts. With inflammatory complications such as rejection, hepatitis and cholangitis, ICAM-1 was *de novo* induced on hepatocyte membranes. They also extended the study to chronic rejection, in which ICAM-1

was found expressed in portal vein, hepatic artery, central vein and sinusoidal endothelia. However, a lack of selectin expression on sinusoidal lining cells during rejection was reported.

A similar pattern of adhesion molecule expression was also observed in the rat. In the acute rejection model, ICAM-1 was induced on the rat sinusoidal and portal vascular endothelial cells within 2 days, and its expression was increased with the advance of rejection (Omura *et al.*, 1992). Strong ICAM-1 expression was also observed in the sinusoidal endothelial cells in graft injury due to long period of cold-ischemic storage (Takei, *et al.*, 1996).

A circulating form of ICAM-1 (cICAM-1) was detected in bile and serum of patients suffering from graft rejection (Adams *et al.*, 1993; Lang *et al.*, 1995). Serum levels were not specific for rejection as they were also elevated in infective complications. However, biliary levels of cICAM-1 were only elevated in rejection. This appeared to be due to local release or secretion within the liver.

The role of ICAM-1 on transplant rejection was further recognized using monoclonal antibodies against the adhesion molecule. Harihara *et al.* (1994) reported a prolongation of rat hepatic allograft survival from 11 days to 24 days after a single dose of antibodies to ICAM-1 and LFA-1 just before the anhepatic phase. Similar results were observed by the Degawa group (1995; 1996) although different strains of rats were used. This immunosuppressive effect was shown to be dose dependent (Harihara *et al.*, 1996). All these experiments were performed on acute rejection models.

The importance of ICAM-1-LFA-1 adhesion has also been demonstrated in the mouse

heterotopic cardiac allograft model. Isobe *et al.* (1992) showed an indefinite survival of the grafts between fully incompatible mice strains after 6 days treatment of monoclonal antibodies to ICAM-1 and LFA-1. To date, minimal amount of literature has reported the role of ICAM-1 in a chronically rejecting liver graft.

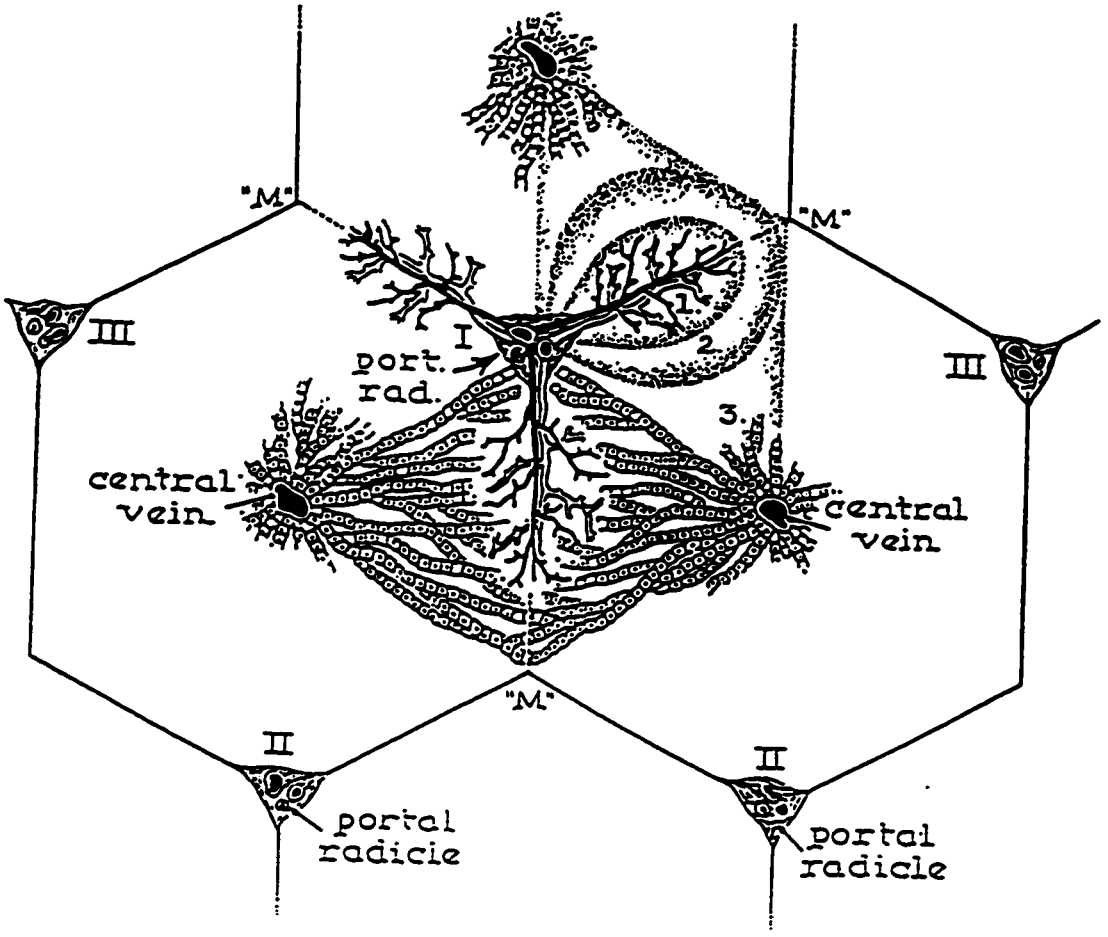


Figure 1.1 Diagram illustrating both the lobular and acinar concepts of hepatic structure. Two hexagonal lobules are shown, each having a central vein and 3 portal triads (radicals) at its periphery. Two diamond-shaped acini are also demarcated. Zones 1, 2 and 3 are shown in one of the acini. Zone 1 has the best blood supply from branches of hepatic artery and portal vein. (Reproduced from Ruebner BH & Montgomery CK. Pathology of the Liver and Biliary Tract. 1st edition. John Wiley & Sons, Inc., New York, 1982. Chapter 1, p.19)

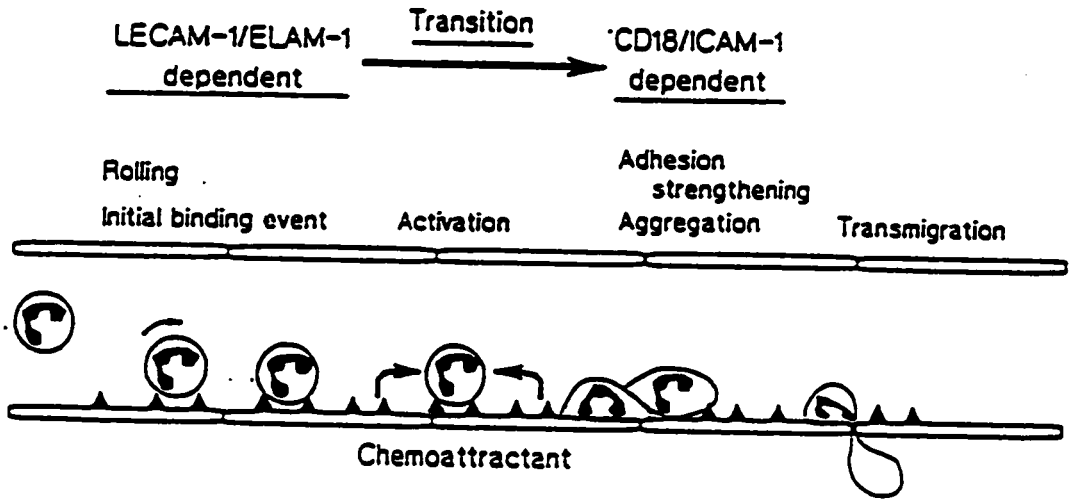


Figure 1.2 Sequential steps in rolling and adhesion of leukocytes. A dynamic model for neutrophil interaction with inflamed endothelium. (From Kishimoto & Anderson. The role of Integrins in Inflammation. In: Gallin JI, Goldstein IM, Synderman R (edi): Inflammation: Basic Principles and Clinical Correlates, 2nd edition. Raven Press Ltd, New York, 1992. Chapter 20, p.384)

Table 1. Differential expression of adhesion molecules on hepatic portal venule (PV), sinusoidal endothelium (SE) and central vein (CV). (From Steinhoff *et al.*, 1992)

	Normal			**Chronic rejection**			**Cholangitis**		
	PV	SE	CV	PV	SE	CV	PV	SE	CV
E-selectin	-	-	-	++	-	+	+	-	+
P-selectin	-	-	-	++	-	+	+	-	+
VCAM-1	-	-	-	++	(+)	+	+	(+)	+
ICAM-2	-	+	-	+	+	-	+/-	+	-
ICAM-1	-	+	-	+	+++	+	-	++	+/-
LFA-3	-	+	-	+	++	+/-	+/-	+	+/-

Staining intensity rated as negative (-), weak (+), moderate (++), or strong (+++).

CHAPTER TWO: HYPOTHESIS AND AIM

HYPOTHESIS

A two step paradigm for leukocyte recruitment has been established in different tissues including the skin, muscle and mesentery and necessitates an initial rolling step via the selectins prior to firm leukocyte adhesion via the integrins. However, a leukocyte recruitment cascade has not been characterized in the venous driven low shear hepatic microvasculature. Under this hydrodynamic condition found in the hepatic sinusoids, the selectins may be of minimal consequence. The role of ICAM-1 in hepatic inflammation and chronic allograft rejection is also not fully understood. Therefore, the following hypotheses were tested:

1. The selectins are not essential for leukocyte recruitment into the inflamed liver microvasculature.
2. ICAM-1 is important for leukocyte recruitment into the inflamed liver.
3. ICAM-1 plays an important role in the pathogenesis of chronic liver allograft rejection.

AIM

The aim of the present study is to test the aforementioned hypotheses on the leukocyte recruitment events in hepatic inflammation and chronic liver allograft rejection. An integrated series of *in vivo* experiments was performed. The hepatic leukocyte recruitment events were investigated using genetic knockout animals, selectin-binding carbohydrate fucoidan and monoclonal antibodies to selectins. The role of ICAM-1 in recruiting white blood cells into the

rat liver transplant chronic rejection model was studied using anti-ICAM-1 monoclonal antibodies and ICAM-1 antisense oligonucleotides.

CHAPTER THREE: MATERIALS & METHODS

3.1 Chemical reagents

Rhodamine 6G, N-formly-Met-Leu-Phe (fMLP), total bilirubin and alanine aminotransferase diagnostic kits were purchased from Sigma Chemical Co. (St. Louis, MO, USA). L-selectin antibody (MEL-14) was purchased from PharMingen (San Diego, CA, USA). ICAM-1 antibody (1A29) was obtained from Pharmacia & Upjohn Co. (Kalamazoo, MI, USA). ICAM-1 antisense oligonucleotide was a gift from Isis Pharmaceuticals, Inc. (Carlsbad, CA, USA). The avidin-biotin complex (ABC) kit and diaminobenzidine tetrahydrochloride (DAB) kit were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

3.2 Leukocyte recruitment into the inflamed liver

A series of *in vivo* experiments were performed in mice and rats in order to investigate the dynamic leukocyte recruitment process during acute hepatic inflammation and chronic allograft rejection.

3.2.1 Hepatic intravital microscopy

Intravital microscopy was used to study the hepatic microvasculature according to the method described by McCuskey (1986), with minor modifications (Figure 3.1). All animals

were fasted for 12 hours before the experiment. Mice were anesthetized with a mixture of ketamine and xylazine administered intraperitoneally (200 mg/kg and 10 mg/kg, respectively). The right jugular vein was cannulated for maintenance of anesthesia and administration of fluorescent dye during the experiment. A midline and a left subcostal incision were made in order to exteriorize the liver. The hepatic ligaments were dissected and the intestine was covered with a moist gauze. Animals were placed in a left supine position with a 30° tilt, with the left liver lobe positioned onto a plexiglass microscopic stage. The liver surface was then covered with Saran Wrap to hold the organ in position. All animals received rhodamine 6G (0.3 mg/kg, i.v.) for leukocytes labelling. Rhodamine 6G associated fluorescence was visualized by epi-illumination at 510-560 nm, using a 590 nm emission filter. An intravital microscope (Optiphot-2 Nikon Inc., Mississauga, Canada) with a X 40 water immersion lens (40/0.55 WI; Nikon, Japan) was used to observe the microcirculatory events on the liver surface. A silicon-intensified fluorescent camera (model C-2400-08, Hamamatsu Photonics, Japan) mounted on the microscope projected the image onto a monitor and the images were recorded for playback analysis using a videocassette recorder. The number of rolling and adherent leukocytes was determined off-line during video playback analysis. Rolling leukocytes were defined as those moving at a velocity less than that of erythrocytes within a given vessel and with an observable rotational movement of the nucleus. Leukocytes were considered adherent to the endothelium if they remained stationary for a period of time equal to or exceeding 30 seconds. After the liver was isolated and placed under the intravital

microscope, the centrilobular (Zone 3) zones were located. Within each field of view (2.1 X 10^4 μm^2) approximately 8-10 centrilobular sinusoids were observed, each of them emptying into a terminal hepatic venule (post-sinusoidal venule). Sinusoids and venules were identified by their diameters and directions of blood flow. In a single animal, 10-15 acinar zones were studied.

3.2.2 Mouse experiments

Mice were used in the first part of the experiment to study the roles of different adhesion molecules in leukocyte recruitment into the acutely inflamed liver microvasculature. Genetic knockout animals were available and their responses to different inflammatory stimuli were investigated.

3.2.2.1 Wildtype and genetic knockout animals

Mice deficient in P-selectin, E- and P-selectin, and both P-selectin and ICAM-1 were generated by gene targeting in embryonic stem cells from Baylor College of Medicine, Houston, Texas, USA. The mice were from a mixed background of 129Sv X C57BL/6. The goal of the gene-targeting (knockout) method was to replace the specific gene of interest with one that is inactive (Majzoub *et al.*, 1996).

In brief, a mouse cDNA clone was used to screen a 129/Sv genomic library and the genomic clones were characterized for preparation of the P-selectin targeting vector. The P-

selectin gene was disrupted by the deletion of a 4.5-kb region containing exons 3-5, and this region was replaced with the neo from Pol2neobpA. The AB2.1 embryonic stem cell line was electroporated with the vector. Digestion with EcoRV was used to identify homologous recombinants on Southern blots. Several clones were isolated which correctly targeted the P-selectin gene. Injection of one of these clones into C57BL/6 embryos, and implantation surgically into foster mothers resulted in generation of chimeric mice (Bullard *et al.*, 1995; Ley, 1995). Chimeras with germ cells derived from the altered embryonic stem cells transmitted the changed embryonic-stem-cell genome to their offspring, yielding mice heterozygous for P-selectin deficiency; the heterozygotes were then bred to each other to create mice homozygous for P-selectin deficiency.

For gene-targeting for E- and P-selectin, an E-selectin replacement construct was generated by replacing 1.4 kb of DNA between the PstI site in exon 4 and the HindIII site for exon 6 with an HPRT minigene. This construct was then electroporated into P-selectin mutant AB2.1 embryonic stem cells carrying the above described P-selectin mutation. Digestion with EcoRI and hybridization with the probe indicated were used to identify homologous recombinants on Southern blots. Injection of the clone correctly targeting the E-selectin gene into C57BL/6 embryos resulted in germline transmission (Bullard *et al.*, 1996).

P-selectin mutant mice were bred to mice containing a mutation in the ICAM-1 gene to generate double homozygotes. P-selectin/ICAM-1 double homozygotes were fertile, viable and with no obvious phenotypic abnormalities (Bullard *et al.*, 1995).

All mutant mice were kept in a specific pathogen-free barrier facility. Animals that exhibited signs of opportunistic infection or lymphadenopathy were not used for experiments.

For completeness, a separate group of E/P-selectin knockout mice received an L-selectin antibody (MEL-14, rat anti-mouse, 3 mg/kg, i.v.; PharMingen) before superfusion with fMLP.

3.2.2.2 fMLP model

In order to study the hepatic inflammatory response, a chemotactic peptide, N-formly-Met-Leu-Phe (fMLP Sigma Chemical Co., St. Louis, MO; 10 μM) at 37°C was superfused onto the surface of mouse liver for 60 minutes. A peristaltic pump (Gilson Miniplus3; Villiers Le Bel, France), set at 3.0 ml/min, was used to control the superfusion rate, while excess fluid was removed via a suction pump. Controls were superfused with bicarbonate-buffered saline (pH 7.4). Recordings were made at the end of the 60-minute superfusion.

In another series of experiments, exploring the role of selectins, a separate group of mice received 10 mg/kg of the selectin binding carbohydrate fucoidan intravenously, before superfusion with fMLP.

3.3.2.3 Lipopolysaccharide model

The acute hepatic response to injury was also tested with another inflammatory stimulus, lipopolysaccharide (LPS, from *Escherichia coli* serotype 0127:B8), a major

constituent of the cell wall of gram-negative bacteria. Mice were injected with 50 μg of LPS intraperitoneally. Four hours later, the hepatic microcirculation was studied using intravital microscopy. The liver was removed for myeloperoxidase activity assay, and blood was taken for liver enzyme measurement.

