THE UNIVERSITY OF CALGARY

Leukocyte Recruitment and Function in Cholestasis

by

Kartika Tjandra

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF GASTROINTESTINAL SCIENCES

CALGARY, ALBERTA

OCTOBER, 1996

ABSTRACT

The mechanisms of the increased incidence of septic complications in jaundiced patients are not well understood. Neutrophils play a central role in the first line of host defense. To fulfill this role, neutrophils must reach inflammatory sites and perform microbicidal functions. Our objective was to determine if neutrophil functions are impaired in cholestatic rats.

We demonstrated that neutrophil recruitment to inflammatory sites was impaired in cholestatic rats and endogenous glucocorticoids were shown to inhibit this process. Neutrophil adhesion, a critical step for cell migration to inflammatory sites, was normal. However, cholestatic plasma exhibited anti-adhesive properties. Furthermore, microbicidal activity was impaired in cholestatic rats and bacterial killing was further reduced in the presence of cholestatic sera.

In conclusion, neutrophil dysfunction in cholestatic rats was manifested as impaired neutrophil accumulation and microbicidal activity. These impairments may, in part, explain the increased incidence of septic complications in cholestatic patients following surgery.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Mark Swain for his scientific guidance, encouragement and continuing support throughout my studies. Additionally, I would like to thank my committee members Drs. Paul Kubes, Ric Woodman and Sam Lee for their valuable insight and support.

I wish to acknowledge the technical assistance of Mirjana Maric, Paul Reinhardt, Dave Kirk and Tai Le. I would also like to thank my husband, Bryn Watson, who has given me strength, courage, and unlimited support throughout these last few years.

Finally, this work was made possible by the support of the Canadian Liver Foundation and Celiac and Colitis Foundation of Canada.

DEDICATION

TO MY PARENTS

for their love, encouragement and unending support

Nitra and Juliana Tjandra

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

^{3}H-LTB4	tritiated leukotriene B4
^{51}Cr	isotope 51 of chromium
^{125}I	isotope 125 of iodine
α	alpha
ACTH	adrenocorticotropic hormone
ADX	adrenalectomized
β	beta
BDR	bile duct resected
Br^-	bromide ion(s)
Ca^{++}	calcium ion(s)
$CaCl_2$	calcium chloride
cc	cubic centimetre(s)
CFU	colony forming unit
Cl^-	chloride ion(s)
cm^2	square centimetre(s)
CO_2	carbon dioxide
cpm	counts per minute
CRH	corticotropin releasing hormone
DAG	diacylglycerol
ddH_2O	deionized distiled water
DHCB	dihydrocytochalasin B
dL	decilitre(s)
ELAM	endothelial-leukocyte adhesion molecule
FACS	fluorescence-activated cell sorter

FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
g	gram(s)
G-6-P	glucose-6-phosphate
G6PD	glucose-6-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour(s)
H_2O_2	hydrogen peroxide
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HMS	hexose monophosphate shunt
HOCl	hypochlorous acid
HVA	homovanillic acid
i.p.	intraperitoneal
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
IP_3	inositol-1,4,5-trisphosphate
IU	international unit(s)
Kd	dissociation constant
kg	kilogram(s)
L	liter(s)
LAD	leukocyte adhesion deficiency
LFA-1	lymphocyte function associated antigen-1
LT	leukotriene
M	Molar

MAb	monoclonal antibody
Mac-1	macrophage-1
µCi	microCurie(s)
mg	milligram(s)
$MgCl_2$	magnesium chloride
min	minute(s)
µl	microlitre(s)
ml	millilitre(s)
mM	millimolar
MPO	myeloperoxidase
MW	molecular weight
$Na^{51}CrO_4$	chromium 51-labeled sodium chromate
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NBT	NitroBlue Tetrazolium
ng	nanogram(s)
nM	nanomolar
nm	nanometre(s)
nmole	nanomole(s)
O_2	oxygen
O_2^-	superoxide anion
OZ	opsonized zymosan
PAF	platelet-activating factor
PBS	phosphate buffered saline
PG	prostaglandin

pg	picogram(s)
PIP_2	phosphatidylinositol-4,5-bisphosphate
PLC	phospholipase C
PMA	phorbol myristate acetate
RIA	radioimmunoassay
ROI	reactive oxygen intermediate
rpm	revolutions per minute
sec	second(s)
SEM	standard error of the mean
sLe^x	sialyl Lewis x
SOD	superoxide dismutase
TNF	tumor necrosis factor
TPCK	L-1-tosylamide-2-phenyl-ethylchloromethyl ketone
TSA	tryptic soy agar
U	units of activity
•OH	hydroxyl radical
°C	degrees Celsius

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

Patients with obstructive jaundice are predisposed to post-operative septic complications. The rate of post-operative septic complications is higher in jaundiced patients than in the general surgical population (Armstrong et al., 1984; Pain et al., 1985; Cainzos et al., 1988). Despite antibiotic pretreatment, improvement in surgical procedures and prophylactic measures such as oral bile salts and pre-operative biliary drainage, infective complications remain a significant factor contributing to mortality rates as high as 10-37% in jaundiced patients following surgery (Armstrong et al., 1984). The mechanism(s) underlying the increased incidence of septic complications in patients with obstructive jaundice are not well understood.

Obstructive jaundice occurs as a consequence of bile duct obstruction; this most commonly occurs when a gallstone or cancer blocks the common bile duct (Guyton, 1991). This results in impaired bile flow, a condition commonly referred to as cholestasis. Hyperbilirubinemia is a hallmark of patients with obstructive jaundice; the rate of bilirubin formation is normal, but bilirubin is unable to be secreted into the intestines for further metabolism and excretion (Guyton, 1991). Therefore, plasma bilirubin levels are elevated in obstructive jaundice and are directly associated with increased severity of disease and risk of infective complications following surgery (Pitt et al., 1981).

Increased septic complications in patients with obstructive jaundice may be associated with an impaired or depressed host immune response to invading pathogens. Roughneen *et al.* have shown that T cell function is impaired in experimental cholestasis by demonstrating impaired host responsiveness to foreign cellular antigen (Roughneen et al., 1986; Roughneen et al., 1987). Furthermore, aberrations in Kupffer cell function in experimental

model of cholestasis and adults with liver failure have also been observed (Drivas et al., 1976; Holman and Rikkers, 1982; Katz et al., 1984). However, little is known of polymorphonuclear leukocyte (neutrophil) function in cholestasis.

Neutrophils are the first line of host defense. They are characterized by their distinctive nucleus which is polymorphous and usually consists of a few sausage-shaped masses as illustrated in Figure 1.1. After several stages of development in the bone marrow, neutrophils are released into the bloodstream. Neutrophils are the most numerous leukocytes in the blood, constituting approximately 50-70% of total white blood cells (Johnson et al., 1992). The circulating half-life of neutrophils is short (approximately 7 hours). Then, they enter tissues for 1-4 days and their ultimate fate is unknown. During pathological conditions such as a bacterial infection, levels of neutrophils in the blood may be elevated for host defense.

The primary role of neutrophils is the ingestion and killing of invading bacteria and other microorganisms. To perform this role, neutrophils need to reach the site of infection and subsequently perform microbicidal functions. Thus, the ability of neutrophils to migrate from the vasculature to affected tissues is essential in targetting and eliminating pathogens.

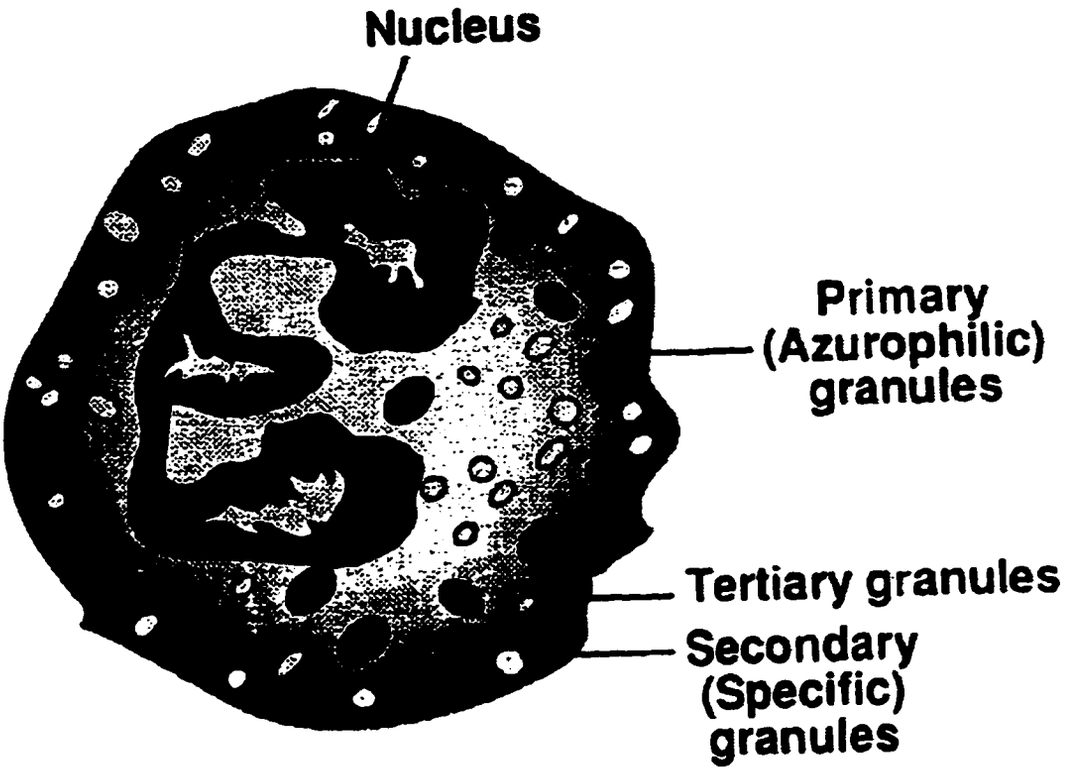

Figure 1.1. Morphology of polymorphonuclear leukocyte (neutrophil).

1.2. NEUTROPHIL RECRUITMENT

Neutrophil recruitment to inflammatory sites generally involves a multiple step process: rolling, firm adhesion and extravasation as illustrated in Figure 1.2. Neutrophil recruitment to a site of inflammation is initiated by tethering/rolling of the neutrophil along the vascular endothelium, followed by firm adhesion, extravasation and migration. This multi-step process is mediated by several adhesion molecules expressed both on the endothelium and on neutrophils. There are three major groups of adhesion molecules: the selectins, integrins and immunoglobulins (Igs). The selectins on endothelial cells and leukocytes are involved in leukocyte rolling along the vascular endothelium. The firm adhesion of leukocytes to the endothelium, as well as transendothelial migration are primarily mediated by the integrin family, mainly the leukocyte β2-integrins, and their ligand receptors on the endothelium, mainly intercellular adhesion molecule-1 (ICAM-1), an immunoglobulin (Harlan et al., 1992).

The upregulation of various adhesion molecules is dependent upon the stimulatory properties of a number of substances generated during the inflammatory response. Chemotactic factors (e.g. C5a, interleukin-8 (IL-8), leukotriene B_4 (LTB_4) and bacterial derived formyl peptides) and inflammatory cytokines (e.g. tumor necrosis factor (TNF) and interleukin-1 (IL-1)) increase expression and/or activity of various adhesion molecules found on neutrophils and endothelium.

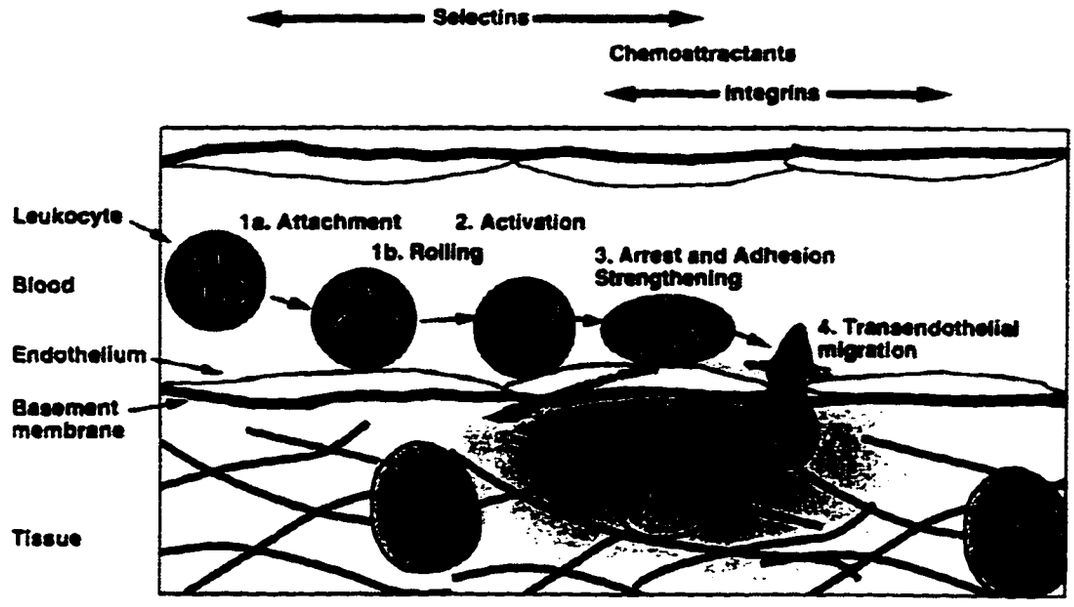

Figure 1.2. The three-step model of neutrophil recruitment to tissues (adapted from Springer, 1994).

1.2.1. Neutrophil rolling

Neutrophil recruitment to tissues is initiated by tethering and rolling of neutrophils along the blood vessel wall. This process is essential for cell adhesion and emigration into tissue. The selectins, single-chain transmembrane glycoproteins, are critical in mediating neutrophil rolling along the vascular endothelium. Three selectins have been described: E-selectin, L-selectin and P-selectin.

L-selectin is constitutively expressed on the neutrophil surface, while P- and E-selectin can be upregulated on the endothelial surface. E-selectin, an endothelial glycoprotein, is synthesized and expressed in response to bacterial endotoxin and the inflammatory cytokines IL-1 and TNF (Weller et al., 1992). E-selectin expression reaches maximal levels by 4-6 h and returns to basal levels over the next 24-48 h. P-selectin is constitutively synthesized and stored in the α granules of platelets and in the Wiebel-Palade bodies of endothelial cells (Bonfanti et al., 1989; McEver et al., 1989). Within minutes of stimulation by a variety of stimuli (e.g. thrombin, histamine, H_2O_2, complement proteins), P-selectin can be redistributed or mobilized to the endothelial surface (McEver et al., 1989; Bonfanti et al., 1989; Patel et al., 1991; Hattori et al., 1989). In contrast to E-selectin, endothelial P-selectin expression is transient. P-selectin levels peak at 5-10 minutes following endothelial stimulation *in vitro* and are absent by 20 minutes (Harlan et al., 1992; Bevilacqua et al., 1994).

Interaction of the selectins with their carbohydrate ligands is essential for the initial contact of leukocytes with activated endothelium. Under conditions of fluid shear stress, this contact is demonstrated as leukocyte rolling along the vascular endothelium. Tetrasaccharide

sialyl Lewis x (sLe^X) and other fucosylated carbohydrates may act as ligands for the selectins (Springer and Lasky, 1991).

1.2.2. Neutrophil adhesion

Neutrophil firm adhesion to the endothelium is critical in mediating neutrophil emigration to tissues. Following their margination along the blood vessel wall, neutrophils adhere to the vascular endothelium in response to various inflammatory mediators and chemoattractants. This process is mediated by the integrins, heterodimeric transmembrane proteins found on neutrophils, and their counter ligands on the endothelial cells as listed in Table 1.1.

1.2.2.1. β2 integrins

Neutrophil integrin contains one of three α chains (α^L, α^M or α^X) which is noncovalently associated with a common β subunit, β2. Various chemoattractants (e.g. C5a, IL-8, platelet-activating factor (PAF)) can increase the adhesiveness and expression of the β2 integrins (Tonnesen, 1989), which favors neutrophil adhesion to endothelium. The importance of the integrins in neutrophil firm adhesion has been demonstrated in studies of patients with leukocyte adhesion deficiency (LAD), an inherited disorder in which the leukocyte β2 integrins are nonfunctional (Springer et al., 1984). Patients with LAD experience recurrent bacterial infections as neutrophils are unable to be recruited to sites of infection. In addition, impaired neutrophil adhesion and subsequently emigration to tissue was also observed using monoclonal antibodies (MAbs) to CD18, CD11a and CD11b subunits (Harlan et al., 1992).

Table 1.1. The neutrophil integrins and their associated ligands on the vascular endothelium (adapted from Springer, 1994).

Subunits	Names	Ligands
$\alpha^{L}\beta 2$	LFA-1; CD11a/CD18	ICAM-1, ICAM-2, ICAM-3
$\alpha^{M}\beta 2$	Mac-1; CR3; CD11b/CD18	ICAM-1, iC3b, fibrinogen
$\alpha^{X}\beta 2$	p150,95; CD11c/CD18	iC3b, fibrinogen

Note: LFA-1, lymphocyte function associated antigen-1; Mac-1, macrophage-1; ICAM, intercellular adhesion molecule.

1.2.2.2. Endothelial adhesion molecules

Several different Ig superfamily members expressed on the endothelium, such as ICAM-1 and ICAM-2, act as ligands for leukocyte integrins. ICAM-1, a transmembrane glycoprotein containing five Ig domains, has been found to bind both lymphocyte function associated antigen-1 (LFA-1) and macrophage-1 (Mac-1) (Marlin and Springer, 1987; Diamond et al., 1991). ICAM-1 is present at low levels under normal conditions, but can be upregulated by endotoxin and inflammatory cytokines (e.g. IL-1 and TNF) (Dustin et al., 1986; Pober et al., 1986). Therefore, increased expression of ICAM-1 is a common feature of inflammation. ICAM-2 contains two extracellular Ig domains that are closely related to the two N-terminal domains of ICAM-1 (Staunton et al., 1989). ICAM-2 is constitutively expressed on endothelial cells and binds to LFA-1 (Staunton et al., 1989), but in contrast to ICAM-1, its expression does not appear to be influenced by inflammatory cytokines (Springer, 1990). Therefore, ICAM-2 may be important for leukocyte trafficking in uninflammed tissues while upregulated expression of ICAM-1 may be important during inflammation by increasing cell-cell interactions, pre-requisites for neutrophil extravasation.

1.2.3. Neutrophil emigration

Neutrophil emigration (also called neutrophil extravasation, diapedesis or transmigration) is a complex process. It is initiated by neutrophil rolling along the vascular endothelium and followed by firm adhesion (sections 1.2.1 and 1.2.2). Several changes take place following integrin-mediated neutrophil attachment to the endothelium. Neutrophils undergo shape change, flatten onto the endothelial surface and polarize themselves towards chemoattractant(s). These changes are facilitated by rearrangement of cytoskeleton and alterations in plasma membranes.

In addition, neutrophil emigration also depends on other cellular processes, mainly the expression and activation of adhesion molecules. Neutrophil transendothelial migration requires β2 integrins, primarily CD11a/CD18 and CD11b/CD18. *In vivo* and *in vitro* studies using MAbs to β2 integrins (anti-CD11a, anti-CD11b, and anti-CD18) demonstrated that CD11a/CD18 and CD11b/CD18 are needed for optimal transmigration (Jutila et al., 1989; Luscinskas et al., 1991). In addition, ICAM-1 and endothelial-leukocyte adhesion molecule-1 (ELAM-1), are also required for transmigration (Furie et al., 1991).

Neutrophils move towards higher concentrations of chemotactic substances (e.g. endothelial-cell derived IL-8, LTB_4, C5a and formylated bacterial products) released at the sites of infection. For access to the site of infection following emigration through endothelial intercellular junctions (Cramer et al., 1980), neutrophils further penetrate the underlying basement membrane and connective tissue. Limited degradation of basement membrane appears to be mediated by lytic enzymes released from specific granules, such as gelatinase (Wright and Gallin, 1979). In addition, neutrophils may use mechanical means to penetrate basement membrane by dilatation of the matrix network (Tschesche et al., 1991).