3.2.2.4 Myeloperoxidase activity

Myeloperoxidase (MPO) is an enzyme found in cells of myeloid origin, and has been used extensively as a biochemical marker of granulocyte (mainly neutrophil) infiltration into tissues. The liver samples were washed, weighed and frozen at -20°C for a maximum period of two weeks. Myeloperoxidase activity was determined using an assay described by Bradley *et al.* (1982), with minor modifications. Liver tissues were placed in plastic test tubes containing 0.5 ml of hexadecyltrimethylammonium bromide (HTAB) buffer. The tissues were homogenized, sonicated and incubated at 60°C for 2 hours in a water bath. The solutions were then centrifuged for 2 minutes at 14,000 g. Samples of 7 μl were placed in microplate wells and then mixed with 200 μl of O-dianisidine solution. Change in absorbance at 460 nm over a 90-second period was determined using a kinetic microplate scanner (Molecular Devices, Canada).

3.2.3 Rat experiments

To ensure that the selectin-independent adhesion in sinusoids was not restricted to

mice, the same principle was examined in rats. The model for intravital microscopy of the rat liver was similar to that of the mice (McCuskey, 1986), except that the rats were anesthetized with pentobarbitol sodium (50 mg/kg, i.p.). The same leukocyte parameters were studied.

3.2.3.1 fMLP model

The rat liver was superfused with 5μM fMLP for 60 minutes. The superfusion procedures were similar to that applied to the mice. The dosage of fucoidan used in the rat model was 25 mg/kg.

3.3 Liver allograft rejection model in the rat

The liver transplantation rejection model was studied in the rat. Orthotopic transplantation (Figure 3.2), with the graft placed in the original anatomical position, was performed in order to achieve maximum physiological and functional resemblance to that of human liver transplant.

3.3.1 Advantages of the rat model

There are several advantages of using the rat liver transplant model for studying rejection. These include (a) the availability of different inbred strains and well defined MHC phenotypes (*Rattus norvegicus*) (Limmer *et al.*, 1980); (b) the availability of different types of rejection models (Zimmermann *et al.*, 1983); (c) the absence of gallbladder in the rat, hence

simplifying the operation; (d) the close resemblance to human liver architecture; (e) the availability of antibodies against rat adhesion molecules; and (f) relatively good size for hepatic intravital microscopy study (McCuskey, 1986).

3.3.2 Orthotopic liver transplantation

All animals were housed in an environmentally controlled vivarium with a 12 hour light/dark cycle and allowed access to rat chow and water *ad libitum*. The protocol was approved by the Animal Care Committee of the University of Calgary under the guidelines of the Canadian Council on Animal Care. Liver transplantation was performed according to the method described by Kamada and Calne (1983), with minor modifications (Hasuike *et al.*, 1988). Food but not water was withdrawn from the cages 12 hours before surgery. The operation was done in the laboratory under clean conditions.

3.3.2.1 Donor operation

The donor rat was anesthetized with intraperitoneal pentobarbital (50 mg/kg). A midline abdominal incision was made and all ligamentous attachments around the liver were divided. The left phrenic, right adrenal and right renal veins were ligated. The portal vein was divided from the left gastric vein. The inferior vena cava was cleared from the connective tissues and dissected down to the level of left renal vein. The hepatic artery and celiac trunk were cleared from all branches. The common bile duct was divided, and a 6-mm length of PE-

50 polyethylene tubing was inserted into the proximal end and secured by 5-O silk suture. The animal was injected intravenously with 200 units of heparin in saline. The inferior vena cava and portal vein were then clamped. The liver was then flushed with 10 ml of cold (4°C) saline containing 50 units of heparin, through the aorta retrogradely. The portal vein, suprahepatic and infrahepatic vena cava were now divided. The liver was then removed from the donor body into a 4°C saline bath.

3.3.2.2 Cuff preparation

The cuff for the portal vein (PE-240) and inferior vena cava (PE-280) consisted of a 0.3-cm length of polyethylene tubing. The cuff preparation of both vessels was performed in the iced saline bath. With a clamp holding the tubing edge, a pair of fine forceps was used to pass the portal vein through the lumen of the tube and everted over it. The cuff was then secured with a circumferential 5-O silk suture. The same method was applied to the inferior vena cava.

3.3.2.3 Recipient operation

The recipient was anesthetized with inhalational halothane. A midline abdominal incision was made. The left phrenic vein and right adrenal vein were ligated. The hepatic artery was ligated and divided. The bile duct was tied close to the liver and divided. Isotonic saline (1 ml) was flushed to compensate for blood loss. The inferior vena cava and portal vein

were clamped with microvascular clips. The suprehepatic vena cava was clamped by a teaspoon-clamp. The vessels were divided and the recipient liver was removed. The donor liver was then placed in the anatomical position immediately. The suprahepatic vena cava was first to be anastomosed end-to-end by 7-O prolene continuous suture. The portal vein and infrahepatic vena cava were then connected to their corresponding cuffs by 5-O silk ties. The vascular clamps were released after the connections were made. Arterialization was performed by diverting blood from the right renal artery into the graft through the celiac trunk. The right kidney was then sacrificed. The bile duct was bridged by inserting the PE-50 tube into the recipient end after bile outflow was observed. The abdominal incision was closed with continuous 3-O silk suture. The clamping time of the portal vein in all animals did not exceed 15 minutes. Immediately after the operation, intramuscular penicillin G (100,000 units/kg) and intraperitoneal gentamicin (1 mg/kg) were administered to the rat. All animals were allowed free access to water after consciousness was regained. Food was resumed 12 hours after the operation.

3.3.3 Post-operative ICAM-1 mAb treatment

The intact ICAM-1 antibody (1A29) reacts with ICAM-1, a 85-kDa surface glycoprotein found on endothelial cells. One group of transplanted rats was treated with 1 mg/kg/day of 1A29 intravenously through the dorsal penile vein for 6 consecutive days after the operation. This dosage has been reported to be effective in blocking ICAM-1 in rats

(Harihara, *et al.*, 1994; Degawa *et al.*, 1995; Nishimura *et al.*, 1996). Control animals received the same volume of normal saline intravenously.

3.3.4 Post-operative ICAM-1 antisense oligonucleotide treatment

ICAM-1 antisense oligonucleotide was provided by Isis Pharmaceuticals, Carlsbad, CA. It was designed to hybridize selectively to the mRNA which encoded this adhesion molecule. Antisense was given to the rats intraperitoneally under sterile conditions at 10 mg/kg/day for 6 days after transplantation.

3.3.5 Liver histology

To verify the pathological changes in the chronic rejection model, hepatic histological examination was performed at day 10 and day 20 after transplantation. Liver sections of 8 μm were stained with hematoxylin & eosin (H & E) and examined under light microscopy.

3.3.6 Liver Biochemistry

To confirm functional liver damage in the transplant rejection group, liver biochemistry tests were performed. Whole blood was collected by cardiac puncture, placed on ice for 30 minutes and centrifuged at 1,000 g. The serum was harvested. All serum samples were frozen at -20°C for a maximum period of two weeks before the experiment.

Alanine aminotransferase (ALT) activity was measured by the methods of Bergmeyer

and colleagues (1978), using commercially available Sigma Chemical Co. diagnostic kits. As a rule, levels of ALT rise whenever liver cells are damaged. Each assay tube contained 0.1 ml of serum, 400 mmol/L of L-alanine, 12 mmol/L of 2-oxoglutarate, 2000 U/L of lactate dehydrogenase and 0.25 mmol/L of NADH in a final volume of 1.1 ml. Alanine aminotransferase catalyzed the transfer of amino group from alanine to 2-oxoglutarate to produce pyruvate and L-glutamate. In the presence of lactate dehydrogenase, the pyruvate formed was then reduced to lactate; with the simultaneous oxidation of NADH to NAD. The rate of decrease in absorbance at 340 nm was directly proportional to the activity of ALT. ALT activity was expressed as Sigma units. Each unit represented 1 μmol of NADH oxidized per minute.

Total bilirubin was also measured by commercially available Sigma Chemical Co. diagnostic kits, based on the method by Hillmann and Beyer (1967). The assay contained 0.1 ml of serum, 1.0 mmol/L of sulfanilic acid and 0.2 mol/L of hydrochloric acid with surfactant at a final volume of 2.6 ml. Determination of serum total bilirubin was based on its reaction with diazotized sulfanilic acid to form a pink coloured product, which intensity was proportional to the bilirubin concentration. The reaction mixture was incubated at 37°C for 5 minutes and measured at a wavelength of 540 nm. Total bilirubin was expressed as SI units of μmol/L.

3.3.7 ICAM-1 immunohistochemistry

To test the effectiveness of the antisense oligonucleotides, an indirect

immunoperoxidase technique (Hsu *et al.*, 1981) was applied to 8-μm-thick liver crystat sections of normal livers, rejecting livers and rejecting livers treated with ICAM-1 antisense oligonucleotides. In brief, liver tissues were fixed in paraformaldehyde overnight after resection from the recipient. The tissues were then rinsed in 20% sucrose in phosphate-buffer. Crystat sections were cut on the following day and stored at -70°C before staining. After rehydration, liver tissues were sequentially incubated for 30 minutes with 5% normal horse serum to block non-specific protein interactions. Sections were then treated with the primary ICAM-1 antibody (1A29) in appropriate dilution for 90 minutes. Endogenous peroxidase activity was blocked by 0.3% H_2O_2. The biotinylated horse anti-mouse IgG secondary antibody and avidin-biotin complex (ABC) (Vector Lab., Burlingame, CA) were applied for 60 minutes and 30 minutes, respectively. The tissues were then incubated with diaminobenzidine (DAB) solution for 5 minutes, and counterstained with hematoxylin. Controls were obtained by omitting the primary antibody, substituted with antibody diluent alone. Staining was studied under light microscopy.

3.4 Leukocyte recruitment in the transplant model

Leukocyte recruitment into the graft was studied on post-operative day 6. Hepatic microcirculation was studied in these transplanted animals using the same intravital microscopy setup as described above.

3.4.1 Hepatic intravital microscopy on transplanted rats

Transplanted animals were anesthetized with pentobarbitol sodium (50 mg/kg, i.p.). The right jugular vein was cannulated for administration of rhodamine dye. A midline and a left subcostal incision were made. Adhesions around the liver were dissected. Neovascularizations arising from the stomach, duodenum, diaphragm, omentum and retroperitoneal tissues were carefully ligated and cleared from the liver capsule. Hemostasis was achieved by gauze packaging and cauterization. The left lobe was then exteriorized and placed onto the plexiglass stage for the experiment. The same parameters for leukocyte recruitment were studied in Zone 3 as described in the mice model.

3.5 Survival study

After liver transplantation, all animals were allowed free access to rat chow and drinking water. They were monitored daily for obvious clinical deterioration until they succumb. Animals that exhibited signs of poor feeding, grossly decreased movements, lack of response to external stimuli were deemed too sick to continue in the protocol and were terminated for humane reasons. Rats that died within the first post-operative day due to obvious surgical complications (e.g. massive hemorrhage or halothane overdose) were not included into the survival study. Animals that have been anesthetized for intravital microscopy or subjected to blood sampling were also excluded from survival study. Post-mortem was performed in all animals.

3.6 Statistical analysis

Statistics were performed by utilizing a commercially available software, INSTAT (Version 2, GraphPad Software Inc., San Diego, CA, USA). All values were reported as mean ± SEM. Differences between the groups were assessed by one-way analysis of variance (ANOVA) followed by a Bonferroni correction for multiple comparisons. Statistical significance was set at $p<0.05$. Statistical analysis for graft survival of each group was performed using the Kaplan-Meier method.

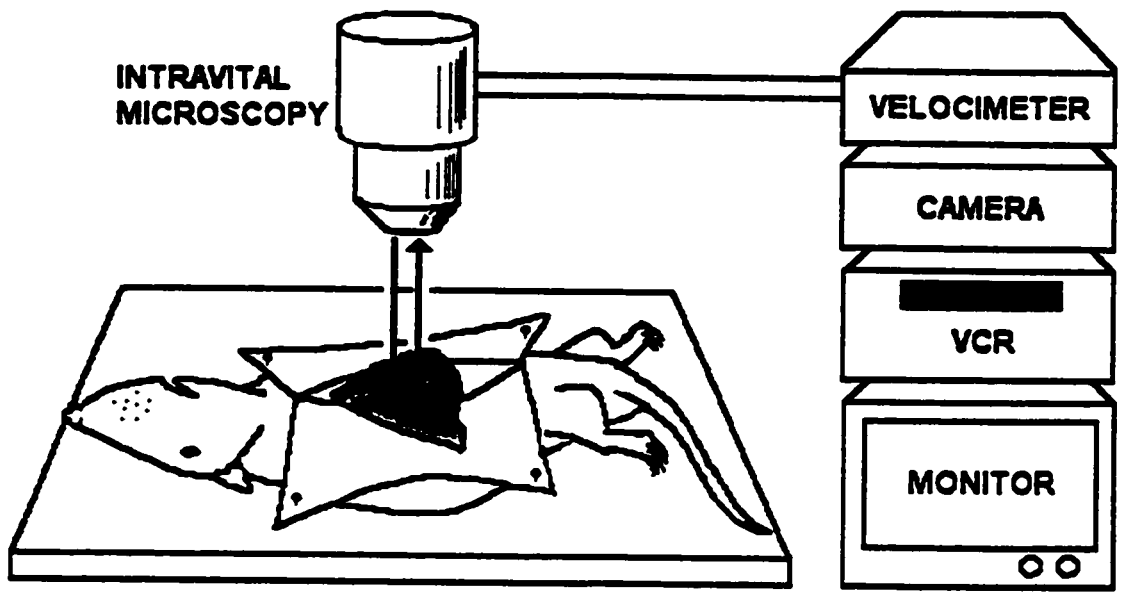


Figure 3.1 Intravital microscopy setup for the mouse or rat liver. The animal is placed supine position, with the left lobe of liver exteriorized. Fluorescence microscopy is performed according to an epi-illumination technique. Images are visualized by means of a video camera and recorded with a video recorder connected to a TV monitor.

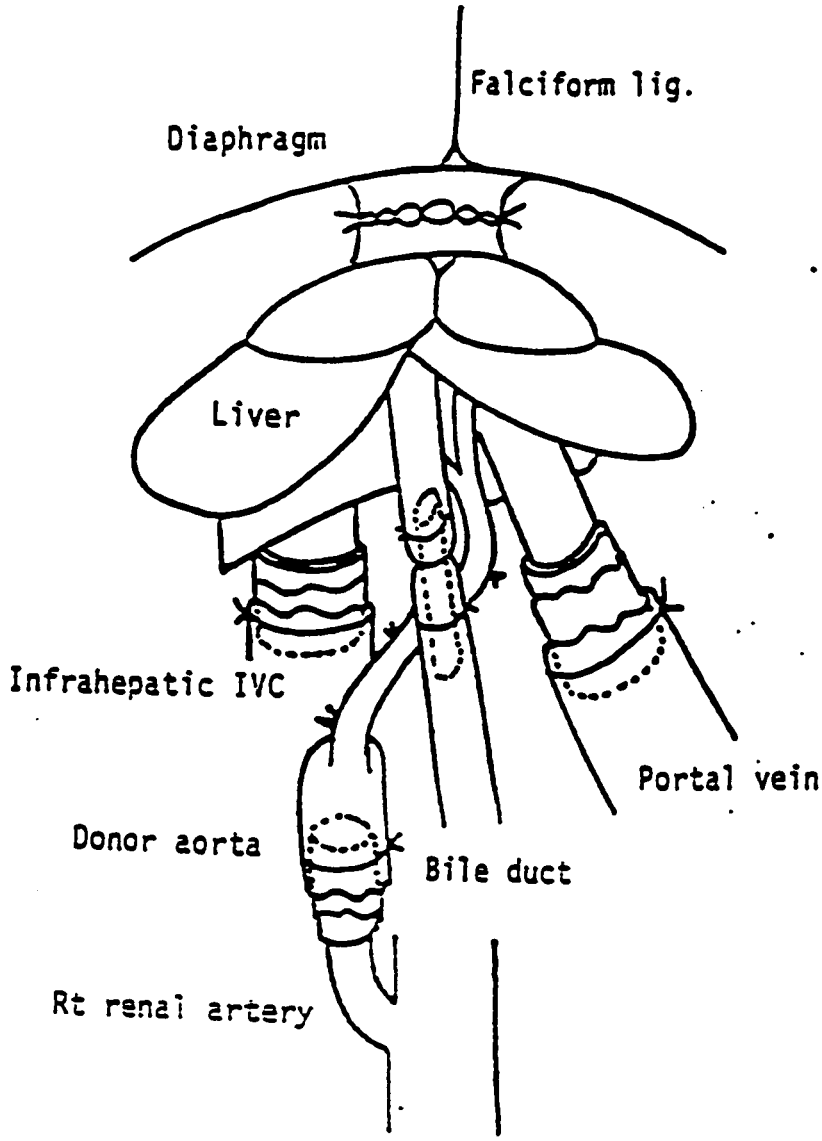


Figure 3.2 Liver transplantation in the rat with arterial reconstruction from the right renal artery. The "cuff technique" is used for connecting the portal vein, inferior vena cava and the bile duct. The suprahepatic vena cava is anastomosed by continuous 7-O Prolene suture. (From Hasuike, *et al.*, Transplantation, 1988.)

CHAPTER FOUR: RESULTS

4.1 Leukocyte recruitment into the inflamed liver microvasculature

4.1.1 Mouse experiments

Visualization of the mouse liver under the intravital microscope revealed an extensive capillary network (sinusoids) that drained into post-sinusoidal venules. Figure 4.1 demonstrated that leukocyte adhesion occurred in both the sinusoids and post-sinusoidal venules in response to the chemotactic factor fMLP. Moreover, adhesion in the sinusoids accounted for 80% of the total number of adherent white cells within the liver microcirculation. However, rolling leukocytes, defined as those cells moving slower than the red cells with detectable rotational motion could only be seen in the post-sinusoidal venules and not in the sinusoids *per se* (Figure 4.2).

Immunoneutralization of selectins by fucoidan failed to inhibit leukocyte adhesion in the mouse hepatic sinusoids (Figure 4.3). This dosage of fucoidan has been shown to block fMLP-induced adhesion effectively in other tissues.

4.1.1.1 fMLP on the genetic knockout model

Figure 4.4 demonstrated that leukocyte adhesion in sinusoids occurred as effectively in P-selectin deficient animals as wildtype littermates in response to fMLP. Mice deficient in both P-selectin and E-selectin still had white cells adhering to the sinusoids in response to fMLP.

Leukocytes could still be seen trapped in the sinusoids of E/P-selectin knockout animals treated with an L-selectin antibody (Mel-14). However, very few leukocytes were seen adhering onto the sinusoids of P/ICAM-1 knockout animals.

Figures 4.5 and 4.6 illustrated that the rise in leukocyte rolling and adhesion in post-sinusoidal venules was modest and returned to control in mice deficient in both E-selectin and P-selectin regardless of whether the L-selectin antibody was added. There was some residual rolling in the P-selectin deficient post-sinusoidal venules which permitted the cells to adhere in these vessels. Rolling and adhesion in post-sinusoidal venules of P/ICAM-1 knockout animals also returned to baseline level.

4.1.1.2 Lipopolysaccharide model

Four hours after intraperitoneal injection of 50 μg LPS, the number of adhering cells in the mice liver sinusoids was studied. We showed that with this amount of LPS in the peritoneal cavity, the insult to the liver was great enough to cause a four fold increase in liver enzyme alanine aminotransferase (Figure 4.7) Hepatic sinusoidal adherent cells increased significantly in the LPS treated animals, compared to the untreated controls (Figure 4.8). There was no difference in sinusoidal leukocyte adhesion between the E/P-selectin deficient and wildtype animals in response to LPS.

4.1.1.3 Myeloperoxidase activity

Myeloperoxidase (MPO) activity, as a marker of leukocyte infiltration into inflamed liver, was expressed as U/mg tissue weight. There was no difference in MPO activity between the untreated wildtype and untreated E/P-sel KO animals (Figure 4.9). MPO levels increased by four fold 4 hours after injection of LPS into the peritoneum in both groups. MPO levels were the same in the E/P-selectin knockout and wildtype animals in response to LPS.

4.1.2 fMLP model in the rat

The hepatic microvasculature of the rat was anatomically and physiologically very similar to that of a mouse under the intravital microscope. Rat hepatic microcirculation also responded to the chemotactic peptide fMLP. Figure 4.10 demonstrated the rise in leukocyte adhesion in the rats sinusoids in response to fMLP. The selectin-binding carbohydrate fucoidan failed to block this leukocyte recruitment process.

4.2 Liver allograft rejection model

The rat liver rejection model was confirmed by histology at different time points; serum alanine aminotransferase levels; bilirubin levels; and a survival study.

4.2.1 Liver histology

Histology of control liver (Lewis to Lewis transplant) was shown in Figure 4.11. The

portal triads were normal. Red blood cells were present inside the arteriole lumen as the graft was arterialized. The hepatic architecture was normal.

Figure 4.12 showed the liver histology of a rejected rat (Lewis to Brown Norway) 10 days after transplantation. Early signs of rejection were present. Dense inflammatory infiltrate was seen in portal spaces with lobular expansion. The lobular architecture was moderately distorted. The majority of hepatocytes were still viable. At high magnification (Figure 4.13), the bile ducts could not be easily visualized because of the damage secondary to rejection. The mononuclear infiltrates were seen in Zone 1. Red cells were present in portal venules.

The representative field of rejected liver at Day 20 was shown in Figure 4.14. Marked edema was seen in the centre of the micrograph which corresponded to destroyed portal space. At high magnification (Figure 4.15), heavy infiltration of mononuclear inflammatory cells was seen. No bile ducts but only newly formed bile ductules were present. These photographs illustrated progression of damage to the biliary tree and hepatocytes suggestive of graft rejection.

4.2.2 Liver biochemistry

Liver enzyme alanine aminotransferase levels of non-operated, control transplant (Lewis to Lewis), and rejection (Lewis to Brown Norway) groups were presented in Figure 4.16. The control transplant group had an elevated enzyme level compared to the non-operated group, but a statistical difference was not reached. The rejection group had a

significantly higher enzyme level compared to the control transplants.

A similar pattern was observed in the bilirubin levels of the three groups of animals (Figure 4.17). The rejection group had a significantly higher bilirubin level than the control transplant animals.

4.2.3 Survival

Lewis to Lewis control transplant rats had indefinite survival of up to a year. Brown Norway rats receiving Lewis livers (rejection group) became very sick at the end of the first month and developed deep jaundice and ascites, with a mean survival of 25.2 ± 2.7 days. There is a statistically significant difference between the survival of these two groups, as compared by the Kaplan-Meier survival estimates, $p<0.01$ (Figure 4.18).

4.3 Leukocyte recruitment during rejection

Leukocyte rolling flux and adhesion onto the post-sinusoidal venules were studied by intravital microscopy on Day 6. A baseline rolling flux of 2-3 cells/minute in the non-operated animals was observed (Figure 4.19). In the control transplant group, the rolling flux was not significantly different from the non-operated animals. A three fold increase in rolling flux was observed in the rejecting rats.

There was also a baseline leukocyte adhesion in the post-sinusoidal venules (1 cell/100 μm) of the non-operated animals (Figure 4.20). In the control transplant group, a threefold

increase in leukocyte adhesion was observed. Leukocyte adhesion in hepatic venules of rejected animals was further increased compared to the isograft controls.

4.4 Leukocyte recruitment after treatment with ICAM-1 mAb or antisense oligonucleotides

After six days of ICAM-1 antibody treatment, leukocyte rolling flux and adhesion on post-sinusoidal venules were both significantly decreased compared to the untreated rejection controls (Figures 4.19 and 4.20). Reduction was greater in adhesive cells which returned to the control isograft level. However, the ICAM-1 antibody could not completely block all adhesions in the rat post-sinusoidal venules.

Another group of rats received ICAM-1 antisense oligonucleotides for 6 days. Leukocyte rolling flux was not reduced (Figure 4.19). Adhesion was significantly blocked, but not to the baseline level (Figure 4.20).

4.5 ICAM-1 immunohistochemistry

ICAM-1 immunohistochemistry was performed in normal livers, rejected livers and rejected livers treated with antisense oligonucleotides on the sixth postoperative day. A weak background staining was observed in the normal hepatic sinusoidal endothelial cells (Figure 4.21). In the rejected livers, intense ICAM-1 staining was observed in the hepatocytes, sinusoidal lining and endothelial linings of portal vein and post-sinusoidal venules, more

prominent in areas with heavy leukocytic infiltration (Figure 4.22). Weak staining was found in sinusoidal endothelium of rejected livers treated with ICAM-1 antisense oligonucleotides (Figure 4.23), suggesting that the antisense blocked ICAM-1 expression to a certain degree but incompletely. However, portal venules and post-sinusoidal venules did not express ICAM-1 in these antisense-treated livers.

4.6 Survival after treatment

Survival curves were presented in Figure 4.18. Rats receiving the ICAM-1 antibody had a short average survival of only 11.8 ± 0.9 days. This is significantly shorter ($p<0.05$) than the untreated rejection group (25.2 ± 2.7 days). Rats receiving ICAM-1 antisense oligonucleotides have prolonged survival up to 42.3 ± 1.2 days, significantly longer than the untreated rejection controls ($p<0.05$).

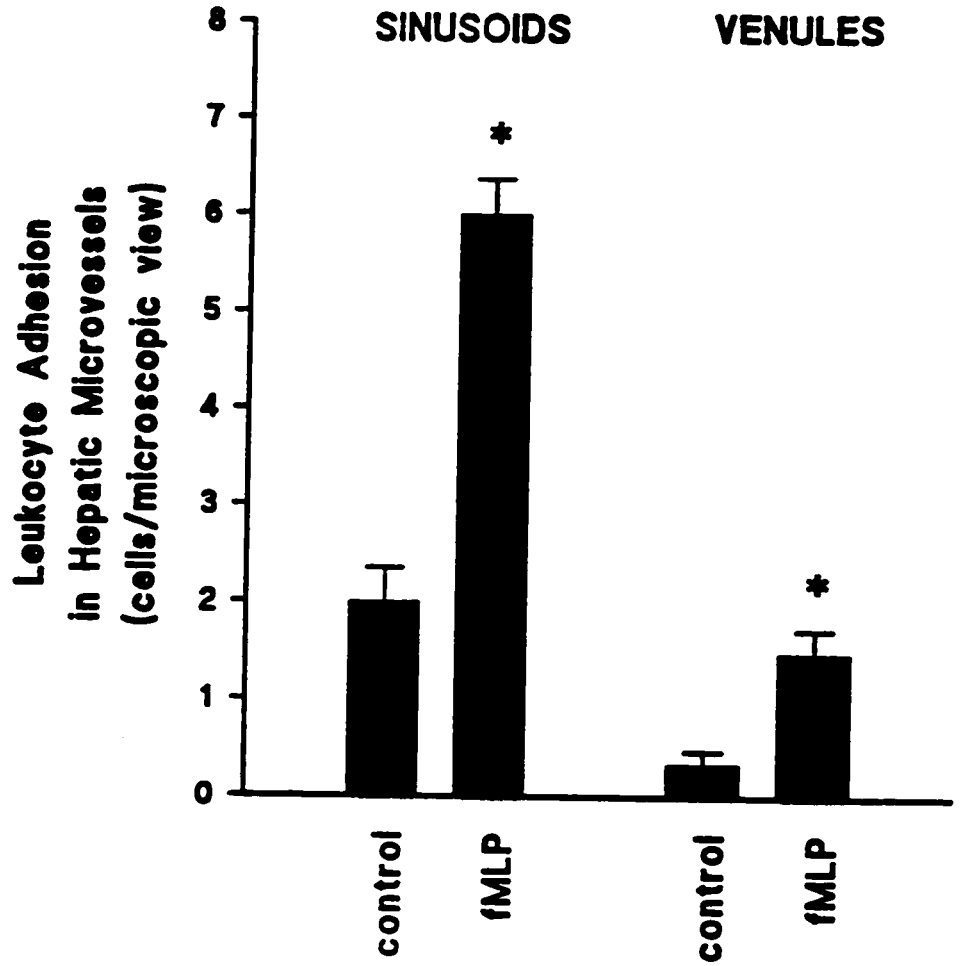


Figure 4.1 Leukocyte adhesion in mouse sinusoids (n=4) & hepatic venules (n=4) superfused with fMLP. Control preparations were superfused with bicarbonate buffered saline (n=4). Adhesion in the sinusoids accounted for 80% of total number of adherent leukocytes per field of view. Data represented as mean ± SEM. * = significantly different from control, $p<0.05$.

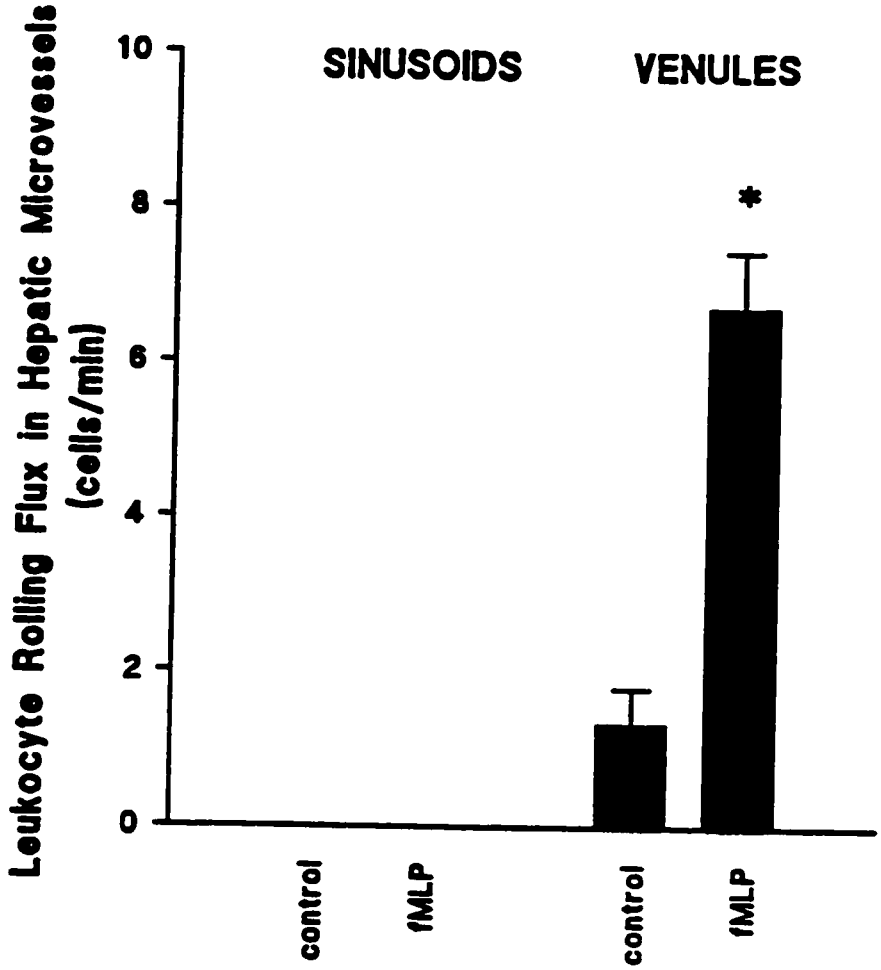


Figure 4.2 Leukocyte rolling flux in mouse sinusoids (n=4) & hepatic venules (n=4) superfused with fMLP or phosphate buffered saline (control, n=4). Rolling is observed in the hepatic venules but not in the sinusoids. Data represented as mean ± SEM. * = significantly different from control, $p<0.05$.

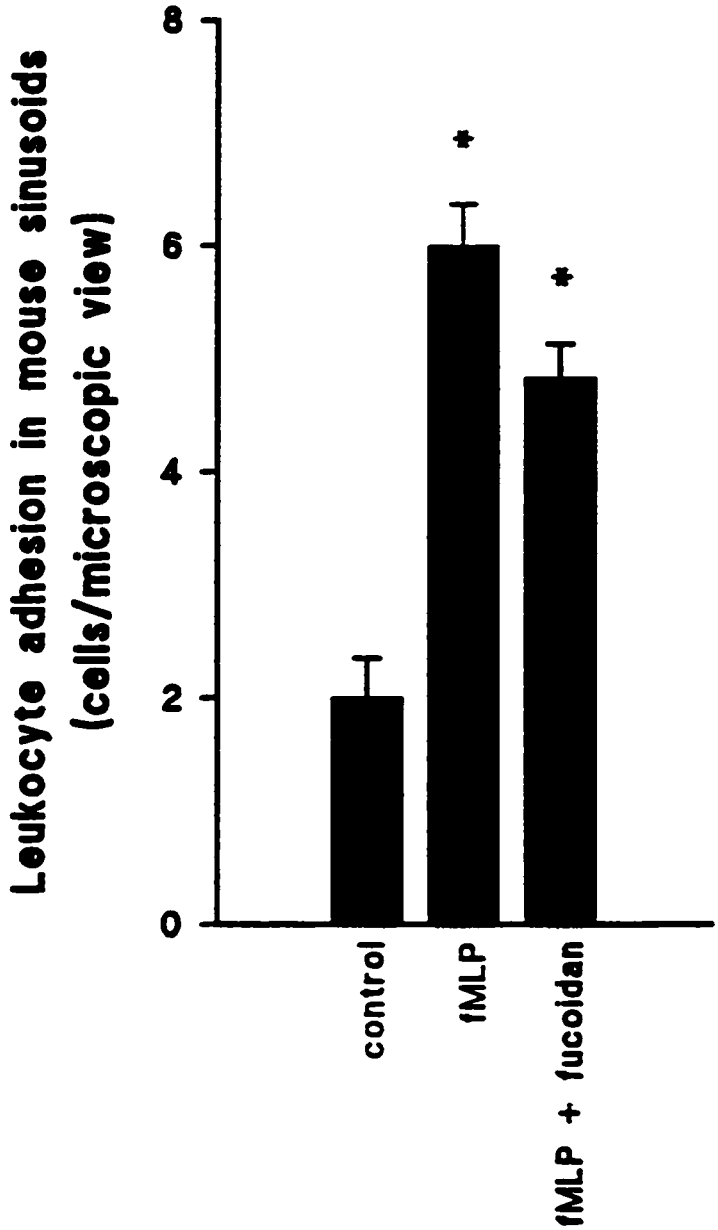


Figure 4.3 Effect of fucoidan on fMLP-induced leukocyte adhesion in mouse sinusoids (n=4). Immunoneutralization of selectins by fucoidan failed to inhibit adhesion in the sinusoids. Data represented as mean ± SEM. * = significantly different from control, $p<0.05$.