1.3. NEUTROPHIL MICROBICIDAL FUNCTION

Neutrophils possess two major weapons for fighting invading pathogens. The first is an oxygen-dependent pathway with the generation of reactive oxygen intermediates (ROIs) and the second is an oxygen-independent pathway involving the release of granular proteins with microbicidal activity (Babior, 1984; Klebanoff, 1992; Elsbach and Weiss, 1992). Following stimulation by an agonist, neutrophils show a sharp increase in oxygen (O_2) uptake and begin to release large quantities of superoxide anion (O_2^-) and H_2O_2. Further reactions involving O_2^- and H_2O_2 result in the formation of more potent microbial

oxidants, such as hypochlorous acid (HOCl) and hydroxyl radical (•OH) (Babior, 1988; Klebanoff, 1992). At the same time, the intracellular granules of neutrophils fuse with the plasma membrane and release their contents extracellularly or into phagocytic vacuoles. Although the microbicidal activity of neutrophils depends largely on ROI formation, several lines of evidence suggest that neutrophils are capable of killing bacteria independent of oxygen radical formation (reviewed in Selsted, 1988). Oxygen-independent neutrophil microbial killing agents (microbicides) have been found in the primary (aruzophil) and secondary (specific) granules of neutrophils (Babior, 1992).

1.3.1. Respiratory Burst

The respiratory burst refers to an abrupt change in O_2 metabolism that occurs when neutrophils are stimulated (Babior, 1978). Some notable changes during the respiratory burst are: 1) increased O_2 consumption, 2) increased glucose metabolism through the hexose monophosphate shunt pathway, and 3) increased production of O_2^- and H_2O_2. Increased oxygen consumption occurs to provide substrate for the formation of the ROIs involved in killing of microorganisms. Nicotinamide adenine dinucleotide phosphate (NADPH), a substrate for the respiratory burst enzyme NADPH oxidase, is generated through the hexose monophosphate shunt from glucose-6-phosphate (G-6-P) oxidation.

Subsequent to neutrophil stimulation, molecular O_2 undergoes a single electron reduction to O_2^-. This reduction process is catalyzed by NADPH oxidase, a multi-component enzyme system central to oxygen-dependent killing (Segal and Peters, 1978). NADPH oxidase, which is found in the plasma membrane of neutrophils, is dormant in resting neutrophils, but is readily activated upon encounter with invading microorganisms or other appropriate stimuli. For its activation, it requires a plasma membrane cytochrome *b*

together with at least 3 proteins which move from the cytosol and bind to cytochrome *b* (Clark et al., 1990; Segal and Abo, 1993). The NADPH oxidase complex mediates electron transfer from NADPH to molecular O_2. In this process, cytoplasmic NADPH is oxidized to $NADP^+$ and two electrons are donated to two molecules of O_2, yielding two molecules of O_2^-.

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2\,O_2^- + NADP^+ + H^+$$

In aqueous solutions, O_2^- is unstable and undergoes spontaneous dismutation to generate H_2O_2.

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

The spontaneous dismutation reaction is relatively slow with a rate of less than 10^2 M^{-1} sec^{-1} (Root and Cohen, 1981) although the rate increases at an optimal pH of 4.8 (8.5×10^7 M^{-1} sec^{-1}; Behar et al., 1970). Superoxide dismutation is accelerated by superoxide dismutase (SOD). The rate of the SOD-catalyzed reaction is approximately 2×10^9 M^{-1} sec^{-1} (Fridovich, 1972).

H_2O_2 is converted by a complex series of secondary reactions to two classes of highly reactive oxidizing agents: hypohalous acids and hydroxyl radicals. Hypohalous acid production is catalyzed by myeloperoxidase (MPO), a heme enzyme released from the primary granules of neutrophils. MPO in the phagocytic vacuole catalyzes H_2O_2 and halide ion (X^-; Cl^-, Br^-) reactions to form hypohalous acids which are more potent microbicidal agents.

$$X^- + H_2O_2 \xrightarrow{\text{Myeloperoxidase}} HOX + H_2O$$

Hypohalous acids, namely HOCl, can further react with nitrogen containing compounds to form nitrogen chlorine derivatives (chloramines; N - Cl):

$$HOCl + R\text{-}NH_2 \longrightarrow R\text{-}NHCl \longleftrightarrow RNCl_2$$

Some of these chloramines are relatively long-lived compounds which may prolong the oxidant activity of HOCl, but the toxicity of these molecules may vary. For example, lipophilic monochloramine (NH_2Cl), formed from the reaction of HOCl and ammonium anion, appears to have increased toxic activity through its ability to penetrate and oxidize biological membranes (Thomas, 1979; Grisham et al., 1984a; Grisham et al., 1984b). On the other hand, taurine ($H_2NCH_2\text{-}CH_2SO_3^-$) found at high concentrations in neutrophils, is relatively harmless and protects certain targets by scavenging HOCl (Weiss et al., 1983; Thomas et al., 1983).

Hydroxyl radical (•OH) can be generated by the superoxide driven Fenton reaction (Haber-Weiss reaction):

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \longrightarrow \text{•}OH + OH^- + Fe^{3+}$$

The source of free iron for this reaction is unclear as levels of free iron are very low in biological systems. One hypothesis is that stimulated neutrophils can release lactoferrin (an iron binding protein) from the specific granules (Cohen et al., 1992). In this case, lactoferrin

may act as the iron donor for •OH formation (Ambruso and Johnston, 1981). In contrast, lactoferrin may play a protective role by preventing iron from triggering hydroxyl radical formation in the vicinity of the activated neutrophil (Britigan et al., 1986). In addition, ferritin may provide free iron for the above reaction by releasing Fe^{2+} when exposed to O_2^- (Blemond et al., 1984). It is also possible, however, that neutrophils use iron from other biological sources (e.g. damaged cells).

The mechanisms by which hydroxyl radicals mediate killing of microorganisms are not well understood. Like superoxide anion, hydroxyl radical is capable of initiating lipid peroxidation, which has been implicated in membrane damage through the oxidation of polyunsaturated fatty acids (Kanner and Kinsella, 1983). In addition, hydroxyl radicals may damage other biological molecules, such as DNA. It has also been speculated that •OH toxicity may result in a loss of membrane selectivity leading to cell lysis, a mechanism similar to complement-mediated killing (Babior, 1984).

1.3.1.1. Scavengers of ROIs

The release of various reactive oxidants following neutrophil stimulation is not only beneficial for killing bacteria and other microorganisms, but may also be detrimental to cells. Granule contents and reactive oxygen metabolites may leak from the neutrophil into extracellular fluid, where they can injure tissues. However, cells are equipped with protective devices to handle reactive oxidants such as O_2^- and H_2O_2. Superoxide anion that escapes from the phagocytic vacuole is reduced to H_2O_2 at an enhanced rate by SOD. This reduction may prevent O_2^- from initiating toxic oxidation reactions with unsaturated lipids and proteins. In addition, neutrophils are protected from the toxic effects of H_2O_2 by

catalase (Roos et al., 1980; Voetman and Roos, 1980) and glutathione (Roos et al., 1979; Spielberg et al., 1979).

Catalase, another heme enzyme, reduces H_2O_2 to water and O_2 as shown below:

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

H_2O_2 can also be neutralized by the glutathione peroxidase-reductase cycle as shown in Figure 1.3.

1.3.2. Non-oxidative killing

Neutrophil microbicidal activity is mediated not only by the activity of reactive oxidants generated through the respiratory burst system, but also by the release of various enzymes with microbicidal potential from neutrophil granules. Following invagination of particles (e.g. bacteria) by phagocytosing neutrophils, the cytoplasmic granules fuse with the phagosome and discharge their contents into it, a process called degranulation. Depending on the stimulus, discharge of granular contents can also be directed to the external milieu.

Based on their biochemical and morphological properties and time of appearance during cell maturation, neutrophils consist of three major types of granules: azurophil (primary) granules, specific (secondary) granules and tertiary granules (Smolen and Boxer, 1995). These granules contain microbicidal proteins and proteases which mediate neutrophil microbicidal activity. A list of the most important constituents of neutrophil granules is given in Table 1.2.

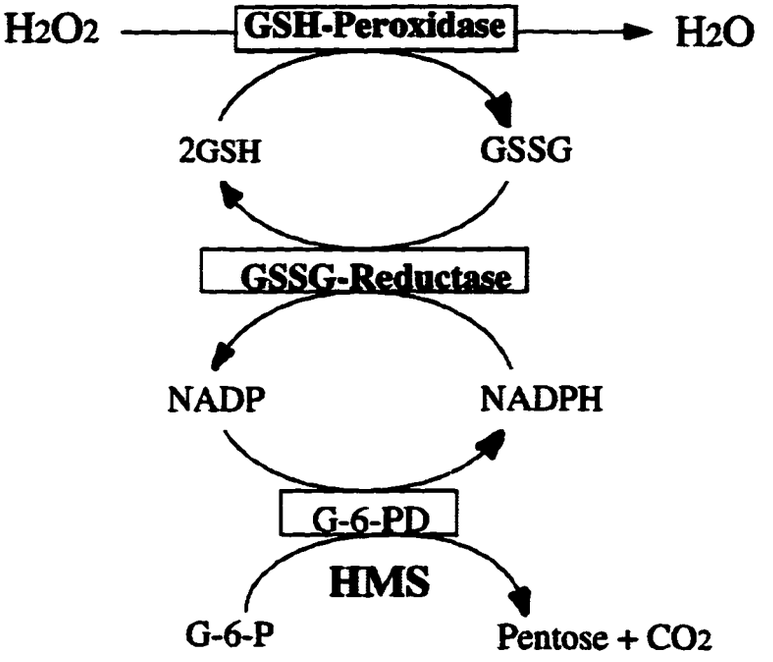

Figure 1.3. Scavenging role of glutathione on H_2O_2. H_2O_2 is reduced to water through the oxidation of glutathione (GSH) by glutathione peroxidase (GSH-peroxidase). Oxidized glutathione (GSSG) is reduced back to glutathione by glutathione reductase (GSH-reductase) using NADPH as a substrate. NADPH is generated by the oxidation of glucose in the hexose monophophate shunt (HMS), which is stimulated by $NADP^+$ (This diagram was adapted from Root and Cohen, 1981). G-6-PD = glucose-6-phosphate dehydrogenase; G-6-P = glucose-6-phosphate; CO_2 = carbon dioxide.

Table 1.2. Neutrophil granule constituents (adapted from smolen and Boxer, 1995).

Constituent	*Azurophil granules*	*Specific granules*	*Tertiary granules*
Microbicidal enzymes	•Lysozyme •Elastase •Cathepsin G •Myeloperoxidase	•Lysozyme	
Antibacterial cationic	•Defensins •Bactericidal/permeability-increasing protein	•Lactoferrin	
Neutral serine proteases	•Proteinases		
Metallo-proteinases		•Collagenase	•Gelatinase
Acid hydrolases	•N-acetyl-glucuronidase •Cathepsin B •Cathepsin D •β-Glucuronidase •β-Glycerophosphatase •α-Mannosidase		
Others	•Kinin-generating enzyme •C5a-inactivating factor	•Plasminogen •Cytochrome *b* •Histaminase •Heparanase •FMLP receptors •C3bi receptors •Vitamin B12-binding protein •Protein kinase C inhibitor •Complement activator •Monocyte-chemoattractant •FcRIII receptors •Thrombospondin receptors •Laminin receptors	•C3bi receptors •Cytochrome *b* •Alkaline phosphatase •FcRIII receptors

Neutrophil degranulation results from a complex biochemical event initiated by the binding of particulate or soluble stimuli to specific cell surface receptors on neutrophils. Various receptors on neutrophils have been described, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) receptors (Williams et al., 1977; Marasco et al., 1983) and C5a receptors (Chenoweth and Hugli, 1978; Rollins and Springer, 1985; Huey and Hugli, 1985). The exact process leading to neutrophil degranulation is not known; however, a model has been proposed for stimulus-response coupling leading to neutrophil degranulation (Figure 1.4).

The binding of a specific ligand to cell surface receptor (R) leads to conformational changes in the receptor and its associated G protein (Gi). This in turn activates Gi to exchange GTP for GDP. Activated Gi (Gi*) subsequently activates polyphosphatidylinositol-specific phospholipase C (PLC). Activated PLC (PLC*) cleaves the endogenous lipid phosphatidylinositol bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 increases intracellular calcium ($[Ca_2^{++}]_{fr}$) by inducing the release of stored calcium from the sarcoplasmic reticulum. Elevations in intracellular calcium alone are known to induce the secretion of granule constituents by fusion of granule membranes with the plasma membranes.

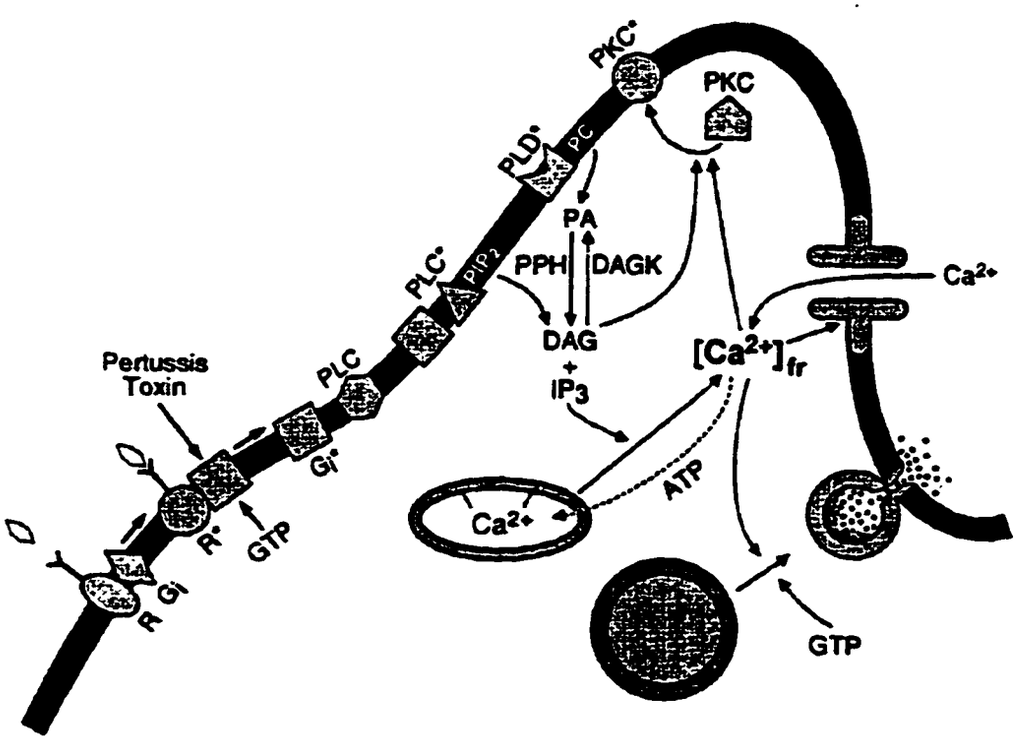

Figure 1.4. A model of stimulus-response coupling leading to neutrophil degranulation (adapted from Smolen and Boxer, 1995).

1.3.3. ROIs and granular proteins

In addition to being microbicidal themselves, ROIs can also potentiate the activity of granular enzymes. For example, HOCl promotes proteolysis by inactivating both α1 antiprotease (Weiss et al., 1986; Hubbard et al., 1987) and α2-macroglobulin (Reddy et al., 1989), plasma inhibitors of the neutral proteases (e.g. elastase and cathepsin G) released from the azurophil granules. In addition, HOCl activates neutrophil collagenase and gelatinase, proteases that are secreted as inactive precursors by stimulated neutrophils. Furthermore, collagenase that has been activated by HOCl destroys α1 antiprotease.

The reverse is also true. Granular proteins potentiate and in some cases mediate the production of reactive oxidants. For example, myeloperoxidase, released from the azurophil granules, catalyzes the formation of hypohalous acids. Lactoferrin, released from the specific granules, has also been shown to facilitate the generation of highly reactive •OH (Ambruso and Johnston, 1981). In addition, granular proteins appear to have modulatory effects on oxygen-dependent mechanisms of killing. This is demonstrated by the mobilization of flavoprotein cytochrome *b*, an important constituent of NADPH oxidase, to the plasma membrane from the specific granules upon neutrophil activation. This may amplify the neutrophil respiratory burst, and enhance neutrophil microbicidal functions. Therefore, microorganism killing is optimized by the actions of both the granule contents and oxidizing agents.

1.4. RESEARCH OBJECTIVES

Surgical septic complications in patients with obstructive jaundice may be related to possible neutrophil dysfunction. This dysfunction may occur at the level of neutrophil recruitment and/or microbicidal function. We hypothesized that neutrophil functions are impaired in cholestasis and contribute to the high incidence of post-operative septic complications in cholestatic patients. To test our hypothesis, *in vivo* and *in vitro* studies were carried out in a rat model of acute cholestasis due to bile duct resection.

The rationale for choosing the bile duct resected rat model of acute cholestasis was that this model has been well characterized and widely used and in addition, it simulates the human condition (Cameron and Oakley, 1932; Kountouras et al., 1984; Greve et al., 1990). The effects of cholestasis on neutrophil functions were studied at day 5 when animals were cholestatic and had recovered from surgical trauma.

The main objectives of the study were:

a) to determine if neutrophil recruitment to inflammatory sites is impaired in cholestasis and to determine the mechanisms contributing to such an impairment. An *in vivo* study of carrageenan-induced acute inflammation was performed to verify if neutrophil recruitment to inflammatory sites was impaired in cholestasis. Carrageenan was chosen because it is a potent inducer of acute inflammation and has been studied extensively (Di Rosa, 1972). Since circulating glucocorticoid levels are elevated in cholestatic rats (Swain and Maric, 1994), we also investigated the effects of endogenous glucocorticoids on neutrophil recruitment.

b) to determine neutrophil adhesion in cholestasis, as firm adhesion is the pre-requisite of neutrophil transmigration. The *in vitro* adhesion of neutrophils to biological substrata was determined using ^{51}chromium-labeled neutrophils.

c) to determine if neutrophil microbicidal functions are impaired in cholestasis. Microbicidal activity was assessed by an *in vitro* time-dependent neutrophil killing of *S. aureus*. Reactive oxidant (e.g. O_2^- and H_2O_2) production and neutrophil degranulation were determined to evaluate factors contributing to an impaired microbicidal activity.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

RU486 (17-β-hydroxy 11 β-(4-dimethylaminophenyl) 17-α (propynyl)-estra 4,9 dien-3-one), a glucocorticoid receptor antagonist, was kindly provided by Roussel-UCLAF, France. The monoclonal antibody to rat CD18 (CL26) was provided by Upjohn, Kalamazoo, MI. The LTB_4 antibody was a kind gift from Merck Frosst (Montreal, QC, Canada). *S. aureus* (strain 502A) was kindly provided by Dr. Woodman, Faculty of Medicine, University of Calgary, Calgary, Alberta. ^3H-LTB_4 was obtained from Amersham Canada (Oakville, ON, Canada), LTB_4 standard was from Cayman Chemicals. Co. (Ann Arbor, MI), rat epithelial cells (IEC-18) were from American Tissue Culture Collection (Rockville, MD), TNF-α was from Knoll Pharmaceutical Co. (Whippany, NJ), Diff Quick Solution was from Baxter Health Care Corp. (Miami, FL), charcoal and gelatin type B were from Fisher Scientific (Edmonton, AB, Canada), safranin-o from BDH, Inc. (Toronto, ON, Canada) and all other reagents were from Sigma Chemicals (St. Louis, MO).

2.2. ANIMAL MODEL OF CHOLESTASIS

Male Sprague-Dawley rats (150-200 g) were obtained from Charles River, St. Constant, PQ, Canada, and either bile duct resected (BDR) or sham resected (sham) as previously described (Cameron and Oakley, 1932; Swain et al., 1995). Briefly, laparotomy was performed under halothane general anesthesia. In BDR rats, the bile duct was doubly ligated and resected between the two ligatures. In sham rats, the bile duct was isolated but without ligation or resection. The animals were housed in a light-controlled room with a 12 hour day/night cycle and were given free access to rat chow and water. Animals were handled humanely under the University of Calgary Animal Care Committee Guidelines.