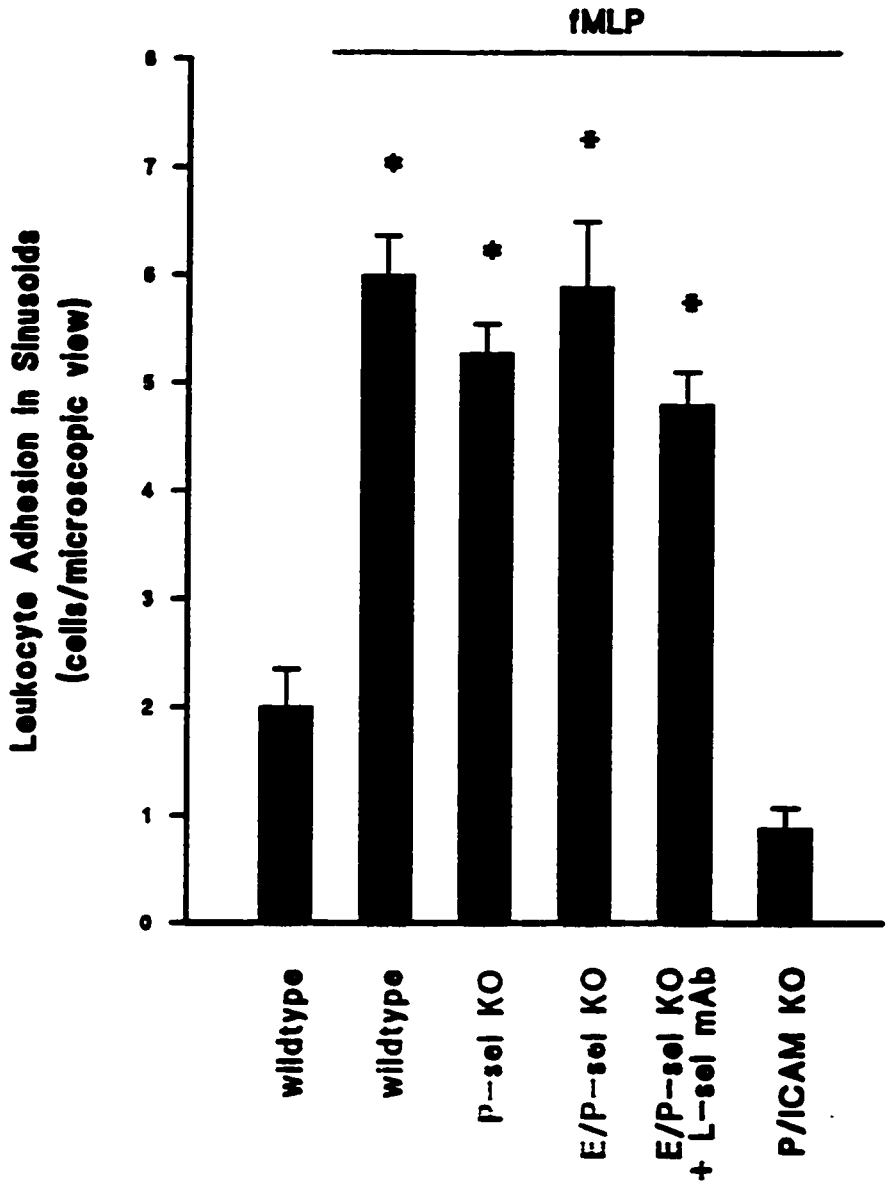


Figure 4.4 Leukocyte adhesion in sinusoids of knockout mice superfused with fMLP. Adhesion occurred effectively in P-selectin knockouts (n=4), E/P-selectin knockouts (n=4) as well as E/P-selectin knockouts pre-treated with an antibody against L-selectin (Mel-14) (n=3). Sinusoidal leukocyte adhesion returned to baseline levels in the P/ICAM-1 knockouts (n=3). Data represented as mean ± SEM. * = significantly different from control, $p<0.05$.

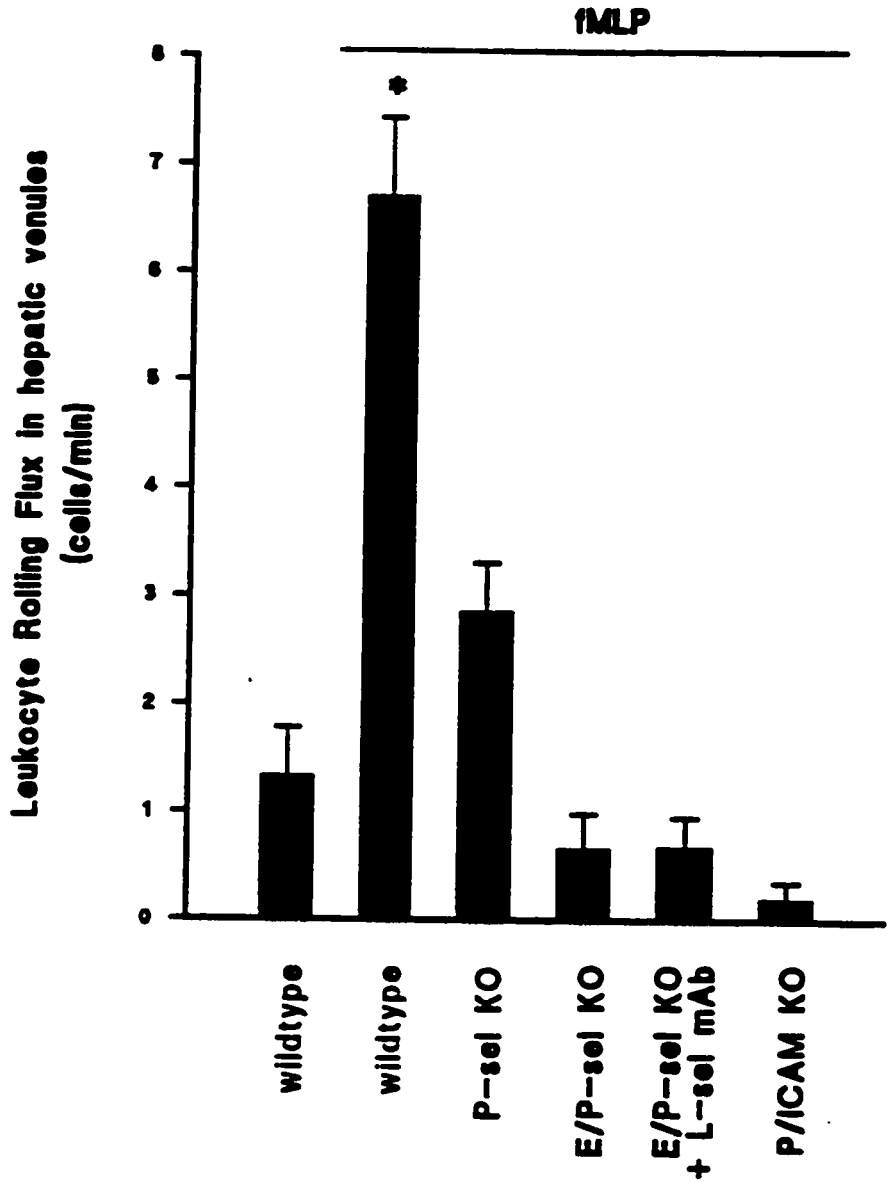


Figure 4.5 Leukocyte rolling flux in hepatic venules of knockout mice superfused with fMLP. Data represented as mean ± SEM. * = significantly different from control, $p<0.05$.

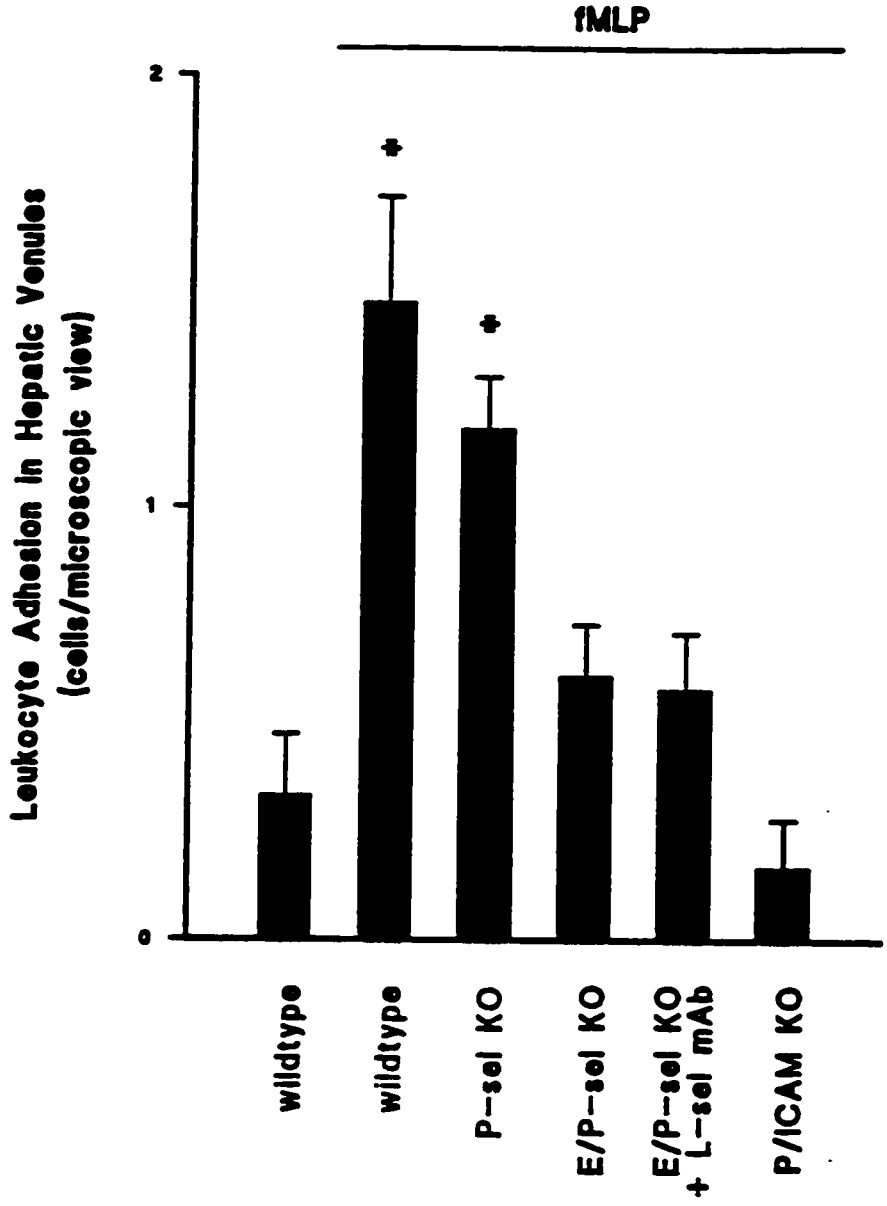


Figure 4.6 Leukocyte adhesion in hepatic venules of knockout mice superfused with fMLP. Data represented as mean ± SEM. * = significantly different from control, $p<0.05$.

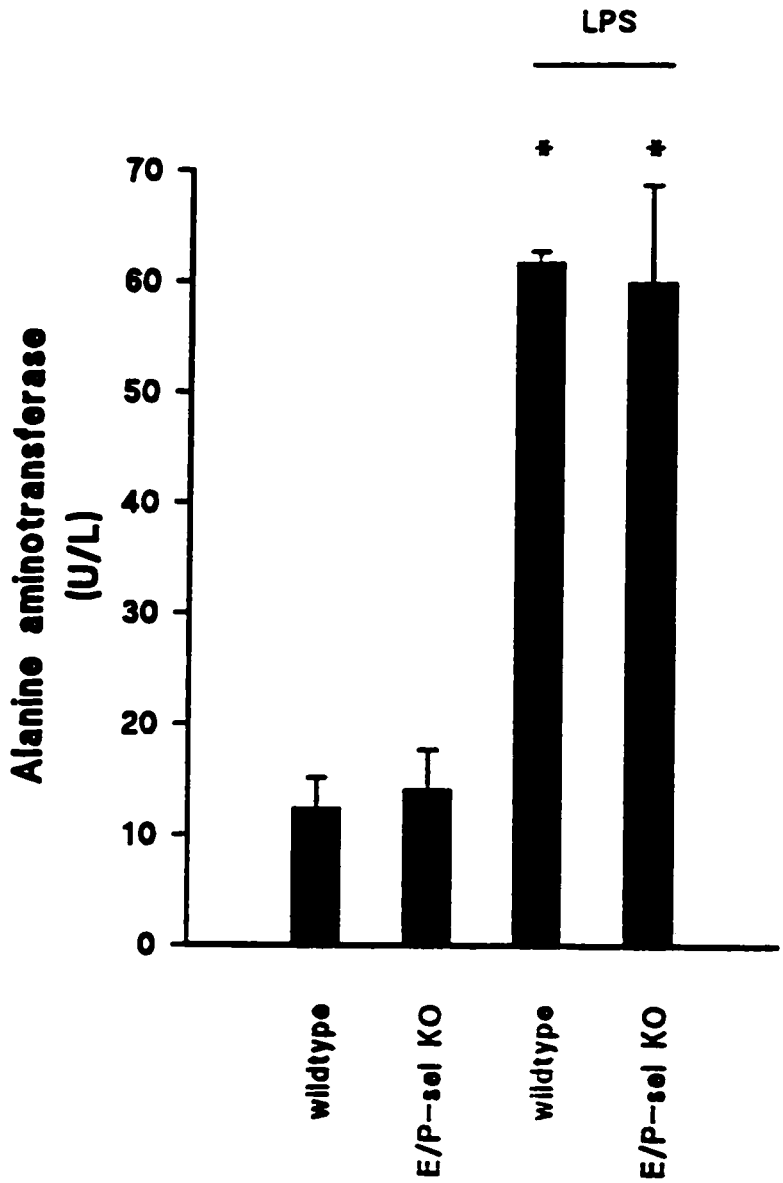


Figure 4.7 Serum alanine aminotransferase levels in LPS treated wildtype (n=3) & E/P-selectin deficient mice (n=3). Blood was taken from the animals 4 hours after intraperitoneal administration of LPS. Values represented as mean ± SEM. * = significantly different from untreated controls, $p<0.05$.

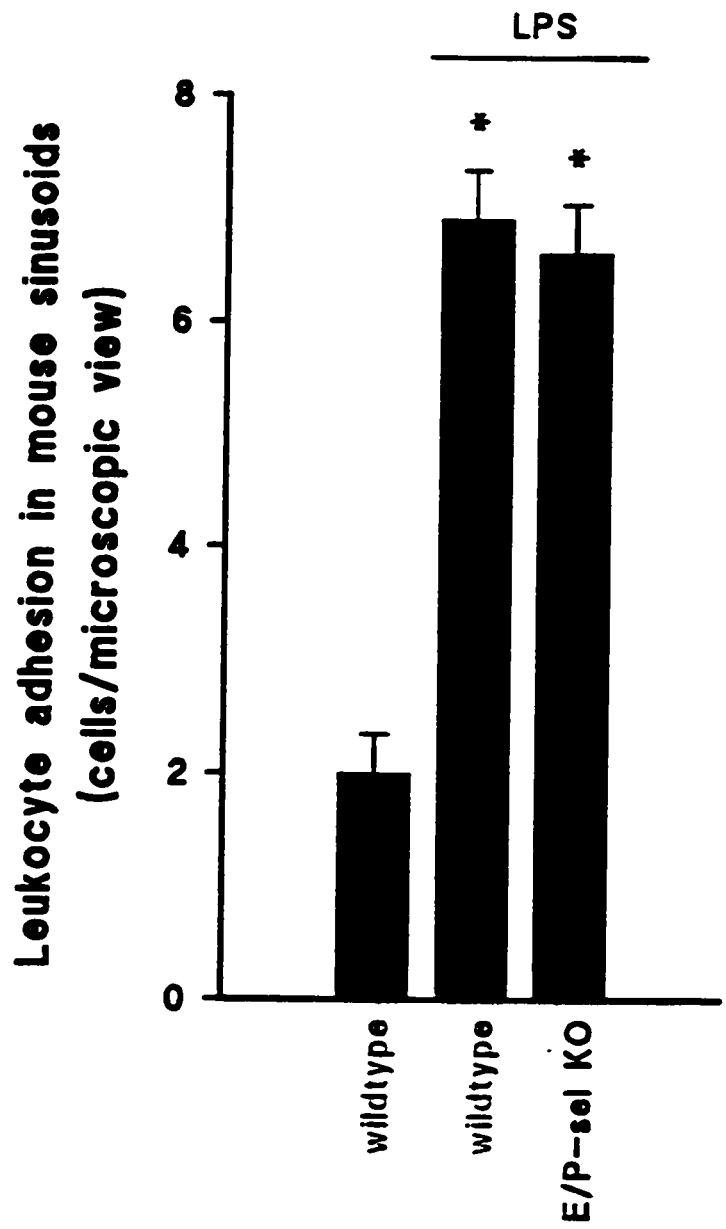


Figure 4.8 Leukocyte adhesion in sinusoids of wildtype (n=4) and E/P-selectin deficient mice (n=4) four hours after LPS adminstration. Data represented as mean ± SEM. There was no difference in sinusoidal leukocyte adhesion between the E/P-selectin deficient and the wildtype animals in response to LPS. * = significantly different from control, $p<0.05$.