Animals were studied five days post-surgery. On day 5, BDR animals showed clinical evidence of jaundice with dark urine and icteric plasma. Cholestasis was confirmed biochemically by a significant increase in plasma bilirubin and alkaline phosphatase levels.

2.3. SERUM BIOCHEMISTRIES

Peripheral blood was collected on day 5 post-surgery from rat inferior vena cavae into syringes containing anticoagulant ACD (0.14M citric acid, 0.2M sodium citrate, 0.22M dextrose). Plasma was obtained following centrifugation at 1000 *g* for 10 minutes at room temperature and stored at -20°C until assayed. Plasma bilirubin and alkaline phosphatase levels were determined using commercially available kits (Sigma, St. Louis, MO).

2.3.1. Alkaline phosphatase

Alkaline phosphatase is a normal constituent of the bile, hence an important diagnostic marker for cholestasis. It was determined by a colorimetric assay which was quick (15 minute incubation) and simple. Upon hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase, p-nitrophenol and inorganic phosphate are formed. When made alkaline with 2-amino-2-methyl-1-propanol buffer solution, p-nitrophenol is converted to a yellow complex readily measured at 410 nm. The intensity of color formed is proportional to alkaline phosphatase activity (International Units per liter = IU/L).

2.3.2. Plasma bilirubin

In this assay, bilirubin is covalently coupled to diazotized sulfanilic acid (p-diazobenzenesulfonic acid) to form azobilirubin. The color of this derivative is pH

dependent: pink in acid or neutral medium and blue under alkaline conditions. The final color is measured at 600 nm, as measurement of the blue form in alkaline medium provides greater sensitivity and eliminates potential problems from protein precipitation (Michaelsson, 1961). The bilirubin level is calculated from a calibration curve prepared from bilirubin reference, described in mg/dL.

Two types of serum bilirubin can be distinguished and quantitated by the diazo reaction. First, the direct form, consisting of conjugated water soluble derivatives, is diazotized in the absence of an "accelerating" agent (caffeine-benzoate-acetate mixture). Second, the indirect form, constitutes free (unconjugated) bilirubin bound to serum albumin, reacts with diazo reagent in the presence of the accelerating agent. This agent facilitates the coupling of albumin-bound bilirubin with the diazo reagent to form azobilirubin. The total bilirubin is the sum of the direct and indirect forms.

2.4. NEUTROPHIL ISOLATION

Isolated neutrophils were used for *in vitro* studies of neutrophil adhesion to biological substrata (Chapter 5) and of neutrophil microbicidal functions (Chapter 6). Rat neutrophils were isolated five days after surgery by dextran (6% dextran in 0.9% NaCl) sedimentation of anticoagulated peripheral blood followed by hypotonic lysis of red blood cells. Cell suspensions were layered on histopaque solution (Sigma, St. Louis, MO) followed by centrifugation at 4°C. Histopaque solution is a polysucrose and sodium diatrizoate solution which facilitates rapid separation of granulocytes from lymphocytes and mononuclear cells (Boyum, 1968). This isolation procedure yielded >95% neutrophils with >90% viability using the trypan blue dye exclusion method (Swain et al., 1995).

Neutrophils were suspended in cold phosphate buffered saline (PBS; pH 7.4) at $2x10^7$ cells/ml for adherence assays. For other functional assays, neutrophils were suspended in cold PBS+++ (PBS containing 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$ and 7.5 mM glucose) at $2x10^7$ cells/ml.

2.4.1. Trypan blue exclusion test

The percentage of viable neutrophils in a cell suspension was determined by staining cell populations with trypan blue. Viable cells exclude the dye, while nonviable cells take up the dye. This contrast allows a direct visual distinction between unstained viable cells and blue-stained nonviable cells. The 0.4% (w/v) trypan blue solution (Sigma, St. Louis, MO) was prepared ahead of time and stored at room temperature. On the day of use, 0.5 ml of trypan blue solution was diluted with 0.3 ml of Hanks' Balanced Salt Solution (HBSS) (Sigma Chemicals, St. Louis, MO). Cells suspensions were diluted 1:5 with trypan blue-HBSS solution mixture, mixed thoroughly and let sit for 5 minutes before placing cells in a hemocytometer. It is critical to count cells immediately, as extended exposure to trypan blue will result in the dye being taken up by both viable and nonviable cells. The percentage of viable cells was calculated by the following formula:

$$\text{Percentage viable cells} = \frac{\text{No. of viable cells}}{\text{No. of viable cells + No. of dead cells}} \times 100\%$$

2.5. STATISTICAL ANALYSIS

Data are expressed as mean $\pm$ SEM. An unpaired student's t-test was used for comparisons between two means, and ANOVA followed by Student-Newman-Keul test for comparisons between more than two means. P value ≤ 0.05 is considered significant.

CHAPTER 3

STUDY I : NEUTROPHIL RECRUITMENT TO INFLAMMATORY SITES IN CHOLESTASIS

3.1. INTRODUCTION

To perform their function in the defense against infection, neutrophils must first reach the site of inflammation through a multi-step process (Springer, 1994). We hypothesized that the increased incidence of post-surgical infective complications in cholestatic patients may be related to the inability of neutrophils to reach inflammatory sites.

To test this hypothesis, acute inflammation was induced subcutaneously in sham and BDR animals with carrageenan, a model commonly used to study mechanisms of cellular infiltration in acute inflammation (Laue et al., 1988; Salvemini et al., 1995).

3.2. AIM

The aim of this study was to determine if cholestasis impairs the development of an acute inflammatory response.

3.3. METHODS

3.3.1. *In vivo* model of acute inflammation

Acute inflammation was induced in BDR and sham rats five days post-surgery by a subcutaneous injection of carrageenan solution according to the method of Laue *et al.* (1988). Briefly, 8 ml of air was injected subcutaneously on the back of the animals 24 h prior to carrageenan administration. Four ml of 2% carrageenan (Type IV, Sigma Chemicals, St. Louis, MO) in 0.9% NaCl were injected into the preformed air pouch. The animals were sacrificed five hours later, the time at which the inflammatory response is

maximal in both BDR and sham resected animals (Swain et al., 1995). The inflammatory exudate in the air pouch was extracted using a 16 gauge needle attached to a 10cc syringe and was quantitated as: a) exudate volume (mls); b) total cell count (cell number x 10^7/ml) as determined using a Coulter Counter (Coulter Channelyzer 256, Coulter Electronics, Hialeah, FL) and c) MPO activity (Units/ml). MPO is an enzyme found in high concentrations in the intracellular granules of neutrophils and is a useful neutrophil marker (Bradley et al., 1982).

3.3.2. Myeloperoxidase activity

The principles behind this assay are outlined below:

$$H_2O_2 \xrightarrow{\text{MPO}} H_2O + O^-$$

$$O^- + AH_2 \longrightarrow H_2O + A$$

This enzyme breaks down H_2O_2, yielding oxygen radical (O^-). This oxygen radical combines with O-dianisidine dihydrochloride, the hydrogen donor (AH_2), which is converted to a colored compound (A). MPO acitivity is determined spectrophotometrically from the appearance of this colored compound over time.

MPO was extracted from cells by suspending the inflammatory exudate in 0.5% hexadecyltrimethylammonium bromide (Sigma Chemicals, St. Louis, MO) in 50 mM potassium phosphate buffer, pH 6.0, before sonication in an ice bath for 20 seconds. Suspensions were then centrifuged at 14,000 rpm for 2 min in an Eppendorf microfuge and the resulting supernatant was assayed for MPO activity using a plate scanner (Titertek Multiskan MCC/340).

3.3.3. Cell differentials

Cell differentials were performed to obtain approximate neutrophil distributions peripherally and in the inflammatory exudate. Peripheral blood leukocyte differentials of BDR and sham rats were determined using blood smears stained with the Diff Quick Method (Baxter Healthcare Corp., Miami, FL). Inflammatory exudate leukocyte differentials were determined on cytospot preparations as described previously (Motyka et al., 1993). Briefly, cytospots of inflammatory exudates were prepared by cytocentrifuging diluted samples at 900 rpm for 5 minutes onto specimen slides using a Shandon Cytospin (Shandon Southern Instruments, Sewickley, PA). Cytospots were fixed and stained with Diff Quick reagent (Baxter Healthcare Corp., Miami, FL). Neutrophil distributions were determined by viewing slides under oil immersion using a light microscope.

3.4. RESULTS

3.4.1. Characterization of cholestasis

Alkaline phosphatase and total bilirubin in sham animals were within normal ranges (39-117 IU/L and 0.2 to 1.2 mg/dL, respectively) (Gambino and Di Re, 1965; Swain et al., 1995). In addition to jaundice, yellow urine and icteric plasma, BDR rats had significantly elevated total plasma bilirubin and alkaline phosphatase levels. Alkaline phosphatase levels in BDR animals were almost twice that of sham animals and total bilirubin was increased by approximately 20 times in BDR rats (Table 3.1).

3.4.2. Inflammatory response

Neutrophil accumulation at inflammatory sites was significantly reduced in BDR animals compared to their sham counterparts. A reduction in the inflammatory response in BDR animals was represented by a significant decrease in carrageenan-induced exudate volume, total cell count and MPO activity (Figures 3.1 - 3.3). Exudate volume of BDR rats was significantly reduced when compared to sham animals (Figure 3.1.; BDR: 2.68 ± 0.2 mls vs sham: 3.63 ± 0.2 mls; $p<0.01$). Furthermore, cell count was reduced in BDR rats compared to sham animals (Figure 3.2; BDR: $(1.58 \pm 0.3)x10^7$ cells/ml vs sham: $(4.91 \pm 1.0)x10^7$ cells/ml; $p<0.01$). Impaired neutrophil accumulation during the acute inflammatory response in BDR rats was confirmed by a significant decrease in the exudate MPO acitivity (Figure 3.3; BDR: 11.72 ± 1.64 U/ml vs sham: 18.25 ± 1.52 U/ml; $p<0.05$)

3.4.3. Cell differentials

The peripheral blood neutrophil count in BDR rats was significantly higher than in sham rats (Table 3.2; $p<0.01$). In contrast, neutrophils in inflammatory exudate were markedly reduced in BDR rats compared with sham-resected rats (Table 3.2; $p<0.001$).

Table 3.1. Serum biochemistries in sham and BDR rats five days post-surgery. Data represent the mean ± SEM of n=4 animals per group.

	Sham	BDR
Alkaline phosphatase (IU/L)	112.8 ± 11.3	206.7 ± 11.4 [a]
Total bilirubin (mg/dL)	0.30 ± 0.11	5.79 ± 0.26 [b]

[a] $p<0.005$ vs sham.

[b] $p<0.0001$ vs sham.

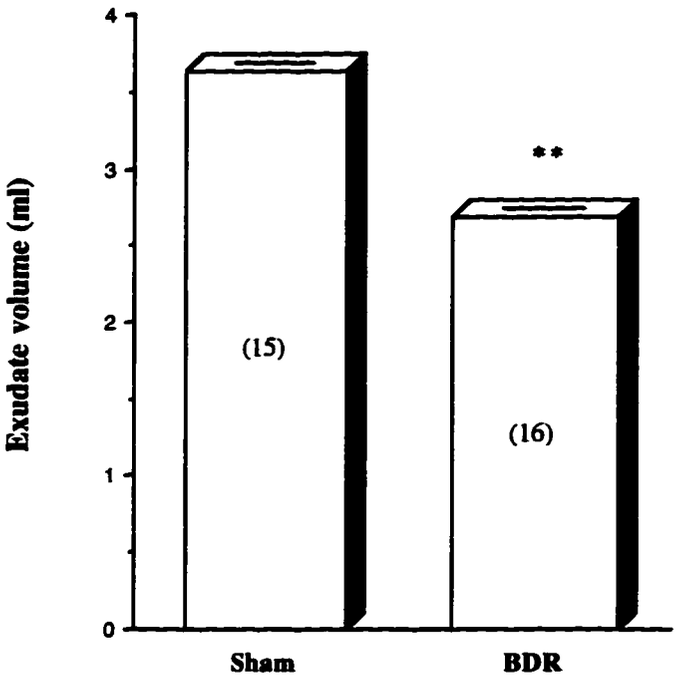

Figure 3.1. Exudate volume 5 hours after the induction of a localized inflammatory response with carrageenan in sham and BDR rats. Data are mean ± SEM of number of animals for each group shown in parenthesis. **$p<0.01$ vs respective sham value.

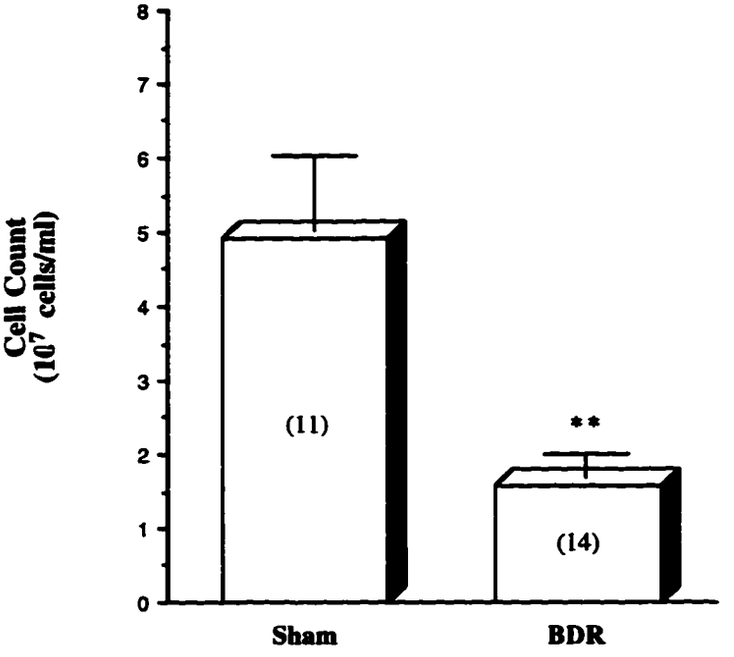

Figure 3.2. Carrageenan-induced leukocyte infiltration 5 hours after carrageenan injection in sham and BDR rats. Data are mean ± SEM of number of rats shown in parenthesis. **p<0.01 vs respective sham value.

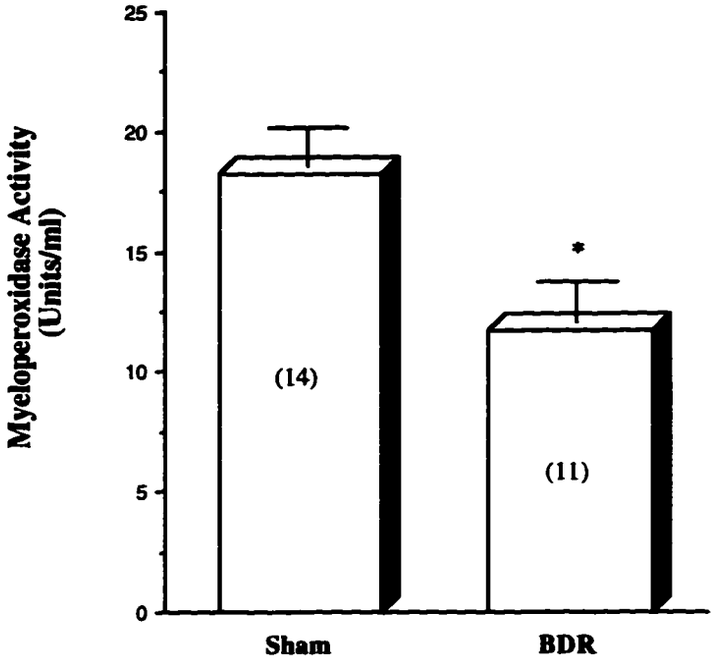

Figure 3.3. Inflammatory exudate myeloperoxidase activity measured 5 hours after carrageenan injection in sham and BDR rats. Data are mean ± SEM of number of rats shown in parenthesis. *$p<0.05$ vs respective sham value.

Table 3.2. Leukocyte differentials of whole blood and inflammatory exudate from BDR and sham resected rats five days after surgery. Data represent the mean ± SEM of n=4 animals per group and are expressed as number of cells x 10^6/ml.

	Peripheral blood neutrophils	Inflammatory exudate neutrophils
sham	5.2 ± 0.7	40.2 ± 1.0
BDR	8.5 ± 0.4 [a]	13.1 ± .0.3 [b]

[a] $p<0.01$ vs sham.

[b] $p<0.001$ vs sham.

3.5. DISCUSSION

BDR animals demonstrated an impaired inflammatory response as indicated by reduced cellular infiltration to inflammatory sites (Figures 3.1-3.3). Two possibilities exist for the significant decrease in the inflammatory response in BDR animals: 1) reduced neutrophil accumulation at sites of inflammation may be related to reduced circulating neutrophil levels, or 2) neutrophils in BDR animals are less capable of migrating to inflammatory sites. To resolve this issue, we performed peripheral blood cell differentials. The levels of circulating neutrophils were significantly higher in BDR than in sham animals (Table 3.2). Thus, it did not appear that the decrease in cellular infiltration to inflammatory sites in BDR animals was contributed to by a lower blood neutrophil count. Despite increased circulating neutrophils in BDR animals, these neutrophils could not be recruited as effectively as sham neutrophils as there were significantly less neutrophils in the inflammatory exudate of BDR than sham rats (Table 3.2).

These results suggest that there are defects in neutrophil recruitment in cholestatic rats. The defect underlying this impairment in neutrophil recruitment is unknown, however, I will speculate in the following paragraphs as to possible mechanisms for this observed impairment.

Plasma membrane physical properties may play an important role in modulating neutrophil movement to inflammatory sites. The composition of cell plasma membranes can influence these properties, as saturated fatty acids and cholesterol affect membrane rigidity (Stryer, 1988). Plasma cell membranes in patients with extrahepatic cholestatic jaundice contain more saturated fatty acids than controls (Scriven et al., 1994). The shift in fatty acid composition to a more saturated form in cholestatic plasma membranes may result in a

stiffer membrane. In addition, Lang *et al.* (1995) documented an increased cholesterol content and membrane rigidity of white blood cell membranes in BDR rats. Increased cell stiffness due to neutrophil activation by fMLP was shown to induce cell retention in lung capillaries (Worthen et al., 1989). In addition, Worthen *et al.* showed that pretreatment with cytochalasin D (which inhibits cell stiffening due to cell activation) appeared to reduce cell retention in capillaries. Therefore, it is possible that the changes in neutrophil plasma membrane composition to a more rigid state may contribute to impaired neutrophil recruitment to inflammatory sites in BDR animals.

Neutrophil transmigration requires adhesion molecules on neutrophils and endothelial cells. Inactivation of CD11/CD18 and ICAM-1 by MAbs significantly reduces neutrophil transmigration (Furie et al., 1991; Harlan et al., 1992). In BDR animals the upregulation of these adhesion molecules may be depressed and thus contribute to impaired neutrophil recruitment to inflammatory sites. Chemotactic factors such as PAF and LTB4 can upregulate CD11/CD18 on neutrophils (Kishimoto and Anderson, 1992), while cytokines (e.g. IL-1 and TNF) stimulate endothelial cells to express ICAM-1 (Dustin et al., 1986; Pober et al., 1986). Therefore, these mediators (e.g. chemoattractants and cytokines) are crucial for the development of an inflammatory response and the production/release of these mediators may be depressed in BDR animals.

BDR animals have been documented to have elevated levels of circulating endogenous glucocorticoids (Swain and Maric, 1994), and glucocorticoids inhibit LTB4 production (Flower et al., 1986), and the release of IL-1 and TNF (Bochner et al., 1987; Zuckerman et al., 1989; Chensue et al., 1991). Furthermore, proinflammatory mediators such as prostaglandins (PGs) and LTs are synthesized from arachidonic acid. Since the levels of polyunsaturated fatty acids (e.g. arachidonic acid) in jaundiced patients were reported to be

lower than in controls (Scriven et al., 1994), the production of LTs and PGs may be correspondingly lower. This, and the possible depressed cytokine release due to effects of increased levels of endogenous glucocorticoids may contribute to impaired neutrophil recruitment to inflammatory sites in BDR animals (further discussed in Chapter 4). In carrageenan-induced pleurisy in rats, neutrophil recruitment was correlated to the levels of inflammatory mediators whose production was mainly controlled by glucocorticoids (Flower et al., 1986). Adrenalectomized rats (devoid of glucocorticoids) had a greater inflammatory response with higher levels of mediators in the inflammatory exudate when compared to sham animals (Flower et al., 1986). In addition, Laue *et al.* (1988) demonstrated that the inflammatory response following carrageenan-induced subcutaneous inflammation was inhibited by dexamethasone in a dose-dependent manner, which moreover was correlated with diminishing levels of inflammatory mediators (e.g. LTB_4 and PGE_2) in the inflammatory exudate.

Therefore, the increased incidence of wound infection in BDR animals and cholestatic patients (Arnaud et al., 1981; Pitt et al., 1981) may be a consequence of impaired neutrophil recruitment to inflammatory sites, and may contribute to the increased incidence of post-surgical septic complications in BDR animals and cholestatic patients.

3.6. SUMMARY

BDR rats demonstrate impaired neutrophil accumulation at inflammatory sites indicated by a reduction in exudate volume, neutrophil count and MPO activity. This impairment, however, was not due to lowered number of circulating neutrophils in BDR animals.

CHAPTER 4

STUDY II : THE EFFECTS OF ENDOGENOUS GLUCOCORTICOIDS UPON NEUTROPHIL RECRUITMENT TO INFLAMMATORY SITES IN CHOLESTASIS

4.1. INTRODUCTION

Glucocorticoids are steroid hormones that are endogenously synthesized and released by the adrenal cortices (Goldstein et al., 1992). The hypothalamus releases corticotropin releasing hormone (CRH) which stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the adrenal cortex to secrete cortisol, the major form of glucocorticoids produced in humans. In response to stress, higher amounts of CRH and consequently ACTH and cortisol are released. Cortisol, in addition to its metabolic, cardiovascular, immunomodulatory, and behavioral effects, exerts a negative feedback effect on the secretion of CRH and ACTH (reviewed in Chrousos and Gold, 1992).

Glucocorticoids are known to suppress the inflammatory response *in vivo* (Laue et al., 1988; Peers et al., 1988; Salvemini et al., 1995). They influence the trafficking of circulating leukocytes and inhibit many functions of leukocytes and immune accessory cells (reviewed in Goldstein et al., 1992). Glucocorticoids also affect inflammatory events (e.g. vasodilation) and alter the production and release of many inflammatory mediators (e.g. PGs and LTs) which results in an inhibition of leukocyte accumulation and plasma exudation at sites of inflammation (Laue et al., 1988; Sternberg et al., 1990).

Inflammatory mediators and cytokines are important in neutrophil recruitment. First, local release of cytokines upregulate the endothelial selectins which facilitate neutrophil margination/rolling. Second, neutrophil integrins are upregulated upon exposure to the local cytokines and chemotactic factors which mediate adhesion; these stimuli also upregulate adhesion molecules on the endothelium. Finally, integrin-endothelial interaction result in morphological changes facilitating emigration into the tissues.

Glucocorticoids have been reported to inhibit the release of several cytokines such as IL-1 and TNF (Bochner et al., 1987; Zuckerman et al., 1989; Chensue et al., 1991). These cytokines participates in the multi-step process of neutrophil recruitment. In addition, glucocorticoids have been shown to down-regulate the cytokine-mediated enhanced expression of ICAM-1 on the endothelium (Cronstein et al., 1992b). ICAM-1, a receptor ligand for adhesion molecules on leukocytes (CD11/CD18), is necessary for the firm adhesion between leukocyte and endothelium. Furthermore, glucocorticoids inhibit the release of LTB_4, an important chemoattractant. LTB_4 has previously been shown to play a central role in neutrophil recruitment in carrageenan-induced acute inflammation (Higgs et al., 1988; Martins et al., 1989; Salvemini et al., 1995). Finally, Peers *et al.* (1988) demonstrated that administration of dexamethasone (a synthetic glucocorticoid) inhibited the formation of inflammatory exudate, leukocyte infiltration and release of inflammatory mediators in the rat carrageenan-induced pleurisy model of acute localized inflammation.

We have shown previously that circulating endogenous glucocorticoid levels are elevated in a rat model of acute cholestasis due to bile duct resection (Swain and Maric, 1994). Since glucocorticoids are anti-inflammatory, and levels of endogenous glucocorticoids are elevated in cholestatic rats, we hypothesized that elevated circulating glucocorticoid levels in cholestasis may impair the development of an acute inflammatory response. We further proposed that elevated levels of endogenous glucocorticoids may impair neutrophil recruitment through inhibition of LTB_4 production.

4.2. AIMS

The aim of this study was: 1) to determine the effects of elevated endogenous glucocorticoids upon neutrophil recruitment during carrageenan-induced acute inflammation in cholestatic rats, and 2) to determine if elevated endogenous glucocorticoids inhibit the release of LTB4 at inflammatory sites in cholestasis.

4.3. METHODS

4.3.1. RU486 treatment

Male Sprague-Dawley rats underwent surgery as described in Chapter 2. However, to study the involvement of circulating endogenous glucocorticoids in the control of the inflammatory response in cholestasis, BDR and sham rats were pretreated 1 hour before carrageenan injection with either the glucocorticoid receptor antagonist RU486 (Roussel-UCLAF, France) given intraperitoneally at 2mg/kg in normal saline, or saline vehicle. This dose of RU486 effectively inhibits glucocorticoid activity *in vivo* (Swain and Maric, 1994). Subcutaneous acute inflammation was induced by 2% carrageenan solution injection into preformed air pouches as described earlier and inflammatory exudates were collected 5 hours later for volume, cell count, MPO activity and LTB4 content.

4.3.2. LTB4 Assay

LTB4 in inflammatory exudates of sham and BDR rats obtained 5 hours after carrageenan injection was measured by radioimmunoassay (RIA) (Wallace et al., 1992). The assay buffer and dextran-coated charcoal were prepared ahead of time and stored at

4°C. The assay buffer consisted of 0.5M Tris buffer containing 0.1% w/v gelatin, pH 8.6. For 500 ml buffer, 39.4 g Tris:HCl was combined with 30.3 g Tris:Base and 0.5 g gelatin in distilled water. Dextran-coated charcoal consisted of 2% charcoal and 0.4% dextran in assay buffer. To make 200 ml of dextran-coated charcoal, 0.8 g dextran and 4.0 g charcoal were mixed in assay buffer.

Polypropylene tubes (12mm x 75mm) were labeled in duplicate for each sample dilution (undiluted and 1:2), total counts, non-specific binding, zero standard and standard curve. The samples were thawed, and centrifuged on high speed in a benchtop Eppendorf microfuge for 2 min. Aliquots of samples were pipetted into test tubes and tubes were placed in a boiling water bath for 2 min to increase the detectable levels of LTB4, presumably by removing a factor that interferes with the assay (Peskar et al., 1986). Assay buffer was added to the tubes to give required sample dilutions, and the samples were vortexed.

The stock solution of tritiated LTB4 (^{3}H-LTB4; Amersham) consisted of 5 μCi of ^{3}H-LTB4 in 250 μl methanol:distilled water:acetic acid (60:40:0.1) and was adjusted to pH 5.6 with dilute ammonium hydroxide. Two μl of stock ^{3}H-LTB4 was added to 1 ml of assay buffer and 100 μl of this solution was used per assay tube to give total counts of approximately 5000 cpm. Approximately 0.5 μl of stock solution of the antibody to LTB4 (α-LTB4) was added to 1 ml of assay buffer. To obtain a dilution of 1:1700, 100 μl of α-LTB4 was added to each assay tube. The LTB4 standard (Cayman Chemicals) was prepared by diluting 5 μl of stock (0.1 mg/ml in methanol) with 1 ml of assay buffer to give 500 ng/ml LTB4. This solution was used for a series of standards.

For the LTB_4 standard curve, 8 polypropylene tubes were labeled A to H and 500 µl of assay buffer was added to each tube except tube H which received 60 µl of 500 ng/ml LTB_4 and 1875 µl of assay buffer. Tube H was vortexed thoroughly and gave rise to 16 ng/ml solution of LTB_4. 500 µl of solution H was pipetted into tube G and this serial dilution was continued successively with the remaining tubes. Aliquots of 100 µl from each serial dilution gave 8 standard levels of LTB_4 ranging from 12.5 pg/tube to 1.6 ng/tube.

Once the reagents were prepared, 200 µl of assay buffer were pipetted into the non-specific binding tubes and the total count tubes, and 100 µl was pipetted into the zero standard tubes. Starting with the most dilute standard, 100 µl of each standard was pipetted into the appropriately labeled tubes as was 100 µl of each diluted sample. 100 µl of ^3H-LTB_4 was pipetted into all tubes, and 100 µl of α-LTB_4 was pipetted into all tubes except the non-specific binding tubes and the total count tubes. Tubes were vortexed thoroughly and incubated for 2 h at room temperature.

Following the 2 h incubation, 200 µl of assay buffer was pipetted into the total count tubes and 200 µl of dextran-coated charcoal, which was being continuously stirred with a magnetic stirrer, was added to all tubes except the total count tubes, and then all tubes were vortexed. Tubes were allowed to sit for 5 min following the addition of dextran-coated charcoal to the last tube and centrifuged at 2000 *g* for 10 min at 4°C. After centrifugation, the supernatant was decanted into scintillation vials and care was taken not to disturb the pellet. Scintillation fluid (4ml) was added to the vials, the vials were capped and shaken, and then placed in a LKB 1214 RACKBETA β-scintillation counter and counted for 5 min. The values were used to calculate percent binding and the amount of LTB_4 in each sample was determined based on a standard curve of percent ^3H-LTB_4 bound.

4.3.3. Plasma corticosterone Levels

Truncal blood was collected five hours after carrageenan injection into prechilled plastic EDTA-containing tubes. Since there is a circadian rhythm of corticosterone release in rats (Hilfenhaus, 1976), truncal blood was collected at similar times in each experiment to minimize inconsistencies between samples. Corticosterone is the principle glucocorticoid secreted by the adrenal cortices of rats (Shimizu et al., 1983). Plasma was separated by centrifugation at 1000 *g* for 10 minutes and frozen at -20°C until assayed. Total corticosterone levels were measured using a sensitive ^{125}I-RIA kit (ImmuChem, ICN Biochemicals, Inc. Costa Mesa, CA). Free plasma corticosterone levels were determined using the Centricon method (Amicon, Beverly, MA; Swain and Maric, 1994).

All reagents in corticosterone ^{125}I-RIA kit were shipped ready to use. Using steroid diluent, rat plasma was diluted 1:200 by adding 10 µl of sample to 2.0 ml diluent. Steroid diluent was added into non-specific binding and zero tubes. Known concentrations of corticosterone (ranging from 25 ng/ml to 1000 ng/ml) were added into separate tubes for the calibration curve. Diluted samples and controls were placed in the pre-labeled unknown and control tubes. 200 µl of radio-labeled corticosterone (^{125}I-corticosterone) was added to all tubes followed by 200 µl of a monoclonal anti-corticosterone antibody. All assay tubes were vortexed and incubated at room temperature for 2 hours. Following incubation, 500 µl of precipitant solution was added to all tubes. All assay tubes were then centrifuged at 1000*g* for 15 minutes. The supernatant was aspirated and the precipitate was immersed in scintillation fluid and counted in an automatic gamma counter (WALLAC Wizard 1480, WALLAC Oy, Turku, finland). This assay was run in duplicate and the % bound was calculated by the following formula:

$$\%B/Bo = \frac{CPM\ (sample) - CPM\ (NSB)}{CPM\ (0\ calibrator) - CPM\ (NSB)} \times 100$$

CPM = average counts of duplicates

NSB = non-specific binding (also known as blank tube)

0 Calibrator = 0 tube (also known as the 100% binding tube)

The percent bound was plotted versus all of the known concentrations of corticosterone to give a standard curve.

4.4. RESULTS

4.4.1. Plasma glucocorticoid levels

Plasma total corticosterone levels in BDR rats were not significantly different from those in sham resected rats (Fig. 4.1.A; BDR: 326 $\pm$ 35.4 ng/ml vs sham: 399 $\pm$ 29.1 ng/ml). However, the levels of free corticosterone (the biologically active form) in cholestatic rats were approximately five times higher than in sham animals (Fig. 4.1. B; BDR: 56.8 $\pm$ 8.4 ng/ml vs sham: 10.4 $\pm$ 1.3 ng/ml; $p<0.001$).

4.4.2. Inflammatory response and the effects of RU486

Pretreatment of sham and BDR rats with RU486 (2mg/kg, i.p.) was performed to evaluate the effect of elevated levels of bioactive endogenous glucocorticoids in BDR rats on carrageenan-induced acute inflammation. Unlike sham rats, BDR rats pretreated with RU486, when compared to saline-pretreated BDR rats, showed a marked increase in the

exudate volume (BDR+saline vs BDR+RU486: 2.69 ± 0.20 mls vs 3.63 ± 0.21 mls; $p < 0.01$) , cell number (1.58 ± 0.3) x 10^7 cells/ml vs (4.48 ± 1.0) x 10^7 cells/ml; $p<0.05$) and myeloperoxidase activity (11.72 ± 1.64 U/ml vs 18.92 ± 2.30 U/ml; $p<0.05$) (Figures 4.2 to 4.4). The inflammatory response in BDR rats pretreated with RU486 during carrageenan-induced acute inflammation was elevated to levels documented in sham resected rats.

4.4.3. LTB_4 Levels

Inflammatory exudate LTB_4 concentrations were similar in all BDR and sham groups (Figure 4.5). The level of this chemoattractant was not reduced in BDR rats compared to sham animals when pretreated either with saline vehicle (BDR: 1514.40 ± 134.90 pg/ml vs sham: 1885.20 ± 199.2 pg/ml; NS) or RU486 (BDR: 1822 ± 225.70 pg/ml vs sham: 2157 ± 201.60 pg/ml ; NS).

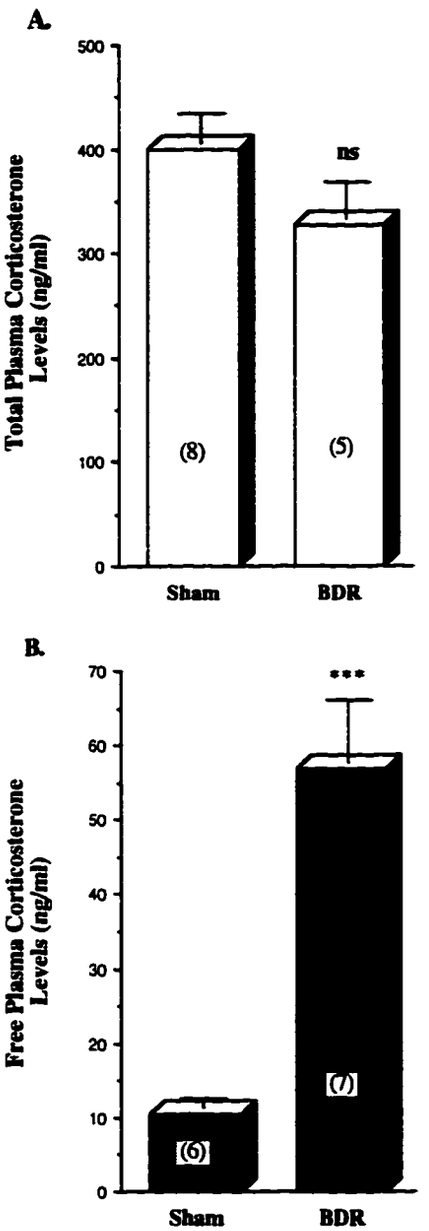

Figure 4.1. Plasma corticosterone levels of truncal blood of sham and BDR rats obtained 5 hours after carrageenan injection. Data are mean ± SEM of number of rats shown in parenthesis. Total plasma corticosterone levels between sham and BDR animals were not significantly different (Fig. 1A). In contrast, the levels of free plasma corticosterone (the bioactive form) were significantly higher in BDR rats (Fig. 1B). ***$p<0.001$ vs sham resected animals.

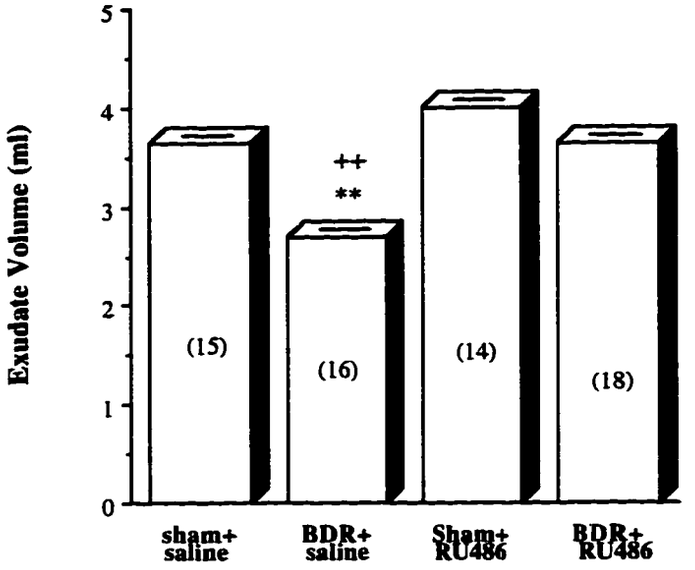

Figure 4.2. Exudate volume 5 hours after the induction of a localized inflammatory response with carrageenan in BDR and sham resected rats. RU486 (2mg/kg; ip) was administered 1 hour prior to carrageenan injection. Data are mean ± SEM of number of animals for each group shown in parenthesis. **p<0.01 vs respective sham value. ++p< 0.01 vs BDR with RU486 pretreatment value.

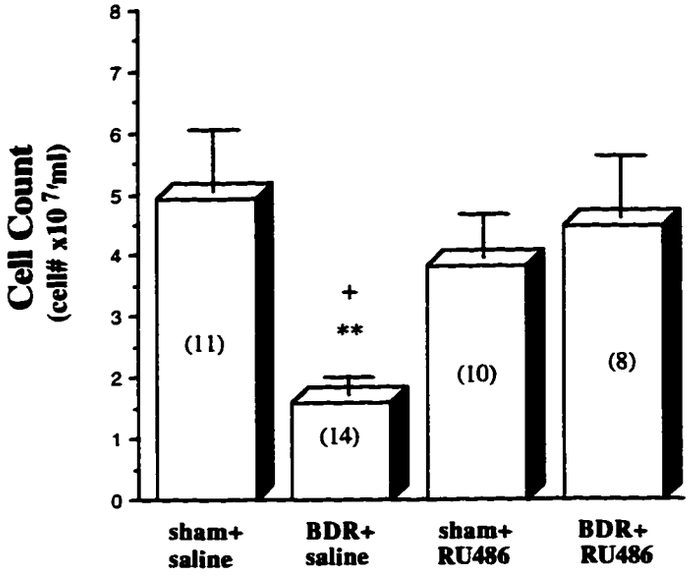

Figure 4.3. Carrageenan-induced leukocyte infiltration 5 hours after carrageenan injection in sham and BDR rats. RU486 (2mg/kg) administered i.p. 1 hour before carrageenan injection. Data are mean ± SEM of number of rats shown in parenthesis. **$p<0.01$ vs respective sham value. +$p<0.05$ vs BDR with RU486 pretreatment value.