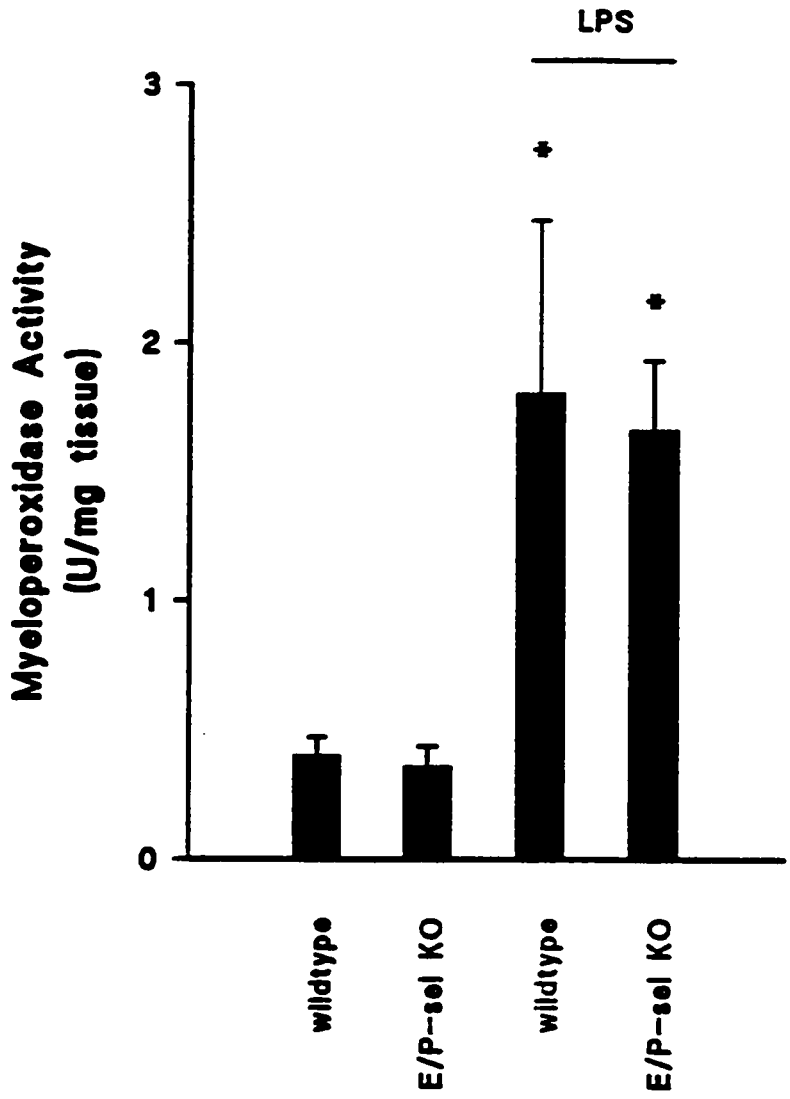


Figure 4.9 Liver myeloperoxidase activity in LPS treated widetype (n=4) & E/P-selectin deficient mice (n=4). Liver samples were taken 4 hours after intraperitoneal administration of LPS. Data represented as mean ± SEM. * = significantly different from control, $p<0.05$.

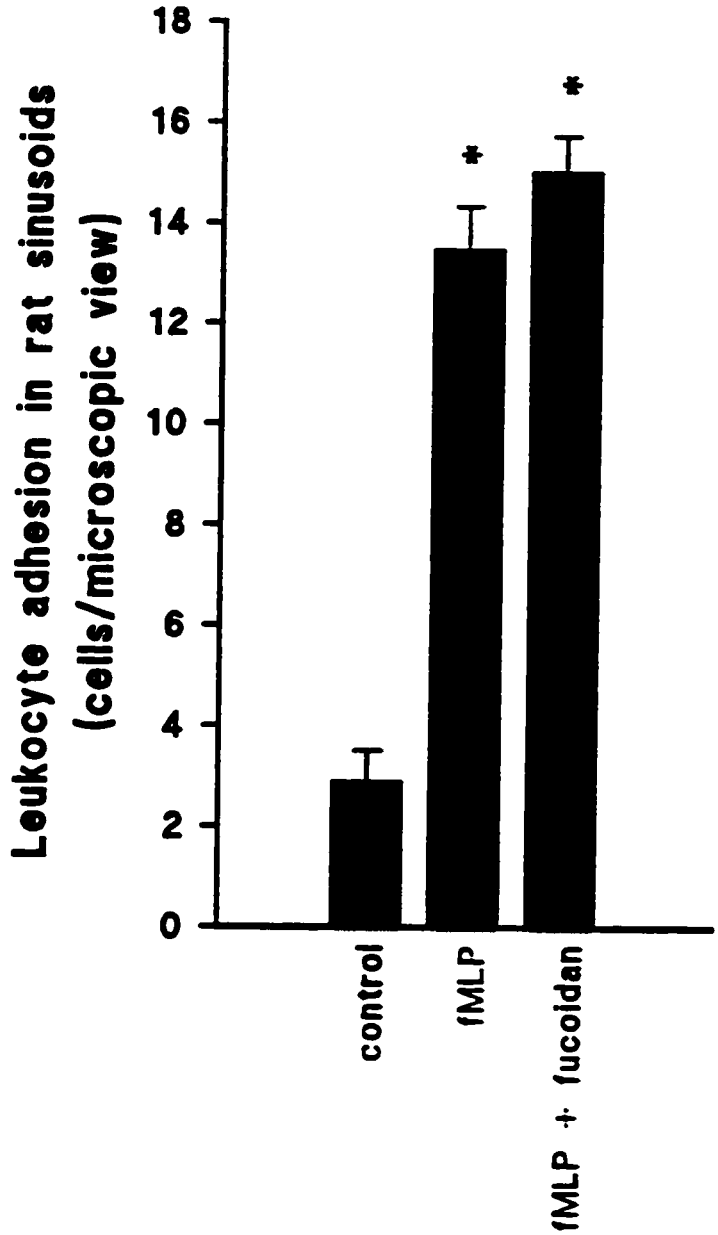


Figure 4.10 Effect of fucoidan on fMLP-induced leukocyte adhesion in rat sinusoids (n=6 per group). Immunoneutralization of selectins by fucoidan failed to inhibit adhesion in the sinusoids. Data represented as mean ± SEM. * = significantly different from control, $p<0.05$.

Figure 4.11 H & E stain of control arterialized liver graft in the rat (Lewis to Lewis). The hepatic architecture is normal. Red blood cells are present inside the hepatic arteriole (A) lumen. P = portal venule. C = central venule.


P

Figure 4.12 H & E stain of liver graft during early rejection (day 10) at low magnification. Liver of a Lewis rat is transplanted into a Brown Norway rat. The lobular architecture is moderately distorted. Inflammatory infiltrate is seen in portal spaces with lobular expansion. The majority of hepatocytes are still viable.

Figure 4.13 H & E stain of Lewis to Brown Norway liver graft during early rejection (day 10) at high magnification. Mononuclear infiltrates are seen in Zone 1. Normal bile ducts cannot be visualized because of the damage secondary to rejection.

Figure 4.14 H & E stain of Lewis to Brown Norway liver graft during late rejection (day 20) at low magnification. Marked edema (E) is present in the centre of the micrograph which corresponds to destroyed portal space.


E

Figure 4.15 H & E stain of Lewis to Brown Norway liver graft during late rejection (day 20) at high magnification. Normal bile ducts are not present but heavy infiltration of mononuclear inflammatory cells is seen.

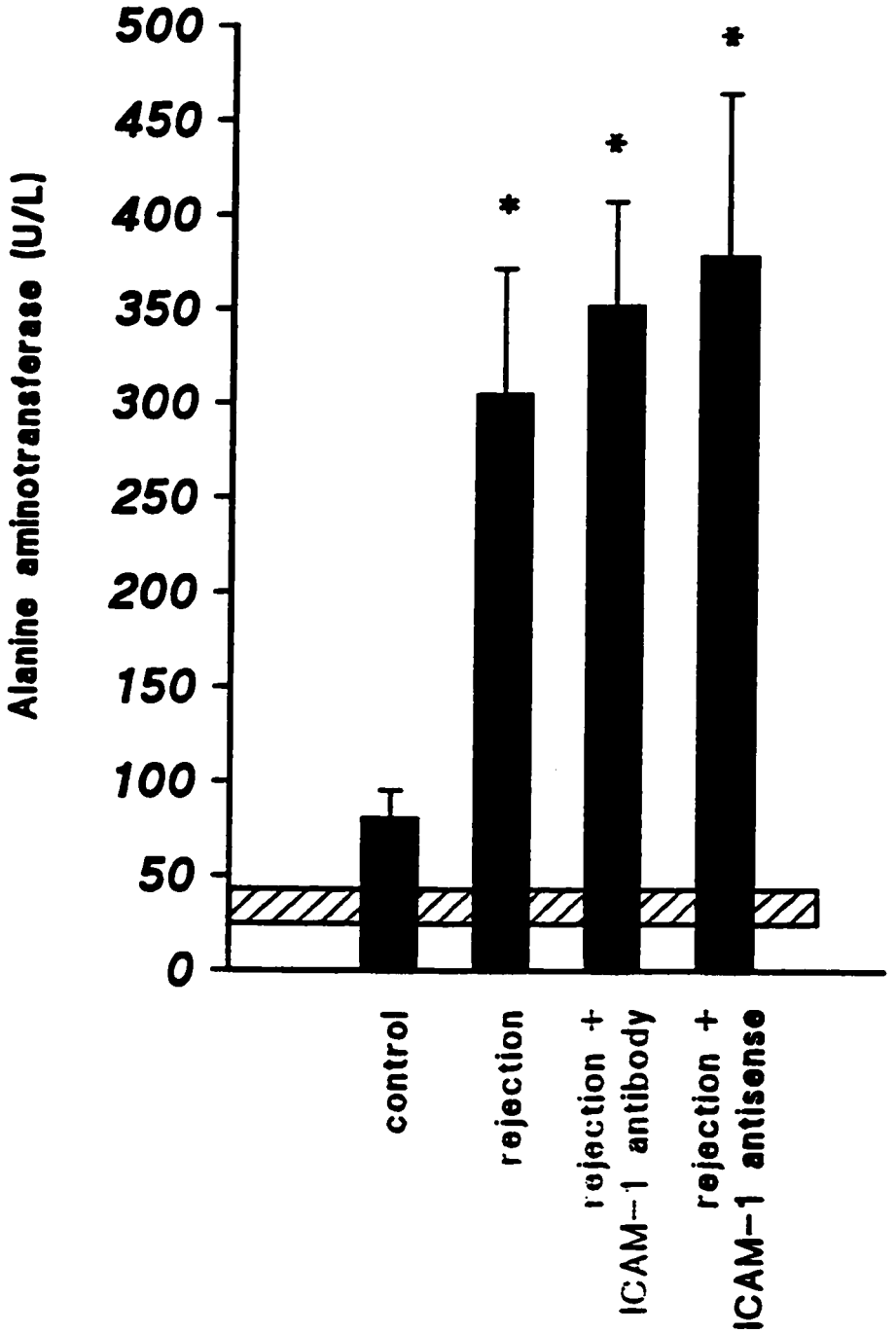


Figure 4.16 Serum alanine aminotransferase levels in 4 groups of animals 6 days after liver transplantation (n=6 per group). Data expressed as mean ± SEM. Shaded area represents the normal range in rats. * = significantly different from isograft control, $p<0.05$.

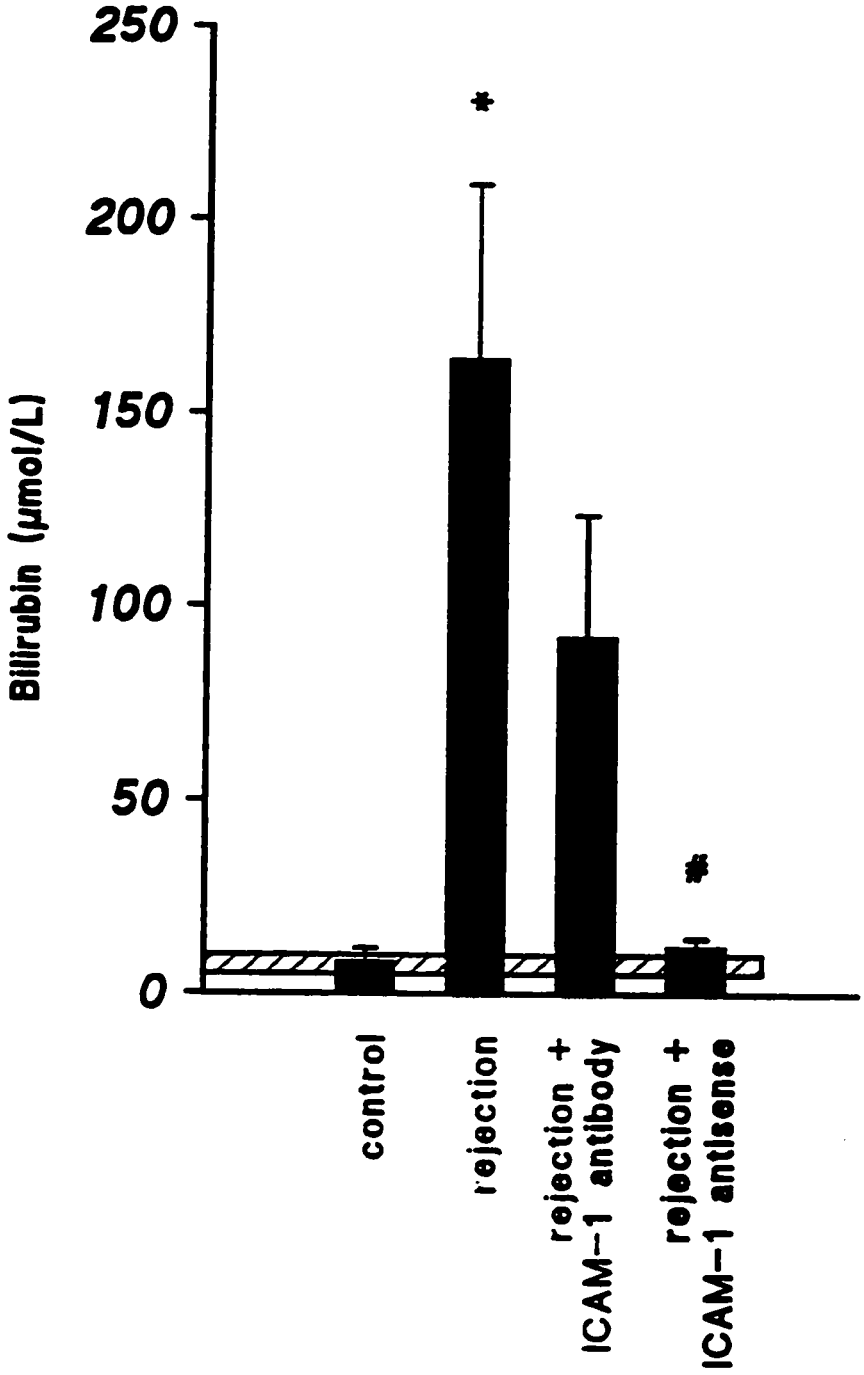


Figure 4.17 Serum bilirubin levels in 4 groups of animals 6 days after liver transplantation (n=5 per group). Data expressed as mean ± SEM. Shaded area represents the normal range in rats. * = significantly different from isograft control, $p<0.05$. # = significantly different from untreated rejection control, $p<0.05$.