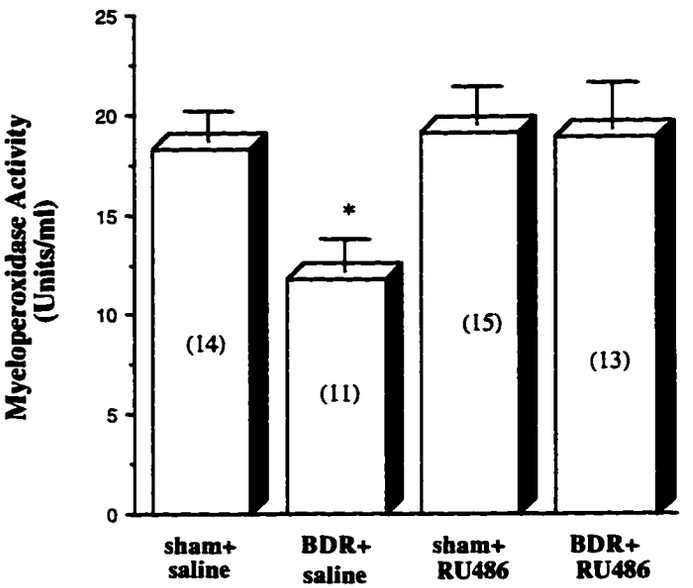

Figure 4.4. Inflammatory exudate myeloperoxidase activity measured 5 hours after carrageenan injection in sham and BDR rats. RU486 (2mg/kg) intraperitoneally injected 1 hour before carrageenan administration. Data are mean ± SEM of number of rats shown in parenthesis. *$p<0.05$ vs respective sham value, and vs BDR with RU486 pretreatment value.

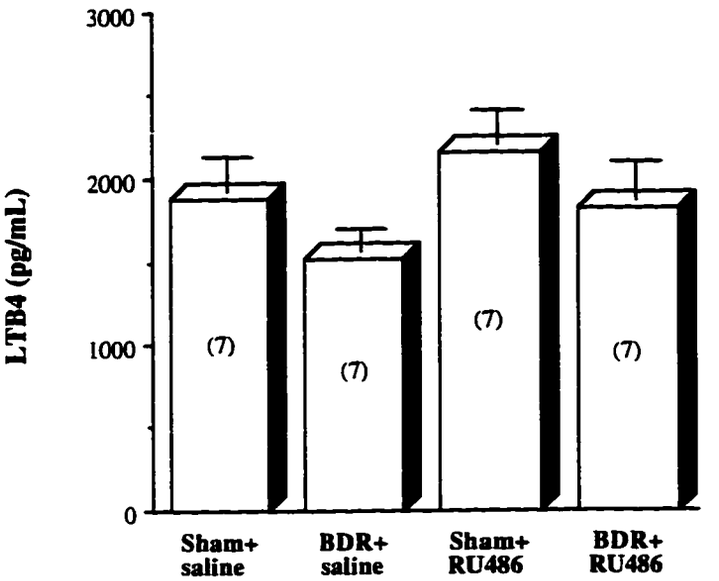

Figure 4.5. The exudate LTB4 levels of sham and BDR rats 5 hours after carrageenan injection determined by RIAs. Data are mean ± SEM of 7 animals per group. When animals were pretreated with either saline or RU486 (2mg/kg i.p.), neither sham nor BDR exudate displayed any significant difference in the levels of LTB4.

4.5. DISCUSSION

The results of this study demonstrate an impaired neutrophil recruitment to a site of acute inflammation in cholestatic rats which appears to be due to anti-inflammatory properties of circulating glucocorticoids in these animals.

Glucocorticoids are capable of inhibiting leukocyte recruitment to inflammatory sites (Laue et al., 1988; Peers et al., 1988; Salvemini et al., 1995). However, most studies in this area have employed the exogenous administration of pharmacological doses of glucocorticoids. Much less is known about the effects of endogenously released glucocorticoids on the inflammatory response.

Over the past several years, however, it has become apparent that the release of endogenous glucocorticoids plays a central role in the control of inflammation (Sternberg et al., 1989; Sternberg et al., 1990). A number of animal models of inflammatory disease are associated with elevated circulating glucocorticoid levels (Sternberg et al., 1989; Sternberg et al., 1990), which appear to dampen inflammatory responses. Specifically, inhibition of endogenous glucocorticoid activity strikingly accentuates joint inflammation in rat models of arthritis (Sternberg et al., 1989). Furthermore, cholestatic rats only develop T-cell dependent adjuvant-induced joint inflammation after endogenous glucocorticoid activity has been blocked (Swain and Maric, 1994).

Therefore, given that circulating endogenous glucocorticoid levels are elevated in BDR rats (Swain and Maric, 1994) and that glucocorticoids inhibit the development of acute inflammation, we examined the role of endogenous glucocorticoids in the control of neutrophil recruitment into an inflammatory site in cholestatic rats. Eliciting an acute

inflammatory response by injecting carrageenan subcutaneously resulted in a significant elevation, of similar magnitude, in total plasma corticosterone levels in both BDR and sham rats. However, levels of free corticosterone (the bioactive form) were more than five times higher in BDR compared to sham rats. Furthermore, inhibition of endogenous glucocorticoid activity by pretreatment with the glucocorticoid receptor antagonist RU486 prior to carrageenan treatment resulted in a significant increase in the acute inflammatory response and neutrophil accumulation in BDR rats, but not sham rats. These results suggest that the high levels of free corticosterone in BDR rats appear to be sufficient to significantly inhibit neutrophil recruitment and the development of an acute inflammatory response in these animals.

The mechanism(s) by which endogenous glucocorticoids inhibit neutrophil recruitment to inflammatory sites are poorly understood. Glucocorticoids may inhibit neutrophil recruitment during inflammation by acting at any stage of the multi-step process of leukocyte recruitment (rolling/adhesion/extravasation). Glucocorticoids have been shown to be capable of down-regulating L-selectin expression on bovine neutrophils (Burton et al., 1995). L-selectin expression is important for the control of neutrophil rolling along vascular endothelium, a pre-requisite for β2 integrin-mediated neutrophil adhesion to vascular endothelium at physiologic shear rates *in vivo* (von Andrian et al., 1992). However, we have previously demonstrated that leukocyte rolling is similar in BDR and sham rats (Swain et al., 1995), suggesting that elevated endogenous glucocorticoid levels are not interfering with rolling in BDR rats.

Glucocorticoids are capable of inhibiting the generation of inflammatory mediators, including proinflammatory cytokines (eg. IL-1, TNFα) and chemoattractants (eg. LTB_4). Inflammatory mediators are important in mediating neutrophil-endothelial interactions,

leading to neutrophil accumulation at inflammatory sites. For example, TNFα stimulates the vascular endothelium to increase adhesion molecule expression (e.g. ICAM-1) thereby enhancing neutrophil recruitment, and LTB_4 acts upon the neutrophil to enhance CD11/CD18 expression thereby augmenting neutrophil adhesion (reviewed in Tonnesen, 1989).

Since LTB_4 has been previously shown to play a central role in neutrophil recruitment in carrageenan-induced acute inflammation, we quantitated LTB_4 in inflammatory exudates of saline or RU486 pretreated BDR and sham rats. LTB_4 levels were similar in the saline and RU486 pretreated BDR and sham rats, suggesting that elevated endogenous glucocorticoid levels in BDR rats do not inhibit leukocyte recruitment by inhibiting LTB_4 generation at the inflammatory site.

Recently, Perretti and Flower (1994) showed that a direct injection of human recombinant IL-1β into murine air pouches increased neutrophil accumulation in the air pouch by approximately five times, and dexamethasone inhibited neutrophil accumulation in a dose-dependent manner. In addition, glucocorticoids inhibit cytokine-induced expression of endothelial adhesion molecules, e.g. ICAM-1 (Cronstein et al., 1992b). Therefore, we speculate that elevated endogenous glucocorticoid levels in BDR rats may inhibit carragenan-induced neutrophil accumulation by blocking cytokine-induced enhanced expression of vascular endothelial adhesion molecules at the site of inflammation. To test this possibility, future studies examining ICAM-1 expression at inflammatory sites in BDR animals would be warranted.

4.6. SUMMARY

We have confirmed an inhibition of the acute inflammatory response and neutrophil accumulation in BDR rats. Furthermore, we have demonstrated that this impairment is due to the anti-inflammatory properties of endogenously secreted glucocorticoids in BDR animals as pretreatment with RU486 increased the inflammatory response in BDR animals to levels documented in sham animals. Endogenous glucocorticoids in BDR rats did not impair neutrophil accumulation at inflammatory sites through inhibition of LTB_4 production.

CHAPTER 5

STUDY III: NEUTROPHIL ADHESION IN CHOLESTASIS: AN *IN VITRO* STUDY

5.1. INTRODUCTION

We demonstrated that neutrophil recruitment is impaired in cholestasis and this impairment is due at least in a major part to the anti-inflammatory effects of endogenous glucocorticoids. Since there are several steps involved in neutrophil recruitment, the impairment could take place at one or several stages during this process. We have previously demonstrated that neutrophil rolling along the mesenteric venules in cholestatic rats is similar to that of control animals (Swain et al., 1995). In addition, the number of adherent neutrophils was similar under basal conditions. However, neutrophil adhesion to the venular endothelium was impaired in BDR rats when the mesenteric vasculature was superfused with 2 nM PAF (Swain et al., 1995). Upon binding to its receptor on neutrophils, PAF upregulates adhesion molecules on neutrophils (CD11/CD18) which mediate neutrophil binding to endothelium (Tonnesen, 1989). In contrast, at supramaximal doses of PAF (100 nM), adherent and emigrated neutrophil numbers were similar between sham and BDR animals (Swain et al., 1995). These data suggest that sham and BDR neutrophils have similar capacities to adhere and subsequently emigrate, but vary in their sensitivity and responsiveness to proinflammatory stimuli. This defect could represent an intrinsic problem with BDR neutrophil responsiveness, or to a factor(s) in the neutrophil milieu (e.g. glucocorticoids) in cholestasis which attenuate neutrophil responsiveness (i.e. adhesiveness) in BDR animals. We undertook to answer this question with the following experiments.

5.2. AIMS

The aims of this study were two-fold: 1) to examine firm adhesion of sham and BDR rat neutrophils to biological substrata *in vitro*, and 2) to determine the effect of endogenous glucocorticoids upon neutrophil adhesion.

5.3. METHODS

5.3.1. Rat epithelial cell monolayers

Rat epithelial cells (IEC18; American Tissue Culture Collection, Rockville, MD) were plated in M199 media containing 10% heat-inactivated fetal calf serum and antibiotics plated in 25cm^2 flasks. Cultures were grown in 5% CO_2 / 95% O_2 at 37°C and 96% humidity, expanded by trypsinization, and grown to confluence in 48-well plates. For these experiments, rat endothelial cells were not available. Monolayer activation by TNFα was carried out by incubating TNFα (30U/mL; Knoll Pharmaceutical Co.) with the monolayers for 3 h in 5% CO_2 / 95% O_2 at 37°C before use.

5.3.2. Serum-coated plastic

In initial experiments, BDR plasma was shown to be anti-adhesive. This anti-adhesive property may be related to the interaction of a component of BDR plasma with adhesion molecules on neutrophils (β2 integrins) or on biological substratum (e.g. ICAM-1). To study if ICAM-1 is involved in the inhibitory action of BDR plasma on neutrophil adhesion, neutrophil adherence to serum-coated plastic was assessed. To obtain serum-coated plastic, 48-well plates were coated with 20 µl of inactivated fetal calf serum per well. The plates

were then incubated in 5% CO_2 / 95% O_2, at 37°C and 96% humidity for 2 hours. Following incubation, wells were washed twice with PBS prior to use.

5.3.3. Neutrophil labeling

Freshly isolated neutrophils ($2x10^7$ cells/ml) were radio-labeled with 30 µCi/ml of $Na^{51}CrO_4$ (Amersham, Oakville, Ontario, Canada) and incubated at 37°C for 30 minutes. Cell suspensions were gently shaken a few times during the incubation to ensure uniform cell labeling. Labeled neutrophils were washed three times and resuspended in cold PBS.

5.3.4. Adherence assay

Neutrophil adherence to rat epithelial cell monolayers and to serum-coated plastic was determined using ^{51}Cr-labeled neutrophils. The neutrophil adherence assay was based on a modified method of Fehr and Dahinden (1979). The labeled neutrophils ($1x10^6$ cells) were allowed to adhere to rat epithelial cell monolayers grown to confluence on a 48-well plate or to serum-coated plastic for 30 minutes at 37°C under control conditions, in the presence of phorbol myristate acetate (PMA, 200 ng/ml) or TNFα (30 U/ml), and in the presence or absence of rat plasma (5%). After incubation, the supernatant of each well was aspirated and the wells gently washed with 500 µl HBSS. The cells that remained adherent were then lysed overnight with 0.5 ml of 2M NaOH. The cell lysate was collected and the lysate and supernatant were assayed for ^{51}Cr activity in a gamma counter (WALLAC Wizard 1480, Turku, Finland). Neutrophil adhesion was calculated as the ratio of radioactivity in the cell lysate versus the radioactivity in the cell lysate plus supernatant.

5.3.5. Glucocorticoids and neutrophil adhesion

BDR plasma was found to inhibit neutrophil adhesion *in vitro*. Our *in vivo* data indicates that endogenous glucocorticoids suppress the acute inflammatory response in BDR rats. Glucocorticoids are known anti-inflammatory agents which have been shown to inhibit neutrophil-endothelial interactions (reviewed in Cronstein et al., 1992a). Therefore, we determined if the anti-adhesive factor in BDR plasma was endogenous glucocorticoids. To test for possible inhibition of neutrophil adhesion by endogenous glucocorticoids, plasma obtained from BDR rats or adrenalectomized BDR rats (BDR+ADX) was added to incubation wells. In addition, plasma incubated with 10^{-5}M RU486 was assessed in the adherence assay.

5.3.6. Characterization of BDR plasma

5.3.6.1. Filtration of cholestatic plasma

Two ml of plasma was filtered through Centricon-10 tubes (Amicon, Beverly, MA) to give an approximate size of the active anti-adhesive factor (10,000 MW cut-off). The tubes were centrifuged at 5000 *g* for 1 hour at 4°C. The supernatant was collected and used immediately in the adherence assay, and the remainder was stored at -20°C.

5.3.6.2. Heat treatment of cholestatic plasma

Plasma collected from BDR rats was incubated in 70°C water bath for 10 minutes to inactivate complement proteins and fibrinogen (Rubens et al., 1992; Worthen et al., 1992).

Heat-treated plasma was centrifuged at 14,000 rpm for 1 h, and the resulting supernatant was used in the adherence assay.

5.3.6.3. Tryptic digestion of cholestatic plasma

BDR plasma was treated with trypsin to inactivate plasma proteins according to Swain *et. al.* (1993). Briefly, 1 ml of BDR plasma was incubated with 1 mg/ml L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK)-treated trypsin (Sigma Chemicals, St. Louis, MO) for 30 minutes at 37°C. Hydrolysis was stopped by adding 2.5 mg/ml trypsin inhibitor (Sigma chemicals, St. Louis, MO), and following a 15 minute incubation on ice, ice-cold TRIS-HCl (pH 7.7) was added to the tube. Trypsin digested plasma remained on ice until use in the adherence assay.

5.3.6.4. Sialidase treatment of cholestatic plasma

To elucidate if the anti-adhesive factor in BDR plasma requires carbohydrate moieties (e.g. sialic acid) for activity, sialidase (0.02 U/ml; Sigma Chemical Co. St. Louis, MO) was added to adhesion wells containing BDR plasma prior to neutrophil addition.

5.3.7. Fluorescence-activated cell sorter (FACS) analysis

CD11/CD18 is central to neutrophil adhesion. The expression of this complex in BDR and sham neutrophils was examined by FACS analysis as described previously (Gaboury and Kubes, 1994). Briefly, 200 µl of anticoagulated whole blood of sham or BDR animals was incubated at 37°C for 10 minutes. Following incubation, 20 µl of CL26 (10µg/ml; a gift from Upjohn, Kalamazoo, MI), a MAb against rat CD18, was added to samples and

incubated at room temperature for 15 minutes. Samples were washed twice with cold PBS and then incubated with 4 µl fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse Ig (2.5 µg/ml; Becton-Dickinson, Mountain View, CA) at room temperature for 15 minutes. The cells were washed as described above. Red cells were lysed with FACS lysing solution (Becton-Dickinson) and incubated at room temperature for 10 minutes. The cells were then washed twice as described above and resuspended in 0.5 ml cold PBS for immediate FACS analysis (FACscan, Becton-Dickinson) using the channel number (log scale) representing the mean flourescence intensity of 10,000 cells.

5.4. RESULTS

5.4.1. Adhesion of rat neutrophils

Sham and BDR neutrophils adhered similarly to biological substrata under basal conditions (control) and in the presence of stimuli (PMA and TNF) (Figure 5.1). Incubation in the presence of PMA (a proadhesive stimulus) resulted in increased neutrophil adhesion to rat epithelial cell monolayers by about 6 fold, whereas endothelial activation by TNF increased neutrophil adhesion by about 2.5 fold (Figure 5.1). As PMA was a stronger proadhesive stimulus, it was used for the remaining adherence studies.

5.4.2. The effect of plasma on neutrophil adhesion

Sham and BDR neutrophils responded similarly to identical treatments in the adherence assay (Figure 5.2). In the presence of sham plasma, adhesion was significantly increased in PMA-stimulated sham and BDR neutrophils compared to their respective controls (neutrophils+sham plasma). However, BDR plasma greatly attenuated the PMA-induced

adhesion of both sham and BDR neutrophils. Adhesion of PMA-stimulated sham or BDR neutrophils in the presence of BDR plasma was comparable to their respective controls (neutrophils+BDR plasma). Since BDR and sham neutrophils had similar adhering abilities (Figure 5.2), only BDR neutrophils were used for the rest of our adhesion studies.

The anti-adhesive effect of BDR plasma was also observed in neutrophil binding to serum-coated plastic (Figure 5.3). PMA-activated BDR neutrophil adhesion was significantly higher in the presence of sham plasma compared to adhesion in the presence of BDR plasma (43.4 $\pm$ 10.9 % vs. 10.97 $\pm$ 4.64 %, respectively, $p < 0.01$).

5.4.3. Characterization of the anti-adhesive agent in BDR plasma

PMA-stimulated neutrophils adhered less well in the presence of adrenalectomized BDR rat plasma compared to adhesion in the presence of rat serum free media (Figure 5.4). This adhesion was similar to that noted for neutrophils in the presence of control BDR plasma (7.6 $\pm$ 0.8 % adhesion vs 10.7 $\pm$ 1.7 %, respectively; NS). Furthermore, BDR plasma coincubated with 10^{-5} M RU486 inhibited neutrophil adhesion to the same degree as untreated BDR plasma (13.7 $\pm$ 2.5 % vs 10.7 $\pm$ 1.7 %, respectively; NS) (Figure 5.4). This data suggests that the anti-adhesive agent contained in BDR plasma is not glucocorticoid.

Upon further investigation of the anti-adhesive agent in BDR plasma, we found that the anti-adhesive property of BDR plasma was sensitive to filtration and trypsin digestion. Filtration through a 10,000 MW cut-off filter, and trypsin treatment of the BDR plasma were able to abolish the inhibitory effect of BDR plasma on adhesion (Table 5.1). Sialidase treatment to BDR plasma resulted in an approximately 60% increase in adhesion compared

to adhesion in presence of untreated BDR plasma. However, heat treatment was ineffective in removing/inactivating the anti-adhesive agent present in BDR plasma (Table 5.1). These results suggest that the anti-adhesive agent in BDR plasma is a heat-stable glycoprotein of greater than 10,000 dalton MW partially requiring sialic acid for its activity.

5.4.4. FACS analysis

FACS analysis (expressed as the mean flouresence in percent) for CD18 expression on sham and BDR neutrophils incubated in the presence of their respective plasma showed similar levels of basal CD18 expression (Table 5.2).

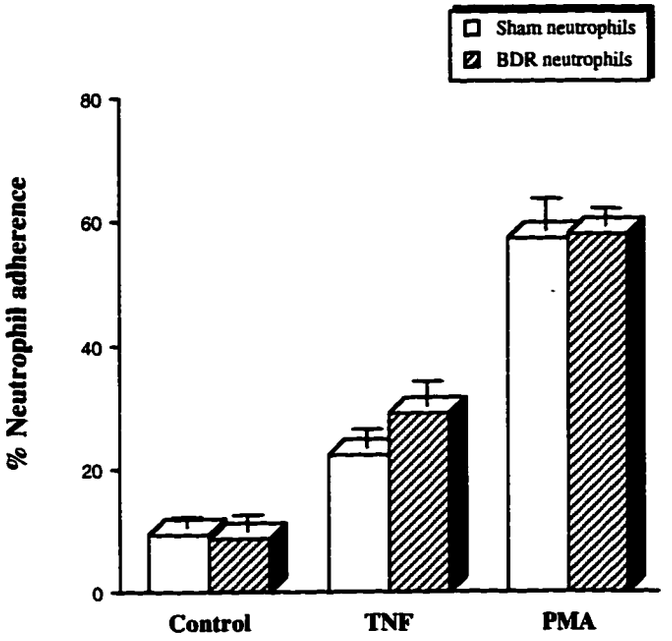

Figure 5.1. *In vitro* adherence of neutrophils isolated from BDR and sham-resected rats to rat epithelial cell monolayers. Adhesion was quantitated in the absence of any stimulation (control), after neutrophil activation with PMA (200 ng/ml for 30 minutes) and after activation of epithelial cells with TNF (30 U/ml for 3 hours). Bars represent the mean ± SEM of data from four experiments. Values for respective BDR and sham neutrophil adhesion are not significantly different.

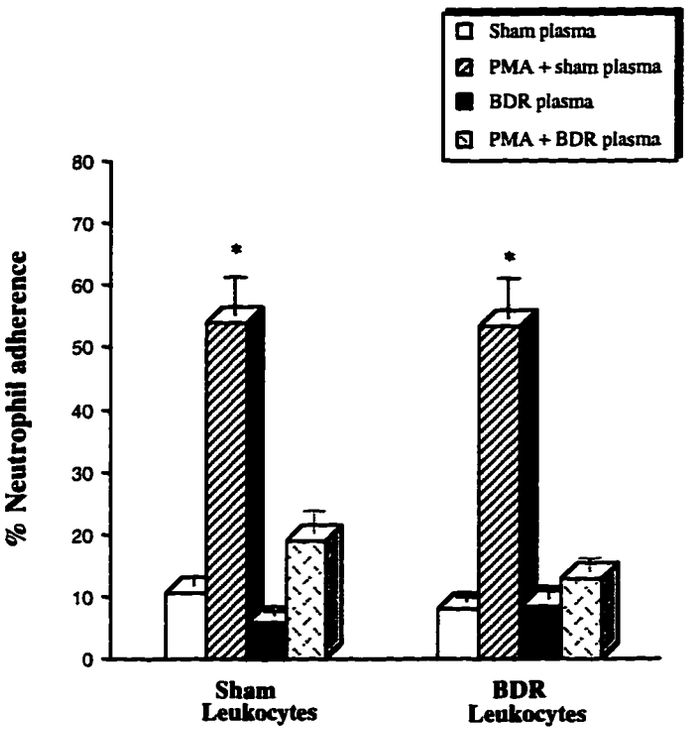

Figure 5.2. *In vitro* adherence of neutrophils isolated from BDR and sham-resected rats to rat epithelial cell monolayers. Adhesion of control and PMA (200ng/ml)-activated neutrophils was assessed in the presence of 5% sham or BDR plasma. Bars represent the mean ± SEM of data from four experiments. * $p<0.01$ vs other treatment groups.

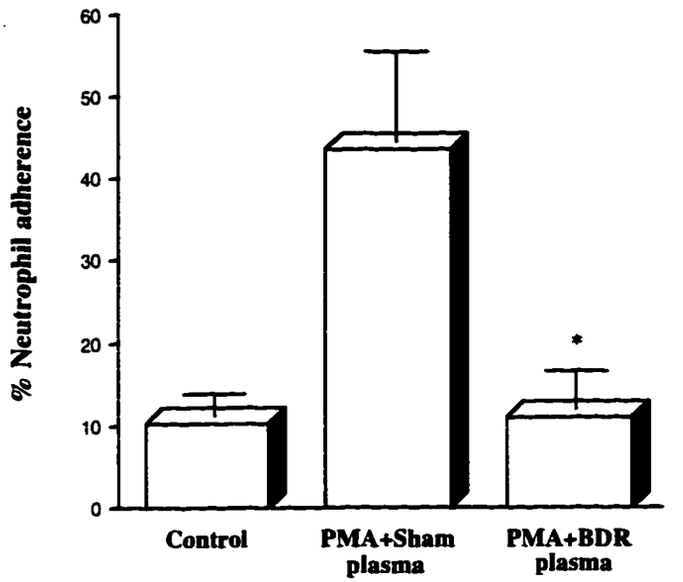

Figure 5.3. The percent adherence of neutrophils isolated from BDR rats to fetal calf serum-coated plastic wells. Adhesion was assessed in nonactivated neutrophils (control) or PMA (200ng/ml)-activated neutrophils in the presence of 5% sham or BDR plasma. Bars represent the mean ± SEM of four experiments. * $p<0.01$ vs PMA+sham plasma.

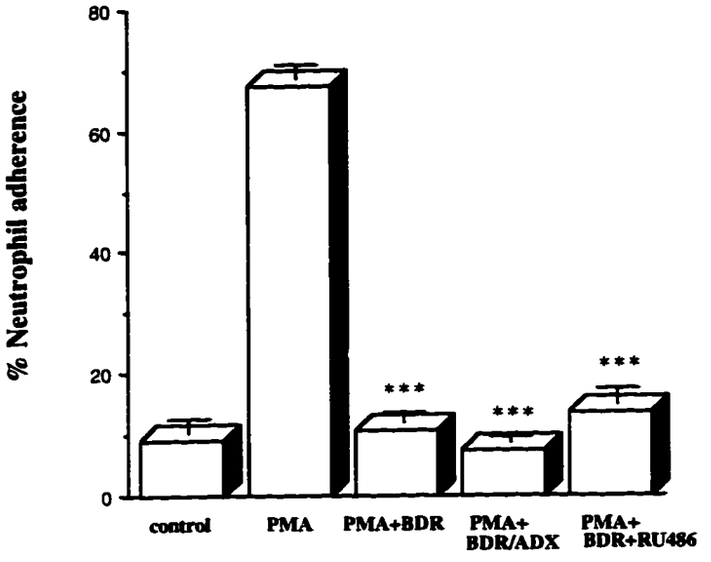

Figure 5.4. The effect of glucocorticoids on neutrophil adherence to a biological substratum *in vitro*. ^{51}Cr-labeled rat neutrophils were coincubated for 30 minutes at 37°C with PMA (200 ng/ml) and plasma from BDR rats (BDR), or plasma from BDR-adrenalectomized rats (BDR/ADX), or BDR plasma plus 10^{-5}M RU486 (BDR+RU486). PMA was absent in the control group. The average plasma corticosterone level of adrenalectomized rats was 0.8125 ng/ml, determined by RIA. Data are mean ± SEM of 4 experiments. ***$p<0.001$ vs adherence in the presence of PMA. Neutrophil adhesion in the presence of PMA and BDR plasma, or BDR/ADX plasma, or BDR plasma+RU486 was not significantly different from each other and from the control value.

Table 5.1. Characterization of the anti-adhesive factor(s) of BDR plasma by exposing plasma to various conditions prior to use in *in vitro* neutrophil adherence assays to rat epithelial cell monolayers. Data are the mean values ± SEM of at least four experiments. The anti-adhesive property of BDR plasma is sensitive to filtration and trypsin and partially to sialidase. [a] $p \leq 0.05$ vs sham plasma value; [b] $p \leq 0.05$ vs BDR plasma value.

Condition	Adhesion (% of PMA + sham plasma value)
PMA + sham plasma (5%)	100
PMA + BDR plasma (5%)	19.82 ± 0.76^{a}
PMA + heat-inactivated BDR plasma	30.32 ± 5.42^{a}
PMA + sialidase-treated BDR plasma	$55.42 \pm 7.41^{a,b}$
PMA + trypsin-treated BDR plasma	$119.37 \pm 4.82^{a,b}$
PMA + filtrated BDR plasma	88.39 ± 5.11^{b}

Table 5.2. Basal CD18 expression on sham and BDR rat neutrophils in the presence of their respective plasma as determined by immunofluorescence flow cytometry. Data are mean ± SEM of 6 experiments. The mean fluorescence of sham and BDR neutrophil basal CD18 expression are not statistically different.

Condition	**Mean flourescence (%) ± SEM**
sham neutrophils + CL26 (Anti-CD18) + sham plasma	71.9 ± 41.3
BDR neutrophils + CL26 (Anti-CD18) + BDR plasma	84.7 ± 42.4

5.5. DISCUSSION

Impaired neutrophil recruitment to inflammatory sites in BDR animals may be associated with impaired neutrophil adhesion, as firm adhesion is a pre-requisite for neutrophil emigration leading to accumulation in tissues. In support of this notion, we have previously demonstrated *in vivo* that neutrophil adhesion was impaired in BDR animals when compared to sham rats (Swain et at., 1995). It was unclear if this impairment was due to a defect in neutrophils themselves or to inhibitory effects of the surrounding plasma. Therefore, we determined adhesion of BDR and sham resected rat neutrophils to biological substrata *in vitro* using radio-labeled cells.

In the presence of proadhesive stimuli (e.g. PMA and TNF), both sham and BDR neutrophils demonstrated increased adherence to rat epithelial monolayers compared to control (Figure 5.1). Both types of neutrophils adhered equally well, although PMA and TNF promote adhesion via different mechanisms. PMA upregulates adhesion molecules on neutrophils (CD11/CD18) while TNF stimulates biological substrata to express adhesion molecules (e.g. ICAM-1). These data indicate normal adhering ability of BDR neutrophils.

To mimic the physiological milieu, neutrophil adhesion was assessed in the presence of plasma. The increased PMA-stimulated adhesion in sham and BDR neutrophils was maintained in the presence of sham plasma. In contrast, adhesion was entirely eliminated for both PMA-stimulated sham and BDR neutrophils in the presence of BDR plasma (Figure 5.2). These data suggest that the inherent ability of BDR neutrophils to adhere is not impaired, but rather that cholestatic plasma impairs adhesion.

Glucocorticoids are known anti-inflammatory agents, which inhibit neutrophil-endothelial adhesive interaction by inhibiting the expression of ICAM-1 (Cronstein et al., 1992b). In addition, our current *in vivo* study (Chapter 4) demonstrated that impaired neutrophil recruitment in cholestatic rats was due, at least in part, to anti-inflammatory properties of endogeneous glucocorticoids (the levels of which are elevated in BDR animals). We speculated that impaired neutrophil accumulation at inflammatory sites in BDR animals may be due to endogenous glucocorticoid inhibition of neutrophil adhesion, possibly through ICAM-1 down-regulation.

To study if glucocorticoids are the anti-adhesive agent in BDR plasma, plasma obtained from adrenalectomized BDR rats was assessed in the adherence assay as well as BDR plasma treated with a known glucocorticoid receptor antagonist, RU486. BDR plasma devoid of glucocorticoids failed to increase PMA-stimulated neutrophil adhesion to rat eptihelial monolayers to levels seen in the presence of sham plasma (Figure 5.4). These data suggest that BDR plasma inhibits neutrophil adhesion independently of glucocorticoids.

We next addressed the issue of whether the anti-adhesive factor in BDR plasma was interfering with neutrophil binding sites on the biological substrata used (i.e. ICAM-1 on epithelial cells). We demonstrated that PMA-stimulated neutrophil adhesion to serum-coated plastic (which lacks ICAM-1) was attenuated in the presence of BDR plasma, but not sham plasma (Figure 5.3). This result was consistent with our previous observations (Figure 5.2) and shows that the anti-adhesive factor in BDR plasma does not interfere with neutrophil ligands on cellular substrata (e.g. ICAM-1).

To further characterize the anti-adhesive factor in BDR plasma, we proposed several possibilities and performed appropriate tests to help identify this factor. Bile acids are

known to have immunomodulatory properties and have differential effects on neutrophil activation *in vitro* (Dahm and Roth, 1990), and have been shown to influence PAF-induced leukocyte-endothelial cell adhesion in postcapillary venules (Russell et al., 1994). Addition of ursodeoxycholic acid, a bile acid used extensively in the treatment of gallstone diseases, to the superfusion buffer led to a significant reduction in the number of adherent and emigrated leukocytes which could not be explained by changes in venular hemodynamics (Russell et al., 1994). We therefore speculated that bile acids may be the anti-adhesive factor in BDR plasma.

Bile acids, however, did not appear to be responsible for the inhibitory effect exhibited by BDR plasma, as filtration of plasma through a selective size filter (10,000 MW) removed the anti-inhibitory effect of BDR plasma and restored the PMA-induced increased adhesion of neutrophils to biological substrata (Table 5.1). In addition, plasma protein degradation by trypsin treatment abolished the anti-adhesive properties of BDR plasma. This suggests that the plasma factor is a protein, or protein-associated molecule. Complement proteins (e.g. C5a and C3b) and fibrinogen are involved in neutrophil-endothelial interactions. The anti-adhesive factor in BDR plasma retained its activity following the removal of these proteins by heat treatment. Therefore, complement proteins and fibrinogen are not suitable candidates as the anti-adhesive factor in BDR plasma. Moreover, degradation of sialic acids and related sugars by treatment of BDR plasma with sialidase partially increased PMA-stimulated neutrophil adhesion, suggesting that the anti-adhesive factor requires sugar moietie(s) for activity. Therefore, in summary our data strongly suggest that the anti-adhesive factor in BDR plasma is a heat-stable glycoprotein. Further investigations may be useful to reveal its specific identity.

Although glucocorticoids do not appear to have direct inhibitory effects on neutrophil adhesion *in vitro*, they attenuate the inflammatory response in BDR animals (Chapter 4). We speculate that *in vivo*, the anti-adhesive factor in BDR plasma is likely to inhibit neutrophil adhesion via a different mechanism than that utilized by endogenous glucocorticoids in inhibiting neutrophil accumulation at the site of inflammation. This "factor" may prevent neutrophil adhesion to vascular endothelium by modulating the expression or adhesiveness of adhesion molecule(s) on neutrophils. In contrast, elevated circulating free endogenous glucocorticoid in BDR rats may prevent leukocyte recruitment *in vivo* by modulating the expression of endothelial adhesion molecules (e.g. ICAM-1), as Cronstein *et al.* (1992b) have shown that glucocorticoids down-regulate ICAM-1 expression *in vitro*. ICAM-1 is an important receptor ligand for neutrophil adhesion molecules during integrin-mediated leukocyte-endothelial firm adhesion. Furthermore, the levels of chemoattractant/inflammatory mediators such as LTB4, which can modulate leukocyte adhesion molecules (e.g. CD11/CD18), do not appear to be influenced by endogenous glucocorticoids. Therefore, the anti-adhesive factor in BDR plasma and endogenous glucorticoids may act together to further inhibit the development of an inflammatory response in BDR rats.

5.6. SUMMARY

Both sham and BDR neutrophils can adhere normally to biological substrata after stimulation. However, BDR plasma, but not sham plasma, impairs both sham and BDR neutrophil adhesion to these biological substrata. Following further characterization of the BDR plasma anti-adhesive factor, our data suggest that the factor is a heat-stable glycoprotein.

CHAPTER 6

STUDY IV: NEUTROPHIL MICROBICIDAL FUNCTION IN CHOLESTASIS

6.1. INTRODUCTION

The high incidence of post-operative septic complications in cholestatic patients may be related to neutrophil dysfunction not only at the level of neutrophil recruitment, but also through defective microbicidal activity. Neutrophil bactericidal activity is mediated through their ability to generate and release oxidants and/or proteases. In addition to reactive oxidants generated through the respiratory burst, the release of lytic enzymes and proteases from the granules of neutrophils may further mediate neutrophil microbicidal activity.

Our *in vivo* study showed impaired neutrophil recruitment to inflammatory sites in cholestatic rats (Chapter 3). There may be additional neutrophil defects which could account for the increased incidence of septic complications in cholestatic patients. We therefore hypothesized that cholestasis may impair neutrophil bactericidal activity.

6.2. AIMS

The purpose of this study was to determine if cholestasis is associated with an impairment of neutrophil microbicidal activity. Specifically, the ability of neutrophils to kill bacteria was tested. Furthermore, we wanted to determine if the neutrophils' respiratory burst and degranulation were impaired in cholestasis.

6.3. METHODS

6.3.1. Bacterial killing assay

Staphylococcus aureus (strain 502A) was prepared in Mueller-Hinton broth (Sigma, MO) and grown overnight at 37°C (Woodman et al., 1995). The following day, the bacteria were centrifuged (2000 *g* for 15 min), washed and resuspended in PBS with 1% gelatin (pH 7.4) to a final volume of 1 ml. To determine bacterial density, the optical density of the bacterial suspension was measured at 620 nm (U-2000 double beam spectrophotometer, Hitachi, Ltd. Tokyo, Japan). The bacterial suspension was adjusted to a density of approximately 2.5×10^7 CFU/ml.

Freshly isolated BDR and sham neutrophils (2.5×10^6 cells/ml) were coincubated with bacteria (2.5×10^6 CFU/ml) in 1 ml of PBS containing 1% gelatin and 10% autologous serum and shaken end-over-end at 37°C for 1 hour (Model 1105 Adams Nutator, Clay Adams, Parsippany, NJ). Aliquots of 50 µl were withdrawn from each tube at 0 and 1 hour of incubation and immediately added to tubes containing 5 ml of distilled water/1% gelatin (pH 7.4). The tubes were held for 10 min to ensure neutrophil lysis, vortexed, and then a further 10-fold dilution was made in 10 mM TRIS buffer/1% gelatin (pH 7.4). Each sample was run in duplicate. Viable bacteria were determined by spreading 50 µl from each dilution tube onto tryptic soy agar (TSA) plates. After an overnight incubation at 37°C, viable bacteria were quantitated as individual colonies. To determine the percentage of *in vitro* bacterial killing by neutrophils, total counts of bacteria on control plates (assuming no killing at time 0) were compared with the number of bacterial colonies remaining on TSA plates after 1 hour incubation.

6.3.2. Superoxide anion assay

Superoxide anion production was quantitated using a continuous kinetic assay of SOD inhibition of ferricytochrome c reduction (Woodman et al., 1988). Superoxide-dependent cytochrome c (cyt c) - reduction was determined spectrophotometrically at 550 nm (U-2000 double beam spectrophotometer, Hitachi Ltd. Tokyo, Japan). Briefly, 100 µl of cell suspension ($1x10^6$ cells) was added to sample and reference cuvettes containing 890 µl of PBS and 0.08 mM cyt c/ddH_2O. Cells were activated by either PMA (200 ng/ml; Sigma, MO), opsonized zymosan (OZ; 0.1 mg/ml) or fMLP (10^{-10}M to 10^{-5}M; Sigma, MO). In some assays, neutrophils were primed with dihydrocytochalasin B (DHCB; 2.5 µg/ml; Sigma, MO) for 2 min at 37°C before fMLP addition. OZ was prepared by incubating 10 mg zymosan A (Sigma, MO) per milliliter of fresh plasma for 30 min at 37°C. The mixture was diluted with HBSS, centrifuged at 450 *g* for 6 min, washed twice with HBSS and suspended at a final concentration of 1 mg/ml; unused portions were stored at -20°C. To examine O_2^- release by neutrophils in a more physiological condition, 10% of either sham or BDR sera was added to each cuvette for some assays. The data from the latter experiments were used to determine whether neutrophil dysfunction in generating ROIs results from a defect in the respiratory burst or possibly a suppressive effect of sera.

6.3.3. NitroBlue Tetrazolium (NBT) slide test

To examine differential release of O_2^- by sham and BDR neutrophils, a NBT slide test was performed (Woodman et al., 1995). Freshly isolated neutrophils were incubated at 37°C on glass slides with NBT dye (Sigma, MO) and PMA (200 ng/ml), or buffer alone (resting). Neutrophils were fixed with absolute methanol before they were stained with safranin-o (BDH Inc., Toronto, Canada). In the presence of O_2^-, the NBT dye is chemically

reduced to yield a dark purple insoluble compound (formazan) which can be clearly discerned microscopically, and the percentage of cells containing the reduced NBT dye (formazan) can be easily determined. A comparison of staining intensity can also be made to control cells.