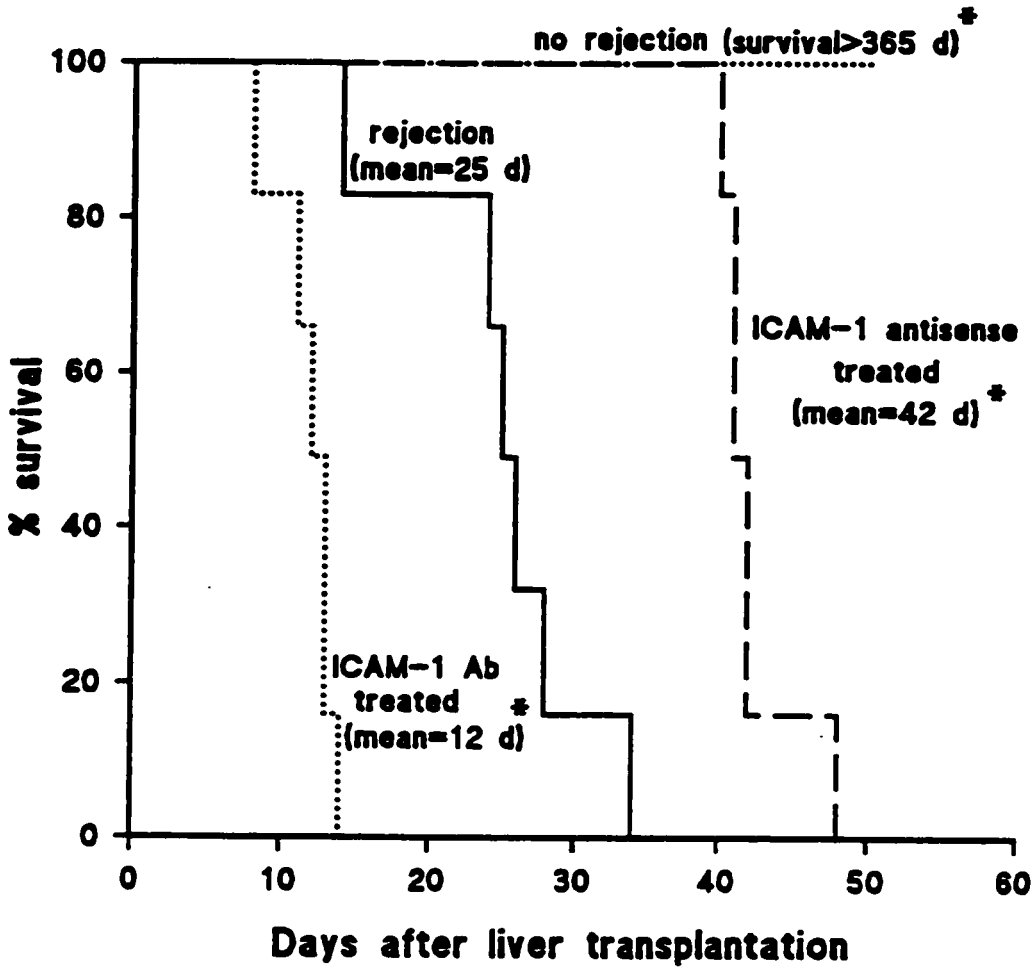


Figure 4.18 Graft survival in the 4 groups of transplanted animals. * = significantly different from rejection group, $p<0.05$. (n=6 per group)

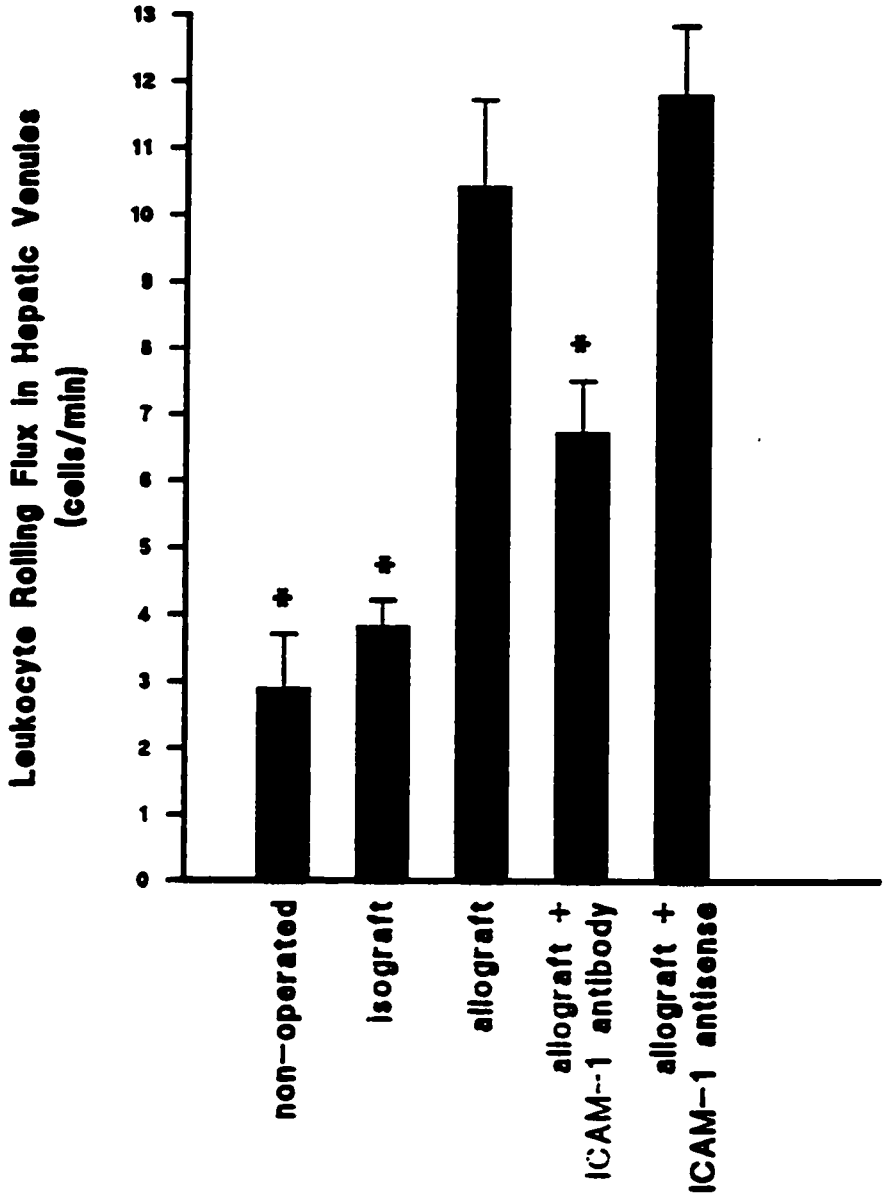


Figure 4.19 Leukocyte rolling flux in transplanted groups with/without treatment with anti-ICAM-1 antibody or ICAM-1 antisense oligonucleotides (n=5 per group). Data represented as mean ± SEM. * = significantly different from allograft, $p<0.05$.

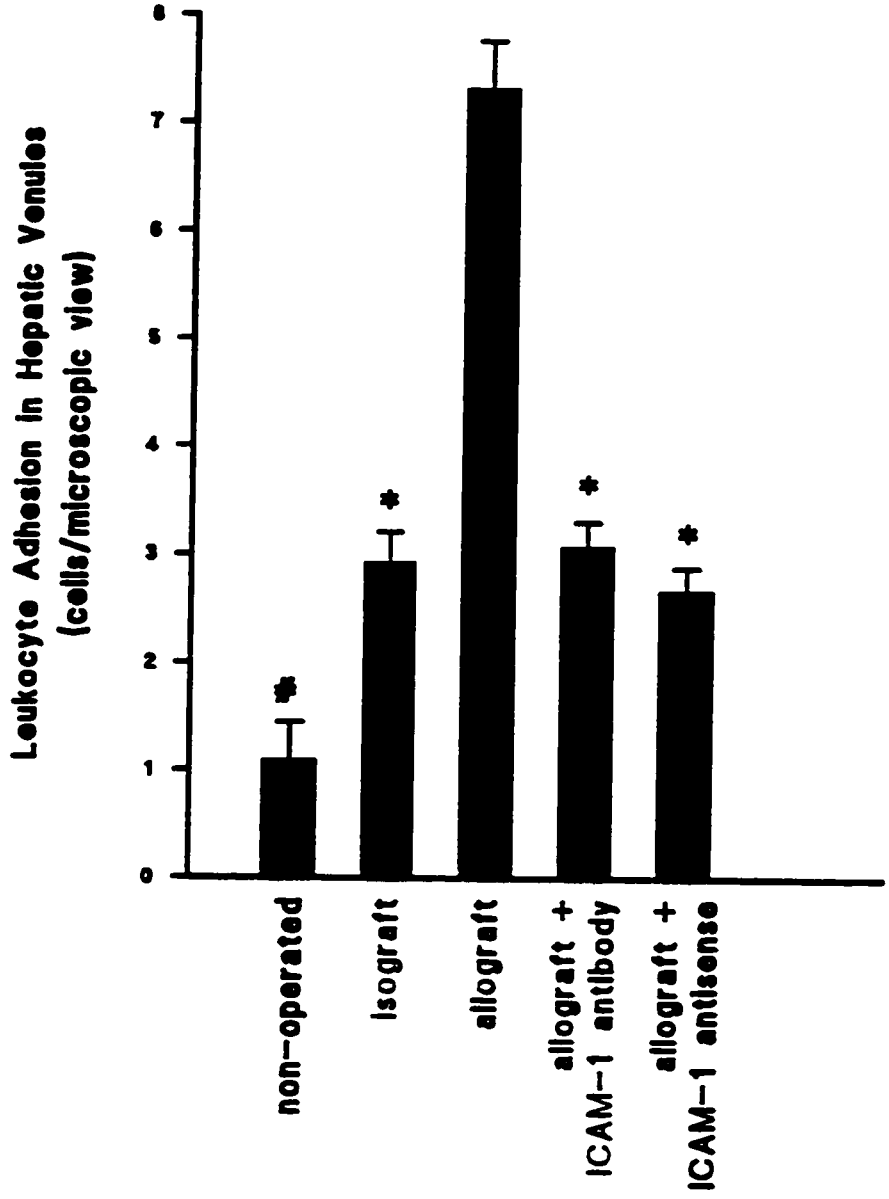


Figure 4.20 Leukocyte adhesion in transplanted groups with/without treatment with anti-ICAM-1 antibody or ICAM-1 antisense oligonucleotides (n=5 per group). Data represented as mean ± SEM. * = significantly different from allograft, $p<0.05$. # = significantly different from isograft control, $p<0.05$.

Figure 4.21 Normal liver tissue stained for ICAM-1. Diffuse staining was observed in sinusoidal lining cells. Hepatocytes were negative for ICAM-1.

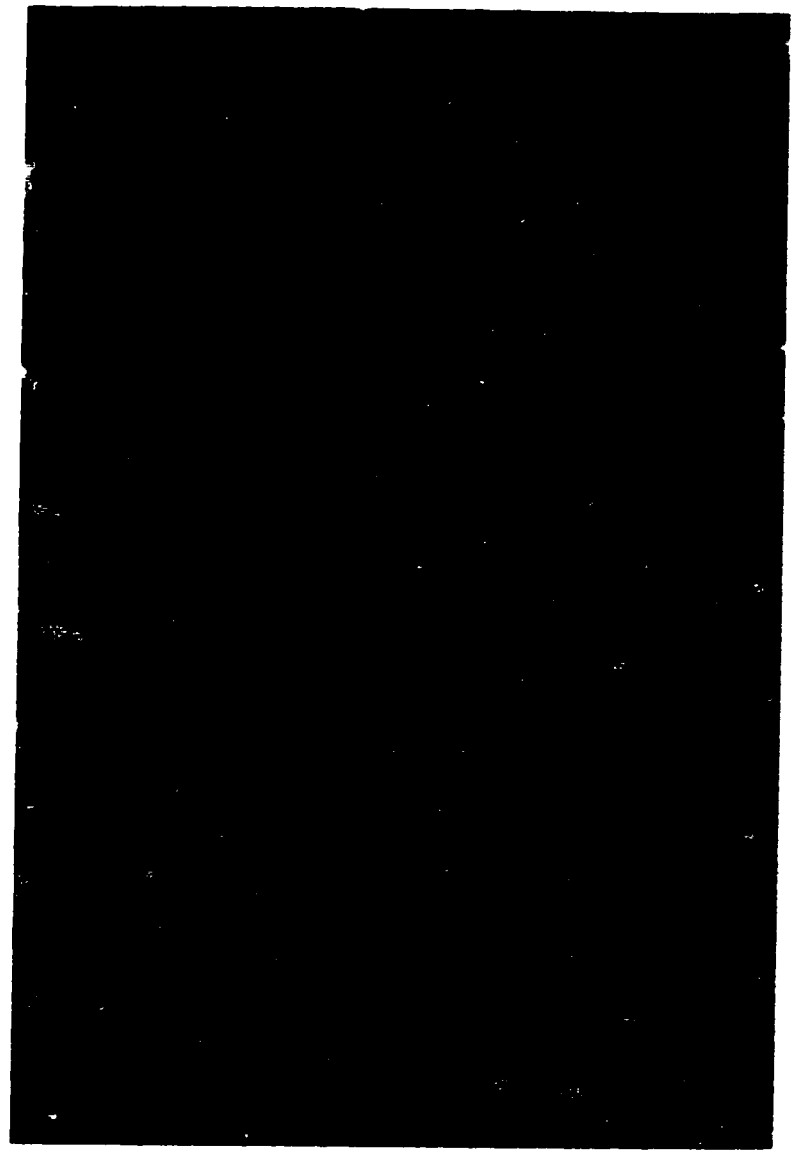
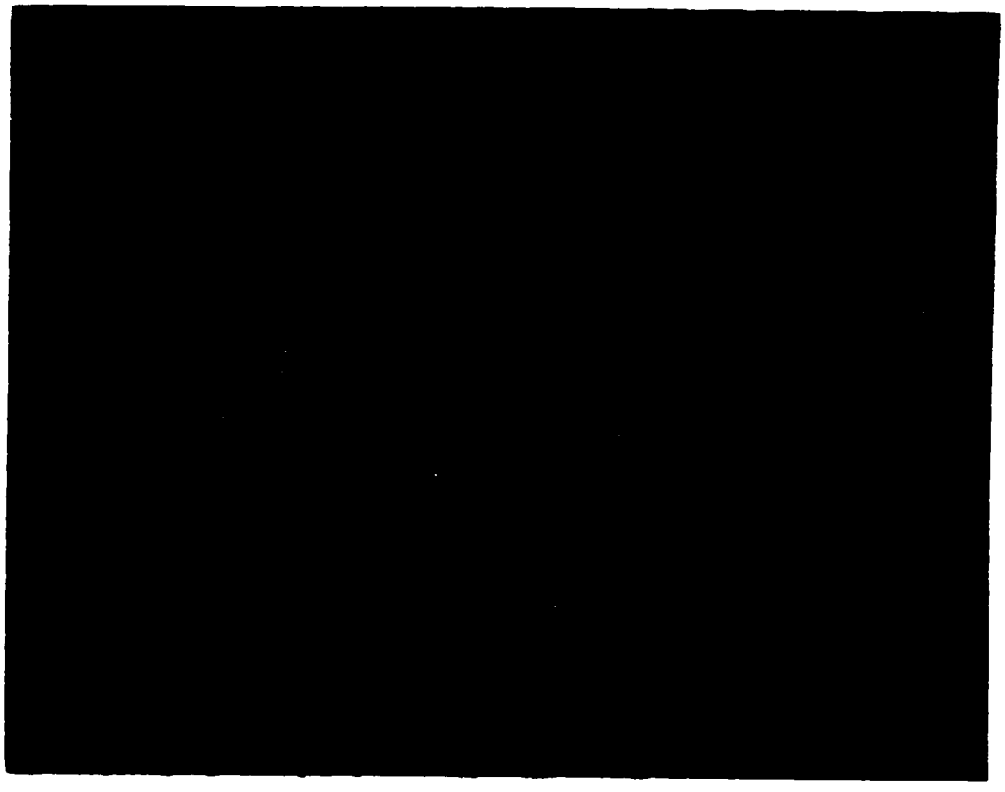


Figure 4.22 ICAM-1 expression in livers undergoing allograft rejection (upper & lower panels). Positive staining was observed in hepatocytes, venular endothelium (V) and bile duct epithelium (B). Leukocytes (L) were seen adhering onto the vessel wall.