6.3.4. Hydrogen peroxide assay

Hydrogen peroxide production by PMA-stimulated neutrophils was measured using a simple assay described by Ruch *et al.* (1983). The assay determines the rate of increase in fluorescence by the horseradish peroxidase (HRP)-catalyzed peroxidation of homovanillic acid (HVA) to a fluorescent dimer (2,2'-dihydroxy-3,3'-dimethoxydiphenyl-5,5'-diacetic acid). A standard curve (without neutrophils) was constructed by measuring the change in fluorescence at various concentration of H_2O_2. A continuous reading of fluorescence was recorded for the experimental system (in the presence of neutrophils plus PMA), and the rates of H_2O_2 production were calculated by comparing the fluorescence change to the standard curve.

Two concentrations of HVA (10 mM and 100 mM) were used to determine if the production of H_2O_2 was HVA-dependent. In addition, the effects of temperature (room temperature and 37°C) on H_2O_2 production were also investigated.

6.3.5. Degranulation assay

Microbicidal proteins and proteases released from the granules of neutrophils may also mediate neutrophil microbicidal activity. We examined exocytic neutrophil degranulation by measuring β-glucuronidase activity in the supernatant of stimulated and unstimulated

neutrophils (Woodman et al., 1993). Briefly, neutrophils ($1x10^7$cells) were incubated at 37°C with DHCB (2.5 μg/ml) and stimulated with either fMLP (10^{-7}M) or PMA (200 ng/ml) for 30 minutes in a shaker bath (Model 2564, Forma Scientific, Marrietta, Ohio). DHCB was added to promote exocytic degranulation. The supernatant from each tube was collected after centrifugation at 420 *g* for 10 min and placed on ice. The β-glucuronidase levels were quantitated spectrofluorometrically (SPF-500 Spectroflourometer, SLM Instruments, Inc. Urbana, IL) against a known β-glucuronidase standard using excitation and emission wavelengths of 365 nm and 460 nm, respectively. Cell degranulation was expressed as a percentage of the amount of enzyme released in the presence of stimuli compared to that released by lysed cells (considered 100% degranulation). Cell lysis was analyzed by measuring glucose-6-P-dehydrogenase (G6PD) activity spectrophotometrically at 340 nm (U-2000 double beam spectrophotometer, Hitachi, Ltd. Tokyo, Japan).

6.4. RESULTS

6.4.1. Neutrophil bactericidal activity

In the presence of autologous sera, sham neutrophils were more efficient at bacterial killing *in vitro* than BDR neutrophils incubated with sham sera (Figure 6.1). The ability of BDR neutrophils to kill bacteria was decreased by approximately 20% relative to sham neutrophils. However, impaired bacterial killing by BDR neutrophils was further reduced in the presence of BDR (cholestatic) sera with a further 50% reduction relative to the percentage of bacterial killing by BDR neutrophils in the presence of sham sera. In addition, the antibactericidal effect of BDR sera was also observed in bacterial killing by sham neutrophils (Figure 6.1). These data suggest that bacterial killing is impaired in cholestatic rats and that BDR sera inhibits neutrophil microbicidal activity.

6.4.2. Superoxide anion production

Neutrophils from BDR rats showed an approximate 30-50% decrease in the rate of O_2^- production when stimulated with either 10^{-7}M or 10^{-8}M fMLP as compared to sham neutrophils (Figure 6.2). However, at higher concentrations of fMLP (i.e. 10^{-6}M and 10^{-5}M), there was no significant difference in the ability of neutrophils to generate O_2^-. There was no appreciable release of O_2^- by either sham or BDR neutrophils when stimulated with lower doses of fMLP (10^{-9}M and 10^{-10}M). When stimulated with either PMA or OZ, sham and BDR neutrophils demonstrated similar rates of O_2^- production (Table 6.1).

The addition of either 10% sham or BDR sera to fMLP-stimulated sham neutrophils decreased the rate of O_2^- production by approximately 35% compared to the rate when neither sera was present, but without a further reduction in the rate of O_2^- release by BDR neutrophils (Figure 6.3). In addition, the rates of O_2^- production were not significantly different between sham and BDR neutrophils in the presence of either sera (Figure 6.3).

6.4.3. NBT slide test

The percentage of NBT-positive sham (93.8 $\pm$ 0.2%) and BDR neutrophils (92.1 $\pm$ 1.1%) was comparable. The NBT staining indicated that the decreased rate of O_2^- production by BDR neutrophils was due to a homogenous reduction of O_2^- release by BDR neutrophils.

6.4.4. Hydrogen peroxide production

There was no significant difference in the rates of H_2O_2 release between PMA-stimulated sham and BDR neutrophils either in the presence of 10 mM or 100 mM HVA (Table 6.2). Although the rates of H_2O_2 production appeared to increase with increasing HVA concentrations, this was not statistically significant. At different temperatures (i.e. 20°C and 37°C), the rates of H_2O_2 production by BDR neutrophils was not significantly different than sham neutrophils (Table 6.3).

6.4.5. Neutrophil degranulation

Neutrophil degranulation, quantitated as the percentage of β-glucuronidase released from primary granules, is shown in Figure 6.4. The percentage of β-glucuronidase released from sham and BDR neutrophils was similar whether they were stimulated with 10^{-7}M fMLP [(23.6 ± 2.1)% vs. (27.4 ± 1.0)%, respectively; ns] or 200 ng/mL PMA [(21.2 ± 3.7)% vs. (25.5 ± 3.1)%, respectively; ns]. The contribution of lysis to neutrophil degranulation, as determined by G6PD activity in the supernatant, was negligible (< 5%).

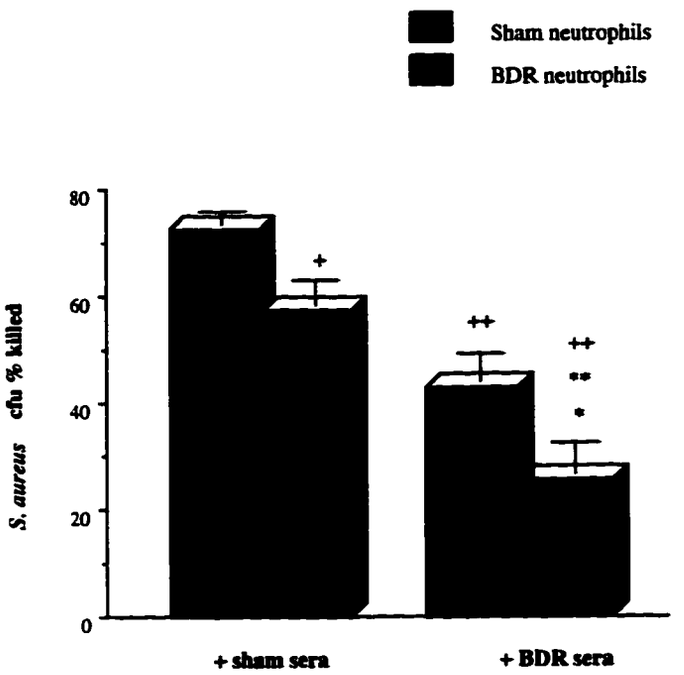

Figure 6.1. The percentage of *S. aureus* killed by sham and BDR neutrophils following a 1 hour incubation. Neutrophils, sera and bacteria were incubated in PBS with 1% gelatin. Aliquots were placed in water containing 1% gelatin for at least 10 minutes to ensure neutrophil lysis. Further dilutions were made into TRIS buffer/1% gelatin. The bacteria remaining in suspension were grown on tryptic soy agar plates overnight and individual colonies counted the next day. Data are mean ± SEM of 5 experiments. * $p<0.05$ vs sham neutrophils + BDR sera; $^{+}$ $p<0.05$ and $^{++}$ $p<0.001$ vs sham neutrophils + sham sera; ** $p<0.001$ vs BDR neutrophils + sham sera.

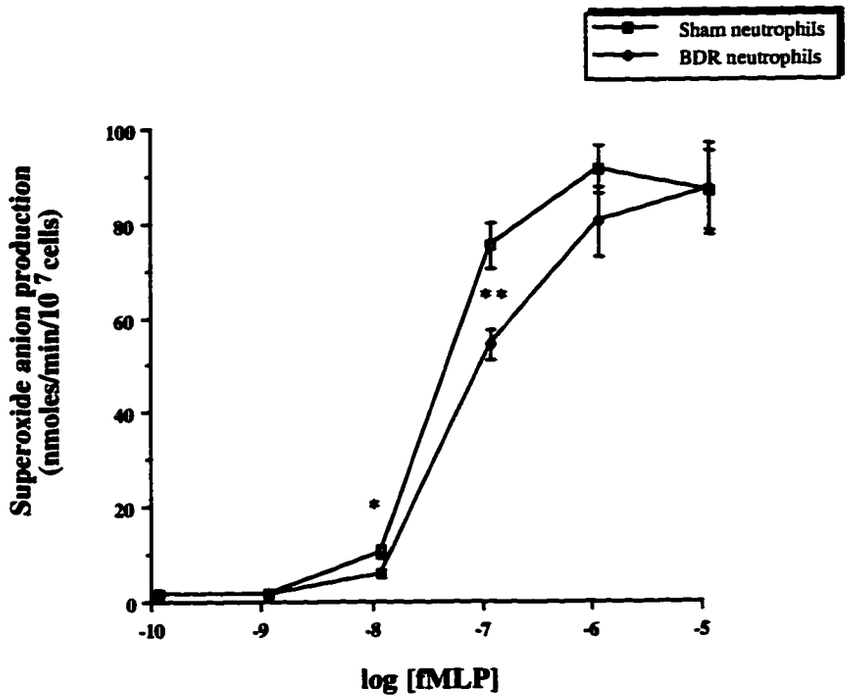

Figure 6.2. Superoxide anion production by sham and BDR rat neutrophils at increasing fMLP concentrations (10^{-10}M to 10^{-5}M). Neutrophils were primed with dihydrocytochalasin B (2.5 mg/ml) for approximately 2 minutes at 37°C prior to fMLP addition. Data are mean ± SEM of 3 experiments (each with duplicate runs). **$p<0.005$ vs sham value, and *$p<0.05$ vs sham value.

Table 6.1. The rates of O_2^- production by sham and BDR rat neutrophils stimulated with PMA (200 ng/ml) and opsonized zymosan (OZ; 0.1 mg/ml). Data are mean ± SEM of 3 experiments (each with duplicate runs).

	Rate of O_2^- production (nmoles/min/10^7 cells)	
	PMA	**OZ**
Sham neutrophils	76.4 ± 5.2	3.24 ± 0.6
BDR neutrophils	64.7 ± 6.6	2.47 ± 0.3

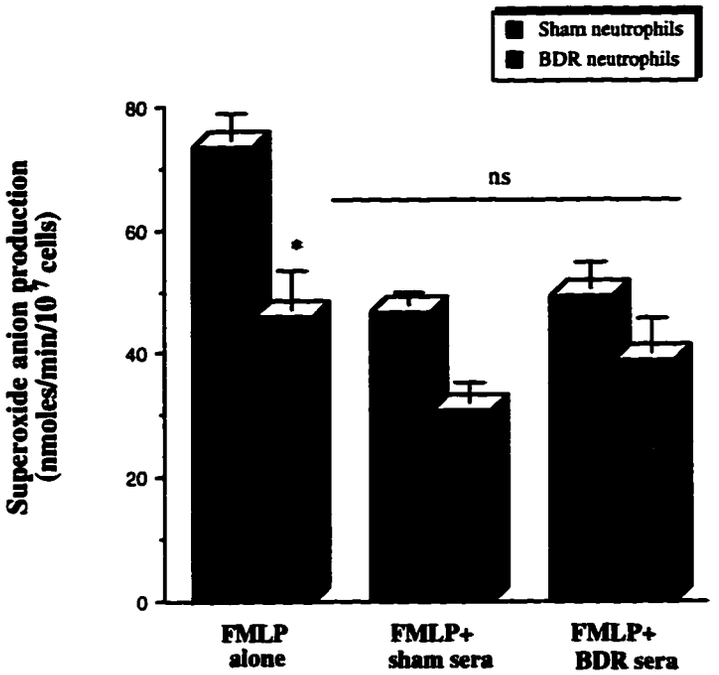

Figure 6.3. The rates of O_2^- generated by sham and BDR neutrophils in the presence of either 10% sham or BDR sera. Neutrophils were primed with dihydrocytochalasin B (2.5 mg/ml) for 2 minutes at 37°C prior to stimulation with 10^{-7}M fMLP. Data are mean ± SEM of 3 experiments (each with duplicate runs). *$p<0.05$ vs sham value; ns=not significant.

Table 6.2. The rates of H_2O_2 production by PMA-stimulated sham and BDR rat neutrophils at 37°C. Neutrophils ($2x10^6$ cells) were incubated for two minutes in an assay solution contaning 10 mM or 100 mM of homovanillic acid prior to cell activation with PMA (100 ng/ml). Data are mean ± SEM of 3 experiments. There was no significant difference in rates of H_2O_2 production between sham and BDR neutrophils.

	Rates of H_2O_2 release (nmoles/min/10^6 cells)
Sham PMN + 10 mM HVA	1.97 ± 0.42
BDR PMN + 10 mM HVA	1.84 ± 0.39
Sham PMN +100 mM HVA	2.92 ± 0.62
BDR PMN + 100 mM HVA	2.38 ± 0.09

Table 6.3. The effects of temperature on H_2O_2 rates of release from PMA-activated sham and BDR rat neutrophils. 10 mM HVA was used in this assay. Data are mean ± SEM of 3 experiments. The rates of H_2O_2 production were similar between sham and BDR neutrophils at either 20°C or 37°C.

	Rates of H_2O_2 release (nmoles/min/10^6 cells)	
	20°C	**37°C**
Sham PMN + 10 mM HVA	1.42 ± 0.05	1.97 ± 0.42
BDR PMN + 10 mM HVA	1.27 ± 0.1	1.84 ± 0.39

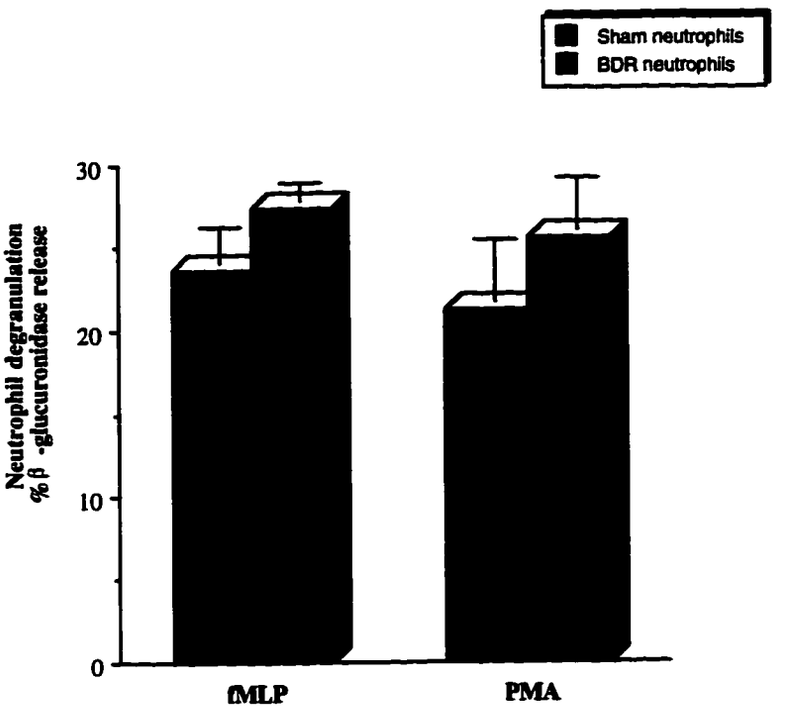

Figure 6.4. β-glucuronidase release from sham and BDR neutrophils following incubation with Dihydrocytochalasin B (2.5 mg/ml) and stimulation with either 10^{-7}M fMLP or 200 ng/ml PMA for 30 minutes at 37°C. Following centrifugation at 420 *g* for 10 minutes, the supernatant of each sample was collected and assayed for β-glucuronidase content using a spectrofluorometer (excitation λ = 365nm ; emmission λ = 460nm). Data are mean ± SEM of 5 experiments.

6.5. DISCUSSION

To examine the ability of sham and BDR neutrophils to eliminate microorganisms, *in vitro* killing of *S. aureus* was analyzed. BDR neutrophils exhibited a significant decrease in bacterial killing compared to sham neutrophils due to a combination of neutrophil functional defects and the antimicrobicidal effects of BDR sera.

To eliminate infectious agents, neutrophils generate ROIs and release granular constituents. Therefore, the impaired *in vitro* bacterial killing of BDR neutrophils may be due to neutrophil defects in generating ROIs and/or releasing microbicidal proteins or a combination of both pathways. BDR neutrophils generated only half of the O_2^- released by sham neutrophils when stimulated with 10^{-8}M or 10^{-7}M fMLP (Figure 6.2). The NBT slide test results suggest that the impairment in O_2^- release by BDR neutrophils occured in the entire population of BDR neutrophils, not just in a specific subset of cells. In contrast, supraphysiological concentrations of fMLP (i.e.10^{-6}M and 10^{-5}M), and PMA and OZ did not result in any significant difference in the rate of O_2^- release between sham and BDR neutrophils suggesting that receptor and soluble agonists can equally activate sham and BDR neutrophils to produce O_2^-.

The similar production of O_2^- between sham and BDR neutrophils when stimulated with high concentrations (i.e.10^{-6}M and 10^{-5}M) of fMLP, PMA or OZ may be explained by their modes of action. PMA activates the respiratory burst by activating PKC, which then phosphorylates cytosolic components of NADPH oxidase complex (Maridonneau-Parini et al., 1986). In contrast, fMLP and OZ bind to receptors on the neutrophil surface, activating G-protein coupled signaling cascades to activate NADPH oxidase (Baggiolini et al., 1993).

As mentioned earlier, higher levels of saturated fatty acids and cholesterol in plasma membranes of BDR neutrophils may yield more rigid membranes and this may influence fMLP receptor mobilization to the cell surface. In human neutrophils, low and high affinity binding sites for fMLP have been determined while in rats, fMLP receptors appear to be of high affinity with a Kd of 1-30 nM (Walker et al., 1991). Therefore, lower concentrations of fMLP would provide more sensitivity with respect to O_2^- production than at higher doses. Similar O_2^- production at higher concentrations of fMLP may suggest a similar overall capacity to produce O_2^- in sham and BDR neutrophils. Since PMA by-passes membrane associated neutrophil activation, similar O_2^- production in BDR and sham neutrophils would be expected if there was a membrane or receptor determined defect. With respect to OZ, interpretation is somewhat difficult. Although there is evidence to suggest that O_2^- production by human neutrophils is OZ-concentration dependent (Cohen et al., 1981), detection of a concentration-dependent increase in O_2^- production by sham and BDR neutrophils stimulated with increasing OZ concentrations was difficult to assess due to increasing solution turbidity with higher OZ concentration.

Our results contrast those of Levy *et al.* (1993) who showed that neutrophils isolated from common bile duct ligated rats generated more O_2^- than sham animals. Despite these differences, it is important to note that the levels of O_2^- produced in their study were much lower than what we observed. For example, using the same stimulus (10^{-7}M fMLP), the amount of O_2^- generated by their BDR neutrophils (which was significantly higher than sham neutrophils) was about 85% lower than what we observed with our BDR neutrophils. Furthermore, the range of O_2^- release in our study falls within a similar range observed for human neutrophils (Woodman et al., 1995). Therefore, we are not certain about the physiological significance of such low levels of O_2^- production reported by Levy *et al.* (1993) on neutrophil microbicidal function. The discrepancies between results may be

explained by the use of different methods of blood collection and neutrophil isolation as well as assays to measure O_2^- generation. We chose the continous kinetic assay, which is more sensitive in quantitating O_2^- release than the static endpoint assay utilized by Levy and coworkers (1993). Unlike the static assay, the kinetic assay allows one to directly observe the kinetics of O_2^- production (lag time, rate of release and plateau of O_2^- production).

The observed decrease in O_2^- production in BDR neutrophils did not appear to completely account for the impaired bacterial killing in BDR animals. We speculate that other activities leading to the killing of microorganisms, such as phagocytosis, may also play a role in impaired killing. Roughneen *et al.* (1989) demonstrated that neutrophils isolated from juvenile cholestatic rats demonstrated an impaired ability to actively phagocytose ^{14}C-labeled *S. aureus*. In contrast, however, others have shown a significant increase in phagocytosis of neutrophils from cholestatic compared to control rats (Levy et al., 1993).