A



B

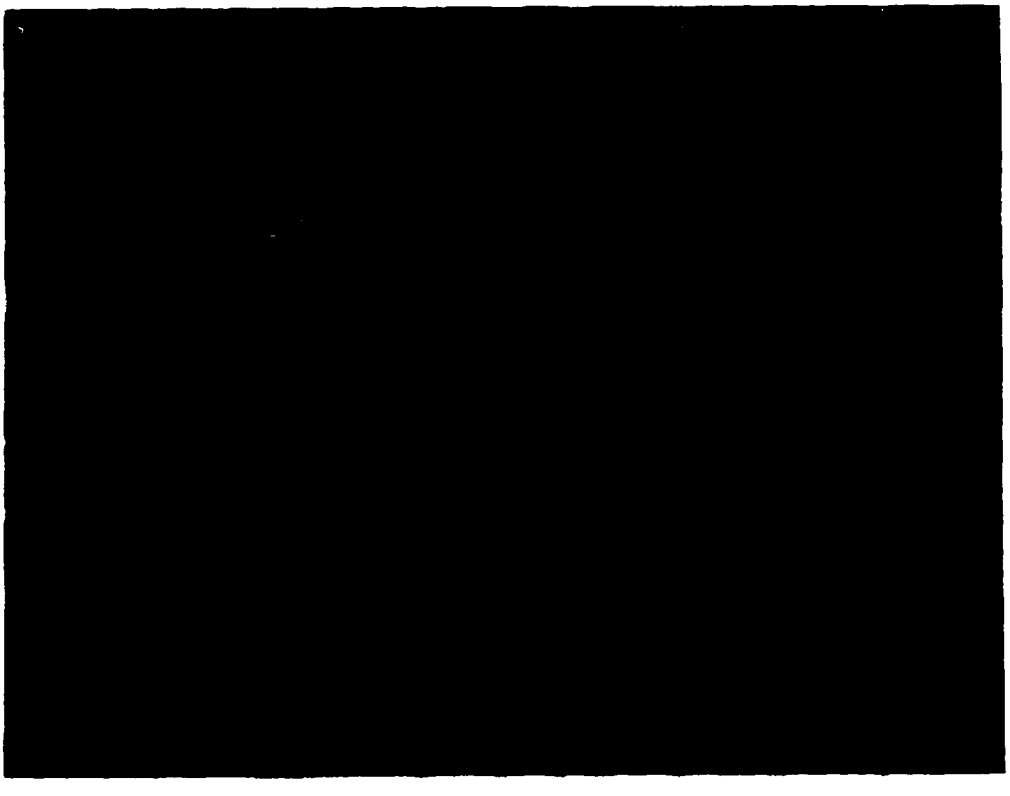
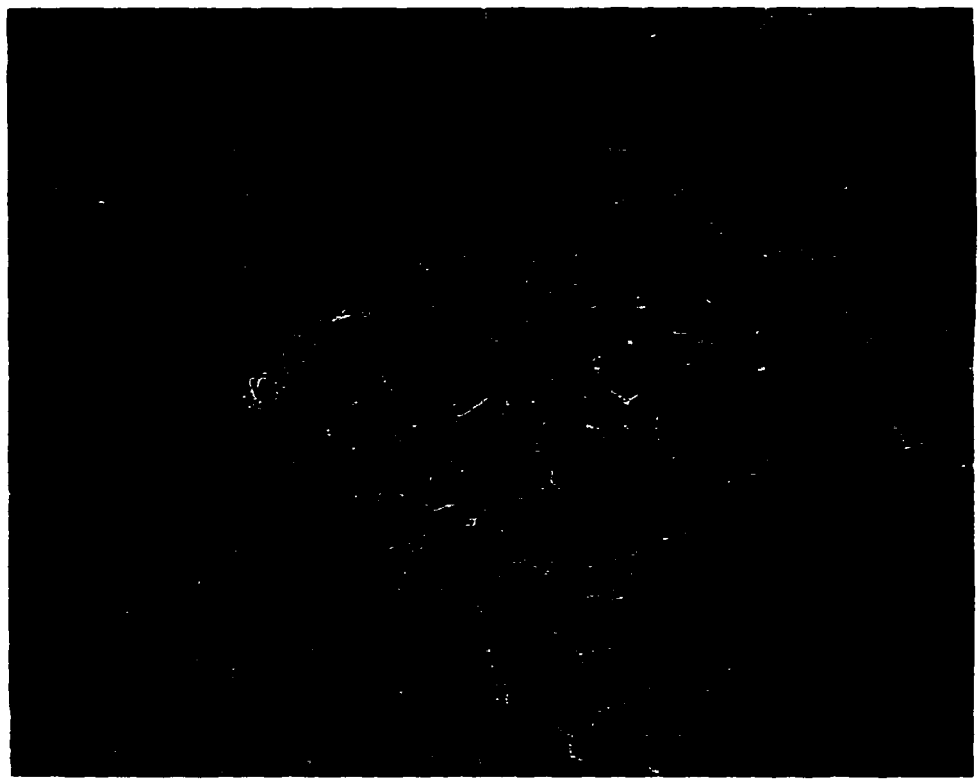


Figure 4.23 ICAM-1 expression in rejecting livers treated with ICAM-1 antisense oligonucleotides for 6 days. Weak staining on sinusoidal linings was observed (upper panel). Lower panel revealed a vein (V) with negative endothelial staining.

A

B



CHAPTER FIVE: DISCUSSION

1. A minimal role for the selectins in leukocyte recruitment into the inflamed hepatic microvasculature

The liver has a distinctive dual blood supply, with 70-75% of blood contributed by the portal vein and 20-25% by the hepatic artery. The portal vein also controls the total hepatic blood flow through intrinsic and extrinsic mechanisms. In the rat, occlusion of the hepatic artery does not affect the systemic or splanchnic circulation (Wong *et al.*, unpublished data). In fact, the hepatic circulation, to a great extent, is a venous driven vascular bed. Under this low shear condition, the hepatic capillary network (sinusoids) may behave differently from microvasculature of other organs in terms of trapping leukocytes from the bloodstream. Direct visualization of leukocyte behaviour within inflamed vessels of translucent tissues such as the mesentery (Lindbom *et al.*, 1992; Kubes & Kanwar, 1994; Kubes *et al.*, 1995) or cremaster (Ley *et al.*, 1995) has revealed that leukocytes tether to the endothelial lining and roll along the length of the venule before firmly adhering to the vasculature. These experiments performed on 20-40 μm postcapillary venules have demonstrated that the rolling event is an important prerequisite for adhesion and migration into inflamed tissues. This has produced a two-step paradigm for leukocyte recruitment: leukocyte rolling via selectins being the initial step, followed by firm adhesion mediated by β_2 integrins (von Andrian *et al.*, 1991; Ley *et al.*, 1991; Lindbom *et al.*, 1992). In the venous driven low shear hepatic microvasculature, leukocyte

may behave differently in the recruitment dynamics. We hypothesized that selectins are not essential for leukocyte recruitment. In fact, adhesion was observed in both sinusoids and post-sinusoidal venules, but rolling was not seen in the sinusoids of hamsters and rats (Menger *et al.*, 1991; Vollmar *et al.*, 1995). Using animal models mimicking acute hepatic inflammation, a minimal role for the selectins in leukocyte recruitment into the inflamed liver microvasculature was demonstrated.

Previous work has clearly pointed out that chemotactic factors (platelet-activating factor, leukotriene B_4, fMLP) as well as cytokines or endotoxins will induce leukocyte infiltration into postcapillary venules (von Andrian *et al.*, 1991; Lindbom *et al.*, 1992; Bienvenu *et al.*, 1992, 1993; Asako *et al.*, 1993; Sriramarao *et al.*, 1994). Intravital microscopy reveals that the mouse cremaster microvasculature displays some rolling but little or no adhesion under control conditions. Exposure of the mouse cremaster to the chemotactic factor fMLP induced a rapid increase in leukocyte adhesion in postcapillary venules of wildtype mice (Wong *et al.*, 1997). It is noteworthy that adhesion only occurred in postcapillary venules and not in other microvascular compartments such as the capillaries and arterioles. In this cremaster model, mice lacking P-selectin had absolutely no rolling or adhesion in response to fMLP. This demonstrated clearly that rolling via the selectin is essential for subsequent adhesion in cremaster muscle vasculature in response to an inflammatory stimulus.

Direct visualization of the mouse liver using intravital fluorescence microscopy with epi-illumination technique revealed an extensive capillary network of sinusoids draining into the

post-sinusoidal venules with low flow rates. We chose to study the centrilobular (Zone 3) sinusoids with post-sinusoidal venules instead of the periportal region (Zone 1) because: (a) the centrilobular zone with the post-sinusoidal venules is more analogous to the postcapillary venules, where leukocyte-endothelial interactions take place preferentially (Menger *et al.*, 1991); (b) immunohistological staining revealed that adhesion molecules tend to express in Zone 3 (Smith & Thomas, 1990); and (c) the complex architectural interconnecting network of portal venules and hepatic arterioles in Zone 1 (afferent zone) can be misleading. Similar to the intravital findings of Menger's group (1991) on the rat, we did not see leukocyte rolling within the sinusoids. A few white cells within the sinusoids were permanently adhesive to the endothelial wall under control conditions. The question of whether this was a physiological adhesion or preparation-induced was not resolved. In response to fMLP, leukocytes adhesion occurred in both the sinusoids and post-sinusoidal venules. Adhesion in the sinusoids accounted for 80% of total number of adherent cells within the liver microcirculation per view of field. This is not surprising since the main bulk of liver parenchyma is comprised of sinusoids. Rolling leukocyte, defined as those cells moving slower than red blood cells with detectable rotational motion could only be seen in the post-sinusoidal venules and not in the sinusoids *per se*, even after administration of fMLP. This finding is in agreement with those of Menger's (1991) and Vollmar's (1995) groups. With the assumption that selectins mediate leukocyte rolling, the lack of expression and inducibility of selectins on the liver sinusoids may explain the absence of leukocyte rolling *in vivo* within this microvascular compartment.

Although these data would suggest that 80% of leukocytes in the inflamed liver microvasculature adhere to the endothelium independent of a rolling motion, it is still possible that selectins may have a functional role in the capturing or tethering of leukocytes within the sinusoidal compartment. With the use of genetic knockout mice, we showed that leukocyte adhesion in sinusoids occurred as effectively in P-selectin deficient animals as wildtype littermates in response to fMLP. Since there is good evidence of selectins overlapping in functions particularly in the single adhesion molecule knockout mice (Labow *et al.*, 1994; Bullard *et al.*, 1996;), animals deficient in both E- and P-selectin were tested. These double mutants still had leukocyte adhesion in sinusoidal vessels in response to the chemoattractant. This is in sharp contrast to previous work that demonstrated that this double deletion prevents recruitment of leukocytes into different tissues. Bullard's *et al.* (1996) showed that in these E- and P-selectin double mutant mice, leukocyte rolling was completely absent in venules of cremaster muscle at all time points. Another remarkable finding was that these double knockouts showed a complete absence of neutrophil emigration in the *Streptococcus pneumoniae*-induced peritonitis model. Leukocyte rolling in inflamed mesenteric venules was also severely impaired in these animals as demonstrated by Frenette's *et al.*(1996). For completeness, a separate group of E/P-knockout mice received an L-selectin antibody (MEL-14) at concentrations that reduced leukocyte rolling in other vascular beds (Ley *et al.*,1995); and we observed that leukocyte adhesion was still not affected in the sinusoidal vessels. Therefore, we showed that leukocyte adhesion in the inflamed sinusoids was independent of all

3 selectins.

As the sinusoids are small vessels with discontinuous endothelial lining, leukocyte adhesion may be entirely independent of endothelial adhesion molecules. In the lungs, it has been shown that neutrophil margination into pulmonary capillaries does not require E- and P-selectins and that streptococcal pneumonia induces an E- and P-selectin-independent increase in white cell interactions with noncapillary endothelium (Mizerd *et al.*, 1996). Hemodynamic forces and biomechanical properties of neutrophils and capillary walls were thought to be involved in this leukocyte-endothelial interactions. To test this possibility in the liver sinusoids, leukocyte adhesion in P-selectin-deficient animals was compared to P-selectin knockouts that also lacked ICAM-1. Our data showed that ICAM-1 is indeed essential for adhesion, and that mechanical trapping within these microvessels (as has been proposed for the lung) is clearly not the mechanism by which cells adhere to the sinusoids in response to fMLP.

The paradigm on post-sinusoidal hepatic venules was different from that of sinusoids. These vessels are bigger (30-40 μm in diameter) than the sinusoids. The increase in leukocyte rolling and adhesion in these venules in response to fMLP was modest and returned to control levels in mice deficient in both E- and P-selectins regardless of whether the L-selectin antibody was added. Interestingly, there was some residual rolling in P-selectin deficient venules which permitted leukocytes to adhere in these vessels. This low level of baseline rolling may be related to constitutive E-selectin expression perhaps as a result of continuous translocation of noxious stimuli from the intestinal lumen. On rare occasions a cell could be seen rolling and

then adhering in the post-sinusoidal venules of all mice including the E/P-selectin knockout animals. This might reflect the low shear forces in these vessels. Indeed, Gaboury and Kubes (1994) have demonstrated that rolling and subsequent adhesion were entirely inhibited in rat mesenteric postcapillary venules by the selectin-binding carbohydrate fucoidan. If shear was lowered by 50%, leukocytes began to roll and adhere despite the presence of fucoidan. This increase in rolling and adhesion was entirely dependent on β_2-integrins and may also underlie the adhesion in liver post-sinusoidal venules of the selectin-deficient animals. Furthermore, we also showed that leukocyte rolling and adhesion were entirely inhibited within fMLP-treated post-sinusoidal venules lacking P-selectin and ICAM-1. This observation is consistent with the previous work by Kunkel *et al.* (1996), showing less rolling in P-selectin/ICAM-1 double mutant mice than in P-selectin deficient mice, and the hypothesis that at lower shear forces, leukocyte rolling and adhesion can occur via non-selectin pathways.

A criticism often levied against the use of gene-deleted mice is that there may be redundancy in these animals. Certainly, one possibility to explain the E/P selectin data in the microvasculature is to invoke the induction of another as yet unidentified adhesion molecule. To test the possibility that selectins were important in the hepatic microcirculation of wildtype mice, some animals received the selectin-binding carbohydrate fucoidan which essentially abolished rolling in the mouse system. The results revealed that immunoneutralization of selectins failed to inhibit leukocyte adhesion in the sinusoids. As this response is identical to that observed in the selectin-deficient animals, it suggests that selectin-independent adhesion is

manifested in all mice and not just the mutant animals. The same conclusion can be drawn in the rat model, as leukocyte adhesion within the rat liver sinusoids was not affected by the selectin-binding carbohydrate.

To confirm that this selectin-independent adhesion in sinusoids was not restricted to fMLP, we tested another stimulus, lipopolysaccharide (LPS), that causes synthesis of adhesion molecules including the selectins, and proved to be a mediator of neutrophil-dependent hepatic injury (Holman & Saba, 1988). Our data revealed that regardless of the inflammatory stimulus, selectins were not necessary for adhesion in the liver sinusoids inasmuch as LPS induced significant adhesion in the E/P-selectin deficient liver microvasculature. A three- to four-fold increase in myeloperoxidase activity and liver enzyme alanine aminotransferase level noted in the LPS treated wildtype and E/P-selectin knockout mice suggested that selectins were neither a prerequisite for leukocyte infiltration into the liver microvasculature nor liver parenchymal injury.

It is intriguing that in the present study, the same number of adhering cells in normal animals and those deficient in P-selectin and E/P-selectins was observed despite the fact that white cell circulating levels were 7-8 fold higher in the E/P-selectin knockouts compared to their wildtype counterparts. Although one could argue that this may support a role for selectins in this model, the argument presumes that the number of adherent cells will be proportional to the number of rolling and circulating leukocytes. Kubes *et al.* (1995) have previously demonstrated that only a fraction of rolling cells adhere, i.e. one must inhibit 90% of

rolling cells to impact on the magnitude of adhesion. In other words, it is the number of "adhesive sites" that regulates the magnitude of adherent cells; and if these sites are saturated, larger rolling cell count will not increase adhesion. An identical relationship may exist for circulating versus adherent cells. Inflamed vessels recruit only a fraction of circulating pool and a very significant number of circulating leukocytes (95-100%) has to be depleted before adhesion and infiltration are affected (Kvietys *et al.*, 1990).

The present study for the first time suggests that anti-selectin therapy with the aim of preventing leukocyte rolling and thereby reducing adhesion to endothelium is unlikely to impact upon the inflammatory process within the liver *per se*. In systemic inflammatory conditions such as trauma-induced shock leading to multi-organ failure, interrupting the inappropriate inf ammatory response with anti-selectin therapy may have minimal effect on hepatic inf ammation and damage. As the liver is a major site for cytokine production in shock, selectin-based therapy is unlikely to be useful in preventing systemic damage induced during shock when the liver is involved. However, anti-ICAM-1 therapy may be advantageous. The present study may also be relevant to designing therapies for liver injury associated with transplantation or various forms of hepatitis. On the other hand, the lack of effect of selectin-based therapy in the liver may be advantageous when targetting specific skin diseases or reperfusion injury to the intestine or skeletal muscle wherein impairment of leukocyte recruitment into the liver may be unnecessary or even detrimental.

2. The importance of ICAM-1 in leukocyte recruitment during chronic hepatic allograft rejection

Transplantation of the liver is now an accepted mode of therapy in end-stage and acute fulminant liver failure. It has proven to be clinically rewarding and often life-saving in terminal and irreversible situations in which no satisfactory medical therapy is available. A cornerstone in the development of liver transplantation in 1979 was the introduction of cyclosporin A as an immunosuppressive agent through inhibition T lymphocyte proliferation by blocking transcription of IL-2 mRNA. Other advances include surgical improvements, such as the use of venous bypass, portal vein grafts, intraoperative cell saver and development of alternative methods of graft arterialization (Maddrey & van Thiel, 1988). However, many aspects of hepatic transplantation physiology and pathophysiology remain poorly understood and a variety of animal models of transplantation has been extensively utilized for experimental studies. The rat orthotopic transplant model in particular has been widely used since the introduction of cuff technique for vascular anastomosis in 1979 by Kamada and Calne. By reducing the time required for portal vein cross-clamping, the success rate of the procedure improved dramatically (Zimmermann *et al.*, 1983). It is now a reliable means of investigating transplant related problems, such as graft rejection (Egawa *et al.*, 1995), ischemia/reperfusion injury (Jaeschke *et al.*, 1993), biliary secretion (Chan *et al.*, 1995) and for analyzing methods of organ preservation (Post *et al.*, 1993).

The rat is ideally suited for studying of transplant pathophysiology due to the availability of a variety of inbred strains with well defined MHC phenotypes. The Lewis to Brown Norway rat hepatic allograft combination used in the present study enables us to examine the pathogenesis of chronic rejection of the organ. The recipient hepatic histopathology, graft survival time, bilirubin level, liver function tests and postmortem findings all correlate well with the human "vanishing bile duct syndrome". However, the rat liver transplant model does not perfectly mimic the human situation in all aspects. Rats have a circulating white cell count predominant in lymphocytes, while neutrophils predominate in man. Rats can survive a nonarterialized liver graft while arterialization is mandatory in human transplants; hepatic artery thrombosis after human liver transplant is often a fatal complication (Hesselink *et al.*, 1989). Immunosuppressive agents are not necessary in rat isograft controls (Lewis to Lewis) since rejection within this purebred strain is never observed, while a rejection is almost always encountered in human transplants without administration of immunosuppressants. Furthermore, transplantation is usually performed in healthy rats but patients requiring a transplant most likely present with a permanently damaged liver and poor pre-operative conditions. It would be interesting to examine the post-transplant course of rats with pre-existing liver disease (e.g. biliary cirrhotic rats in the bile duct-ligation model), though technically this would be very demanding.

Ischemia/reperfusion injury after solid organ transplant is inevitable. All major blood vessels connecting the liver have to be transiently clamped for vascular anastomosis. During

this anhepatic period, the recipient suffers from three major undesirable effects: loss of circulatory preload due to decreased venous return to the heart from the lower body; impediment of renal venous outflow that can compromise renal function; and, most importantly, venous congestion of the intestines owing to transient obstruction of the portal vein. Because of these concerns, the Pittsburgh group reinstated the use of venovenous bypass in liver transplant patients. In the rat model, this external shunt may not be necessary, provided that the anhepatic phase does not exceed 26 minutes (Kamada 1992). This procedure was not performed in our transplant rats as the longest clamping time did not exceed 15 minutes. After resuming vena caval and portal blood flows, we observed macroscopic improvements in renal function (return to normal kidney colour and size, positive urine output); in gut function (noticeable peristalsis; regression of splanchnic venous congestion and bowel edema) as well as in the systemic hemodynamics (maintenance of normal blood pressure and heart rate). However, the ischemia/reperfusion injury to the liver itself was unavoidable. Vollmar and his group (1995) showed that this leukocyte-mediated manifestation of post-ischemic liver damage was ICAM-1-dependent, and exhibited in the first few hours after transplantation. As we were interested in the microvascular events of rejection, animals were examined under the intravital microscopy on post-operative day 6. We assumed that the acute ischemia/reperfusion insult to the liver had ceased after 6 days and that the leukocyte recruitment process was primarily due to rejection of the graft. On the other hand, we did not attempt to study the hepatic microcirculation at a very late time point (e.g. after a month) when most of the leukocyte-

mediated destruction would have been permanent. A sequential quantitative measurement of hepatic oxidative stress at different post-operative time points may be useful in determining the ideal period for this chronic hepatic microvascular study.

The arterial hemodynamic characterizations of liver transplant recipients have been extensively studied on the systemic and splanchnic circulations. Several works have revealed that human liver transplantation is associated with persistent hyperdynamic circulation up to one to two years after successful grafting (Henderson *et al.*, 1992; Hadengue *et al.*, 1993). In the rat liver transplant model, we also demonstrated a significantly higher cardiac output, lower systemic vascular resistance, higher hepatic arterial blood flow and hyperemia in the splanchnic circulation with augmented mesenteric blood flow using the radioactive microsphere method (Wong *et al.*, 1998, submitted). However, these hyperkinetic afferent circulations to the liver did not result in increased leukocyte rolling flux in the post-sinusoidal hepatic vascular bed of the isograft (Figure 4.19). The extensive sinusoidal network might have buffered this augmented arterial vascular load, such that no increase in cellular flux was noted at the venous end.

Few studies have directly examined (visualized) the microcirculation of the liver after transplantation. Menger's group investigated the immediate leukocyte events after grafting (Marzi *et al.*, 1990) and the effects of different preservation solutions on the liver microvasculature (Post *et al.*, 1993). These acute studies mainly focused on ischemia/reperfusion injury of the grafted liver. They showed that these leukocyte-mediated

events were predominantly affecting the sinusoids and postsinusoidal venules. Morphologically, these models were similar to acute rejection but not chronic rejection. Long term *in vivo* studies after transplant are to a great extent restricted by the formation of a thick opaque capsule surrounding the graft after one week. In the present study, we managed to visualize hepatic venules at the surface of liver on the sixth postoperative day using an epi-illumination technique. The results represented a relatively early cellular recruitment in a chronic rejection model. We believed that intervention of leukocyte adhesion to the endothelium should take place at a early time point so as to prevent chronic rejection. A slight increase in leukocyte adhesion in the grafted livers could be attributed to the transplant procedure, as the isograft controls exhibited a higher number of adhesive cells compared to unoperated animals. However, the majority of adhesive leukocytes observed in the Lewis to Brown Norway allografts was most likely due to the rejection process. We did not attempt to block leukocyte adhesion by anti-selectin therapy since we have already demonstrated a minimal role of selectins in the recruitment of white cells into the inflamed liver. Blocking ICAM-1 seems more reasonable in preventing rejection injury. However, the incomplete inhibition of adhesion by either ICAM-1 antibody or antisense oligonucleotides suggests that alternative adhesion pathways that do not require ICAM-1 may exist for leukocyte recruitment into the liver microvasculature during rejection.

As the sinusoids behave differently from the hepatic venules in terms of leukocyte recruitment, it would be of interest to extend the present study to examine leukocyte adhesion

in the sinusoids of rejecting grafts as well. Due to technical difficulties, we could only visualize the larger hepatic venules on the surface. Leukocyte adhesion in sinusoids, unfortunately, could not be clearly demonstrated due to the edematous microvasculature and the thick opaque capsule developed during rejection. Improvements could be achieved by the use of higher resolution microscopy (Suematsu, 1987).

In the present model, liver enzymes remained derranged in the allografted animals after treatment with anti-ICAM-1 antibody or ICAM-1 antisense oligonucleotides, suggesting that hepatocellular injury persists despite blockade of the ICAM-1 pathway. This is not surprising since the pathogenesis of allograft rejection is multi-factorial, some of which may not be mediated by ICAM-1. Humoral response, ischemia, low grade infection and Kupffer cell-mediated injury may perhaps contribute partly to the deterioration in liver function after transplantation.

Intrahepatic bile ducts are known to be the major targets for immune destruction during chronic rejection. Biliary drainage has been called the "technical Achilles' heel of liver transplant" because biliary complications have significantly contributed to postoperative morbidity and mortality. The gradual development of cholestatic jaundice accompanied by increasing levels of serum bilirubin are clinical manifestations of the process. Possible etiologic factors include ischemia, infection, graft rejection and poor operative techniques. Since the sole blood supply to the intrahepatic bile ducts is derived from the hepatic artery, an arterialized model is preferable and employed in this study of chronic rejection. Intrahepatic bile duct loss

is predominantly due to direct lymphocytotoxic attack, although ischemia due to obliterative vasculopathy may also be a cofactor in the pathogenesis of bile duct injury. Before a leukocyte is able to destroy the biliary system, it must (a) migrate out of the bloodstream (a process proven to be highly dependent on ICAM-1); and (b) attach to the bile duct epithelial cells. Whether the second step is also mediated by ICAM-1 is unknown. Expression of ICAM-1 on bile duct epithelium and close proximity of the lymphocytes to the bile ducts during rejection strongly suggest that it is a ICAM-1-mediated process. In the present rejection model, bile duct injury and cholestasis were confirmed by histology and hyperbilirubinemia, respectively. The role of ICAM-1 in protecting the biliary tree is demonstrated by the normal bilirubin level and lack of clinical jaundice in the ICAM-1 antisense treated rats.

The survival study serves as a simple but reliable way of studying graft rejection. It is not surprising to see long term survivors in the Lewis to Lewis isograft control transplant group, since no evidence of rejection has yet been documented among this purebred strain. On post-mortem, these animals did not exhibit signs of hepatic destruction or portal hypertension induced by the vascular cuffs. The rejection group (Lewis to Brown Norway rats) had a mean survival time of 25 days. Post-mortem of these animals revealed grossly damaged liver with evidence of fluid retention (ascites and occasionally pleural effusion). Deep jaundice was also observed in some animals. Rats treated with the antibody to ICAM-1 (1A29) had a short mean survival time of only 12 days. This is in contrast to the findings by Degawa's group (1995) and Harihara's group (1996) who demonstrated an immunosuppressive effect of the antibody on rat

hepatic allografts. However, other investigators showed results in agreement to our data. A shortened survival was observed in transplanted rats treated with 1A29 by Omura *et al.* (1992, 1993), but no explanation was given to the cause of death. Kamada's group also demonstrated severely aggravated liver injury and massive hepatic necrosis in rats treated with this intact ICAM-1 antibody, with evidence of complement system activated by immune complex on the surface of endothelial cell membrane. However, when treated with the $F(ab')_2$ fragment of 1A29 alone, liver injury was reduced and complement was not activated in the absence of Fc portion (Takei *et al.*, 1996; Nishimura *et al.*, 1996). In the present study, intact 1A29 was administered intravenously to the recipient rats. It is therefore not surprising to see a shortened survival if such an unfavourable immunologic reaction was also activated in the host.

In the present study, rats tolerated the ICAM-1 antisense oligonucleotides well, with a prolonged mean survival of 42 days, suggesting an important role of ICAM-1 in the event of chronic rejection. Although survival of these animals was significantly longer than the untreated group (25 days), permanent tolerance could not be induced after 6 days of treatment. This is probably due to the presence of newly synthesized ICAM-1 after the 6-day course of antisense oligonucleotides. Interestingly, in the mouse heterotopic heart transplant model, a 7-day treatment of ICAM-1 antisense oligonucleotides in combination with anti-LFA-1 monoclonal antibody resulted in permanent tolerance (>150 days) (Stepkowski *et al.*, 1995). This suggests that inhibition of ICAM-1 and its ligand LFA-1 simultaneously may produce potent synergistic *in vivo* immunosuppressive activity leading to transplant tolerance.

The use of monoclonal antibodies in solid organ transplantation dated back 17 years ago. The first trial of monoclonal antibody therapy in human allograft recipients was the use of OKT3, a pan-T-cell suppressive agent, for renal transplant patients in 1980 (Cosimi *et al.*, 1981). It is known that OKT3 does not lead to donor-specific tolerance and result in increased risk of viral infection and development of human anti-murine antibodies, as it is a mouse monoclonal. Later, anti-CD4 monoclonal antibodies were introduced. These agents react with a more restricted subset of lymphocytes and hence provide a more selective suppression of the immune system. Clinical trials using these antibodies have been carried out. One of these agents, 16H5, was shown to significantly deplete CD4+ T lymphocytes and improve allograft function in renal transplant recipients (Sablinski *et al.*, 1991). Afterwards, monoclonal antibodies against interleukin-2 receptor (anti-CD25; IL-2R) were developed to selectively suppress cytotoxic T cells from activation and proliferation. Unfortunately, only minimal beneficial evidence was found (Friend *et al.*, 1991). Recently, a phase-I dose-seeking study of anti-ICAM-1 antibody (BIRR1) was performed in cadaveric renal allograft recipients by Cosimi at the Massachusetts General Hospital with promising results, suggesting the importance of ICAM-1 in transplant rejection. Patients having a high risk for post-transplant complications such as acute rejection or delayed graft function were given BIRR1 prophylactically in addition to conventional immunosuppressive therapy. The incidence of complications was lowered in patients with adequate BIRR1 levels (Haug *et al.*, 1993). Results from liver transplant trials were also encouraging. A phase-I trial has been carried out

by Neuberger's group in Birmingham, studying hepatic survival after liver transplant. A trend towards reduced rejection was observed in patients receiving high dose BIRR1 and standard triple immunosuppression (Davies *et al.*, abstract 1993).

Several limitations of monoclonal antibodies were identified during these clinical trials. Immunization against the murine immunoglobulin molecule was one of the first drawbacks (especially in the early trials of OKT3). This xenosensitisation effect was due to the xenogeneic origin of the antibodies. Upon administration of the antibody, patients were found to have very high titres of anti-OKT3 IgM and IgG antibodies by day 5 to 7 of treatment (Chatenoud *et al*, 1986). This humoral response had a potent neutralizing effect essentially mediated by the IgG antibodies.

Moreover, *in vivo* cell activation can be induced by injection of monoclonal antibodies. This phenomenon may be exacerbated if the antibody is injected into a patient or animal with pre-existing immune activation such as a rejection process. For instance, anti-CD3 monoclonal antibodies can induce polyclonal T-cell activation (van Wanwe *et al.*, 1980). Within few hours after anti-CD3 administration, massive release of several cytokines is associated with membrane expression of activation markers (IL-2R) on T-cells. These cytokines include TNF, IFN-γ, IL-2,3,4,6,10 and GMCSF. The release induces an acute flu-like syndrome including high fever, chills, headache, vomiting, respiratory distress and sometimes hypotension.

Another important side effects of monoclonal antibodies in clinical transplant setting is over-immunosuppression resulting in increased susceptibility to malignant tumours such as

non-Hogkins lymphoma (Chatenoud, 1995). The frequency of lymphoma correlates directly with the potency of overall immunosuppressive regime rather than to the use of a particular immnosuppressant.

The use of antisense oligonucleotides is another therapeutic strategy for the intervention in leukocyte-endothelial cell interaction. By hybridizing to the mRNA coding for the specific protein, antisense oligonucleotides reduce the expression of the functional protein. Selective gene expression may be affected at several molecular levels, including induced hydrolysis of target RNA by RNase H, inhibition of 5'-cap formation, inhibition of RNA splicing, inhibition of polyadenylation of the mRNA, or inhibition of RNA transport out of the nucleus and arresting translation of target mRNA (Bennet & Crooke, 1994). In animal models, ICAM-1 antisense oligonucleotides have been proved useful in prolonging the grafts in heart allograft rejection (Stepkowski *et al.*, 1994) and pancreatic islet transplantation (Katz *et al.*, 1995). In the present rat liver transplant model, ICAM-1 antisense oligonucleotides show promise as a therapeutic agent to block allograft rejection.

Future prospects for transplant research could also be directed to the use of anti-oxidant therapy in rejection. Several groups have proposed that oxygen free radicals contribute to organ injury after liver transplantation (Marzi *et al.*, 1992; Gonzalez-Flecha *et al.*, 1993; Goode *et al.*, 1994) though most of the work emphasized on the effects of anti-oxidants in ischemia/reperfusion injury. It has been shown that superoxide dismutase infusion would result in long-term hepatic allograft survival in the presence of subthreshold immunosuppressive

dosages of FK-506 (Rao *et al.*, 1995). Lipid peroxidation of the reoxygenated liver tissue was suppressed effectively by the pre-ischemic administration of the antioxidant ascorbic acid (Ozaki *et al.*, 1995). In kidney transplantation, a beneficial effect of superoxide dismutase on acute and chronic rejection events has been demonstrated by Land *et al.* (1994). Ascorbic acid and α-tocopheral can delay cardiac allograft rejection when combined with cyclosporine therapy (Slakey *et al.*, 1993). Adminstration of high dose ascorbic acid orally in our chronic liver rejection model also prolonged survival significantly (Lee & Wong, unpublished data). These antioxidants may provide a basis for the development of specific therapeutic interventions that can be applied to chronic hepatic transplant rejection.

Orthotopic liver transplantation in the mouse model was recently described by Qian *et al.* (1991). Although the surgical principles and techniques employed were similar to those in the rat, the procedures were much more difficult with higher mortality and morbidity due to the smaller size of the animal. There are many advantages to using this mouse liver transplant model for immunologic research. The mouse genome is more thoroughly characterized than the rat or any other species. The mouse also have a histocompatibility system which bears a striking resemblance to the human HLA complex. Furthermore, there are numerous genetically defined inbred strains and commercially available anti-mouse monoclonal antibodies for research purposes. With the recent development of genetic knockout mice, further insights into the pathophysiology of hepatic transplant rejection will be gained in the near future.

CHAPTER SIX: CONTRIBUTIONS TO SCIENTIFIC KNOWLEDGE

It is clear that recruitment of white cells from the bloodstream into areas of injury or infection is essential for host defense, but also that many acute and chronic allergies, autoimmune, or transplant graft rejection conditions are exacerbated by the presence of excessive leukocytes (Osborn, 1990). In the present study, we examined the importance of different adhesion molecules, namely P-, E-, an L-selectins and ICAM-1, in terms of leukocyte-endothelial interactions in the hepatic sinusoids and the post-sinusoidal venules. Defining the mechanisms underlying leukocyte recruitment in the liver is extremely important not just for our understanding of the inflammatory process in the organ, but also in directing our attempts to design anti-adhesion therapies to reduce injury to the liver caused by leukocytes.

The second set of experiment demonstrating the importance of ICAM-1-mediated leukocyte recruitment into the liver graft during chronic transplant rejection may provide a mechanistic basis for the beneficial action of anti-ICAM-1 therapy for this irreversible condition.

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