To examine the effects of BDR sera on O_2^- release, 10% of either sham or BDR sera was included in the assay system. In contrast to Ohshio's results (Ohshio et al., 1988), but in agreement with Levy *et al.* (1993), we were unable to see any increase in O_2^- production by neutrophils when incubated with either sham or BDR sera. In fact, we observed a 35% reduction in O_2^- release by sham neutrophils when exposed to either sera compared to its absence; there was comparable O_2^- production by sham and BDR neutrophils in the presence of either sera (Figure 6.3). A significant reduction in O_2^- release by sham neutrophils in the presence of either sera may be due to antioxidant activity of the sera as physiological antioxidants (e.g. glutathione) and bystander antioxidants (e.g. plasma proteins) scavenge ROIs (Dobrinich and Spagnuolo, 1991; Bucurenci et al., 1992; Kim et al., 1995).

Cytokines have been shown to be capable of priming neutrophils to enhance O_2^- release (Weisbart et al., 1987; Woodman et al., 1988; Bajaj et al., 1992; Wozniak et al., 1993). Woodman *et al.* (1988) documented that preincubation of human neutrophils with GM-CSF caused an augmentation in the rate of O_2^- production, but without a significant increase in the maximal amount generated. Since glucocorticoids can inhibit cytokine release (Bochner et al., 1987; Zuckerman et al., 1989; Chensue et al., 1991), the elevated levels of endogenous glucocorticoids previously documented in BDR animals (Swain and Maric, 1994) may depress cytokine production. This in turn may affect neutrophil priming/activation. The role of cytokines in neutrophil O_2^- release in BDR rats may be of interest for future studies.

During the respiratory burst, O_2^- spontaneously dismutates to form H_2O_2. In addition to being potentially harmful themselves, O_2^- and H_2O_2 undergo further reactions which produce reactive oxidants with even greater microbicidal activity, such as hypohalous acids and hydroxyl radicals. Therefore, in addition to O_2^-, we also determined H_2O_2 production. PMA-stimulated sham and BDR neutrophils produced similar amounts of H_2O_2 for all conditions tested (Tables 6.2 and 6.3). These results were expected as similar amounts of O_2^- were produced in PMA-stimulated sham and BDR neutrophils (Table 6.1). Although these results indicate that lowered microbial resistance observed in BDR rats is not associated with lowered production of H_2O_2, caution must be taken in interpreting data using a non-physiologic stimulus such as PMA. Unfortunately, we were unable to detect H_2O_2 production following sham or BDR neutrophil stimulation with a more physiologic stimulus such as fMLP.

Evidence for the contribution of granule constituents in neutrophil microbicidal activity has been demonstrated in studies utilizing neutrophils obtained from patients with chronic

granulomatous disease who are deficient in functional NADPH oxidase (Kaplan et al., 1968; Root and Cohen, 1981). Therefore, we examined the degranulation process in sham and BDR neutrophils. The release of granule contents by stimulated sham and BDR neutrophils, measured as β-glucuronidase release, was not significantly different (Figure 6.4). These data suggest that lowered microbial resistance in cholestasis is not due to impaired neutrophil degranulation.

6.6. SUMMARY

In summary, we have demonstrated that neutrophil function is impaired in cholestatic rats. Specifically, we have identified two functional defects which may contribute to lowered resistance to microbial invasion in cholestasis; (1) decreased O_2^- generation and bacterial killing, and (2) BDR sera exhibits anti-microbicidal effects which further reduces the ability of neutrophils to perform bacterial killing.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Our findings are summarized in the following schematic which illustrates what may take place during host response to infection in BDR animals.

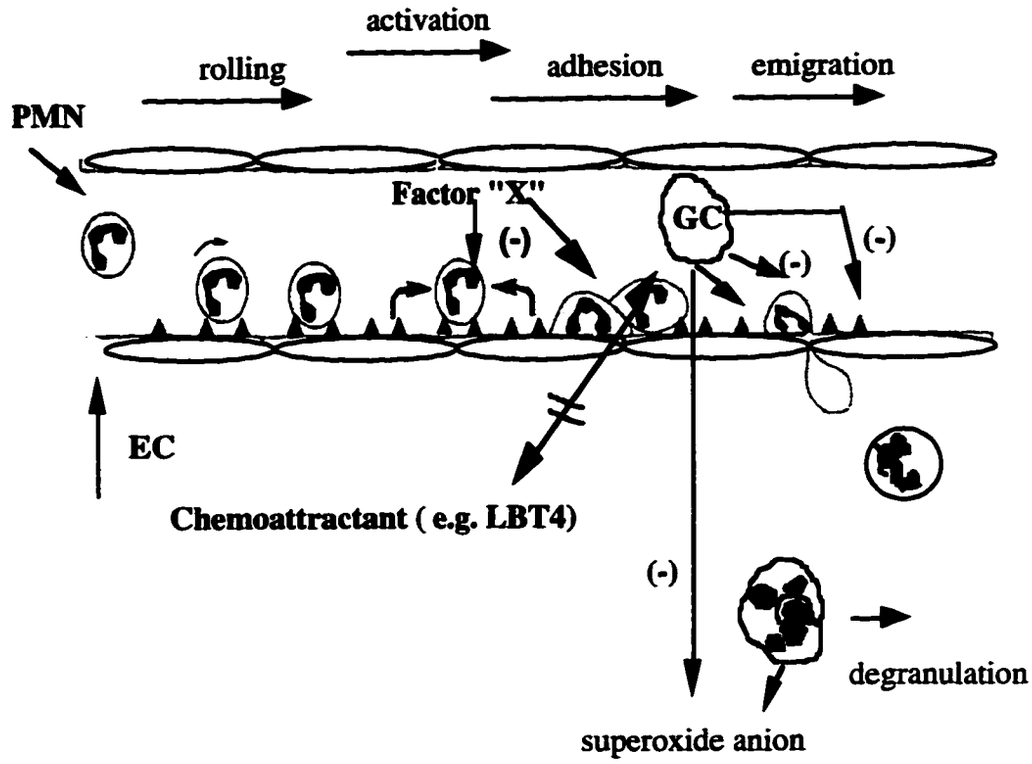

Figure 7.1. A schematic diagram of neutrophil function during host response to bacterial invasion in cholestatic animals. *Abbreviations:* PMN= polymorphonuclear leukocytes; EC=endothelial cells; GC= glucocorticoids; Factor"X"= anti-adhesive factor in BDR plasma; negative sign indicates inhibitory effect; double lines indicate no apparent effect.

We demonstrated that in carrageenan-induced acute inflammation, neutrophil recruitment to inflammatory sites was impaired in BDR animals. This impairment is due, at least a major part, to the anti-inflammatory effects of elevated levels of circulating endogenous glucocorticoids. Endogenous glucocorticoids in BDR rats did not appear to inhibit neutrophil recruitment through inhibition of LTB4 production at inflammatory sites. We speculate that endogenous glucocorticoids may inhibit neutrophil recruitment in BDR rats through downregulation of ICAM-1 expression on endothelial cells.

Neutrophil recruitment to tissues is mediated by a cascade of events (rolling, adhesion and emigration). Impaired neutrophil recruitment in cholestatic rats appeared to occur at a post-rolling stage since our previous study showed that neutrophil rolling is similar in sham and BDR neutrophils (Swain et al., 1995). Although neutrophil adhesion under basal conditions was similar in sham and BDR animals, we observed an impaired neutrophil adhesion following activation with 2nM of PAF. BDR neutrophils were able to overcome this deficit when maximally activated with 100 nM PAF. These data suggested that impaired neutrophil adhesion in BDR animals may be due to an intrinsic problem with BDR responsiveness to proinflammatory stimuli, or to a factor(s) in the neutrophil environment which attenuates neutrophil adhesiveness. To further investigate these possibilities, an *in vitro* study of neutrophil adhesion was performed. From these studies, we found that the ability of BDR neutrophils to adhere to biological substrata is not impaired compared to sham neutrophils, but rather BDR plasma impairs both sham and BDR neutrophil adhesion to these substrata. The anti-adhesive factor in BDR plasma (factor "X") is a heat-stable glycoprotein, not glucocorticoid, and does not interfere with neutrophil ligands on cellular substrata (e.g. ICAM-1). Therefore, this plasma factor is likely to act on neutrophils by downregulating the expression or activity of adhesion molecules (e.g. CD11/CD18). Since there were no significant effects of factor X on adhesion molecule expression on resting

neutrophils, this factor is likely to downregulate adhesion molecules on activated neutrophils. Future studies using flow cytometry will be useful in determining this possible downregulation. Decreased activity or expression of neutrophil adhesion molecules might result in reduced neutrophil-endothelial interactions, which subsequently attenuate neutrophil migration into tissues. Therefore, impaired neutrophil recruitment in BDR rats appears to be mediated by factors (endogenous glucocorticoids and factor X in BDR plasma) that target both the endothelium and neutrophils, respectively.

In addition to impaired neutrophil recruitment, neutrophil microbicidal activity was also depressed in cholestatic rats. Moreover, cholestatic sera contains anti-bactericidal agents which further impair bacterial killing by both sham and BDR neutrophils. These data suggest that there is an intrinsic defect in neutrophil bacterial killing, and a plasma-derived inhibitor of bacterial killing in cholestatic animals. The mechanisms of impaired killing are not known. However, our studies suggest that it is, in part, due to impaired O_2^- production. Impaired O_2^- production in BDR rats is not likely due to a defect in NADPH oxidase, as indicated by the NBT test results. Cytokines have been shown to be capable of priming/activating neutrophils to augment the rate of O_2^- production (Woodman et al., 1988). Elevated circulating glucocorticoids in BDR animals may indirectly suppress O_2^- production by inhibiting the release of cytokines used for priming/activating neutrophils. In addition, glucocorticoids may have a direct effect on O_2^- production. Coates *et al.* (1983) documented that dexamethasone inhibited the production of O_2^- from human peripheral neutrophils in a dose-dependent manner following its incubation for 20 min at 37°C.

Impaired bacterial killing in BDR neutrophils however, cannot be explained by a defective degranulation process as a similar degree of degranulation was observed in sham and BDR neutrophils. Furthermore, degranulation data also indicate that cell signalling

through receptor-mediated events is not impaired in BDR neutrophils. We propose that in addition to impaired O_2^- production, there may be other mechanisms which contribute to the observed impaired killing in BDR neutrophils that are yet to be determined.

In conclusion, neutrophil recruitment to inflammatory sites in BDR animals is impaired and attributed to an impairment at multiple levels. In addition, microbicidal function was also impaired in BDR animals. Therefore, the combined effects of these impairments may explain the increased incidence of post-surgical septic complications in cholestatic patients.

CHAPTER 8

LITERATURE CITED

Ambruso R, Johnston RB. Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. J. Clin. Invest. 1981; 67:352-360.

Armstrong CP, Dixon JM, Taylor TV, Davies GC. Surgical experience of deeply jaundiced patients with bile duct obstruction. Br. J. Surg. 1984; 71: 234-238.

Arnaud J-P, Humbert W, Eloy M-R, Adloff M. Effect of obstructive jaundice on wound healing. Am. J. Surg. 1981; 141: 593-596.

Babior BM. Neutrophil function as related to neutrophil-endothelial cell interactions. Nouv. Rev. Fr. Hematol. 1992; 34 (suppl):S29-S35.

Babior BM. Oxidants from phagocytes: agents of defense and destruction. Blood. 1984; 64(5):959-966.

Babior BM. Oxygen-dependent microbial killing by phagocytes. N. Eng. J. Med. 1978; 298:659-668, 721-725.

Babior BM. The respiratory burst. p.132-134. In: Lehrer RI. moderator. Neutrophils and host defense. Ann. Intern. Med. 1988; 109:127-142.

Baggiolini M, Boulay F, Badwey JA, Curnutte JT. Activation of neutrophil leukocytes: chemoattractant receptors and respiratory burst. FASEB J. 1993; 7: 1004-1010.

Bajaj MS, Kew RR, Webster RO, Hyers TM. Priming of human neutrophil functions by tumor necrosis factor: enhancement of superoxide anion generation, degranulation, and chemotaxis to chemoattractants C5a and F-Met-Leu-Phe. Inflammation. 1992; 16(3):241-250.

Behar D, Czapski G, Rabani J, Dorfman LM, Swartz HA. The acid dissociation constant and decay kinetics of perhydroxyl radical. J. Phys. Chem. 1970; 74: 3209-3213.

Bevilacqua MP, Nelson RM, Mannori G, Cecconi O. Endothelial-leukocyte adhesion molecules in human disease. Annu. Rev. Med. 1994; 45: 361-378.

Blemond P, van Eijk HG, Swaak AJ, Koster JF. Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes: possible mechanism in inflammation disease. J. Clin. Invest. 1984; 73: 1576-1579.

Bochner BS, Rutledge BK, Schleimer RP. Interleukin 1 production by human lung tissue. II. Inhibition by anti-inflammatory steroids. J. Immunol. 1987; 139(7): 2303-2307.

Bonfanti R, Furie BC, Furie B, Wagner DD. PADGEM (GMP-140) is a component of Wiebel Palade bodies of human endothelial cells. Blood. 1989; 73: 1109-1112.

Boyum A. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 1968; 21(Suppl 97): 77.

Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J. Invest. Dermatol. 1982; 78(3): 206-209.

Britigan BE, Rosen GM, Thompson BY, Chai Y, Cohen MS. Stimulated human neutrophils limit iron-catalyzed hydroxyl radical formation as detected by spin-trapping techniques. J. Biol. Chem. 1986; 261: 17026-17032.

Bucurenci N, Blake DR, Chidwick K, Winyard PG. Inhibition of neutrophil superoxide production by human plasma α1-antitrypsin. FEBS Lett. 1992; 300(1):21-24.

Burton JL, Kehrli ME, Kapil S, Horst RL. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. J. Leukoc. Biol. 1995; 57: 317-325.

Cainzos M, Potel J, Puente J. Anergy in jaundiced patients. Br. J. Surg. 1988; 75:147-149.

Cameron GR, Oakley CL. Ligation of the common bile duct. J. Path. Bact. 1932; 35: 769-798.

Chenoweth DE, Hugli TE. Demonstration of specific C5a receptor on intact human polymorphonuclear leukocytes. Proc. Natl. Acad. Sci. USA. 1978; 75: 3943-3947.

Chensue SW, Terebuh PD, Remick DG, Scales WE., Kunkel SL. In vivo biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia. Kinetics, Kupffer cell expression, and glucocorticoid effects. Am. J. Path. 1991; 138(2): 395-402.

Chrousos GP, Gold PW. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA. 1992; 267: 1244-1252.

Clark RA, Volpp BD, Leidal KG. Nauseef WM. 2 cytosolic components of the human neutrophil respiratory burst translocate to the plasma membrane during cell activation. J. Clin. Invest. 1990; 85:714-721.

Coates TD, Wolach B, Tzeng DY, Higgins C, Baehner RL, Boxer LA. The mechanism of action of the anti-inflammatory agents dexamethasone and auranofin in human polymorphonuclear leukocytes. Blood. 1983; 62: 1070-1077.

Cohen HJ, Newburger PE, Chovaniec ME, Whitin JC, Simons ER. Opsonized zymosan-stimulated granulocytes-activation and activity of the superoxide-generating system and membrane potential changes. Blood. 1981; 58(5): 975-982.

Cohen MS, Mao J, Rasmussen GT, Serody JS, Britigan BE. Interaction of lactoferrin and lipopolysaccharide (LPS): effects on the antioxidant property of lactoferrin and the ability of LPS to prime human neutrophils for enhanced superoxide formation. J. Infect. Dis. 1992; 166: 1375-1378.

Cramer EB, Milks LC, Ojakian GK. Transepithelial migration of human neutrophils: an in vivo model system. Proc. Natl. Acad. Sci. USA. 1980; 77:4069-4073.

Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G. Corticosteroids are transcriptional regulators of acute inflammation. Trans. Assoc. Am. Physicians. 1992a; 105: 25-35.

Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann GA. Mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. Proc. Natl. Acad. Sci. USA. 1992b; 89: 9991-9995.

Dahm LJ, Roth RA. Differential effects of lithocholate on rat neutrophil activation. J. Leukoc. Biol. 1990; 47: 551-560.

Di Rosa M. Biological properties of carrageenan. J. Pharm. Pharmac. 1972; 24: 89-102.

Diamond MS, Staunton DE, Marlin SD, Springer TA. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell. 1991; 65: 961-971.

Dobrinich R, Spagnuolo PJ. Binding of C-reactive protein to human neutrophils. Arthr. Rheum. 1991; 34(8):1031-1038.

Drivas G, James O, Wandle N. Study of reticuloendothelial phagocytic capacity in patients with cholestasis. Br. J. Med. 1976; 1: 1568-1569.

Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA. Induction by IL-1 and interferon-γ: tissue distribution, biochemistry, function of a natural adherence molecule (ICAM-1). J. Immunol. 1986; 137:245-254.

Elsbach P, Weiss J. Oxygen-independent antimicrobial systems of phagocytes. In: Inflammation: Basic Principles and Clinical Correlates. 2nd. ed. Gallin JI, Goldstein M, Snyderman R, eds. Raven Press, New York. 1992. pp. 603-636.

Fehr J, Dahinden C. Modulating influence of chemotactic factor-induced cell adhesiveness on granulocyte function. J. Clin. Invest. 1979; 64: 8-16.

Flower RJ, Parente L, Persico P, Salmon JA. A comparison of the acute inflammatory response in adrenalectomised and sham-operated rats. Br. J. Pharmac. 1986; 87: 57-62.

Fridovich I. Superoxide radical and superoxide dismutase. Accts. Chem. Res. 1972; 5: 321-326.

Furie MB, Tancinco MCA, Smith CW. Monoclonal antibodies to leukocyte integrins CD11a/CD18 and CD11b/CD18 or intercellular adhesion molecule-1 inhibit chemoattractant-stimulated neutrophil transendothelial migration *in vitro*. Blood. 1991; 78: 2089-2097.

Gaboury J, Kubes P. Reductions in physiologic shear rates lead to CD11/CD18-dependent, selectin-independent leukocyte rolling *in vivo*. Blood. 1994; 83: 345-350.

Gambino SR, Di Re J. Bilirubin Assay [Revised]. In: Standard methods of clinical chemistry. Volume 5. Meites S. ed. Academic Press, New York. 1965. pp. 55-64.

Goldstein RA, Bowen DL, Fauci AS. Adrenal corticosteroids. In Inflammation: Basic Principles and Clinical Correlates, 2nd. edition. Gallin JI, Goldstein M, Snyderman R, eds. Raven Press, New York. 1992. pp. 1061-1081.

Greve JW, Gouma DJ, Soeters PB, Buurman WA. Suppression of cellular immunity in obstructive jaundice is caused by endotoxins: a study with germ-free rats. Gastroenterology. 1990; 98: 478-485.

Grisham MB, Jefferson MM, Melton DF, Thomas EL. Chlorination of endogenous amines by isolated neutrophils. Ammonia-dependent bactericidal, cytotoxic and cytolytic activities of the chloramines. J. Biol. Chem. 1984a; 259:10404-10413.

Grisham MB, Jefferson MM, Thomas EL. Role of monochloramine in the oxidation of erythrocyte hemoglobin by stimulated neutrophils. J. Biol. Chem. 1984b; 259: 6757-6765.

Guyton AC. Textbook of Medical Physiology. 8th Edition. W. B. Saunders Company, Toronto. 1991. pp. 774-775.

Harlan JM, Winn RK, Vedder NB, Doerschuk CM, Rice CL. *In vivo* models of leukocyte adherence to endothelium. In Adhesion: Its Role in Inflammatory Disease, Harlan JR, Liu D, eds. W. H. Freeman and Company, New York. 1992. pp. 117-150.

Hattori R, Hamilton KK, McEver RP, Sims PJ. Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. J. Biol. Chem. 1989; 264: 9053-9060.

Higgs GA, Follenfant RL, Garland LG. Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: effects on acute inflammatory responses. Br. J. Pharmacol. 1988; 94: 547-551.

Hilfenhaus M. Circadian rhythm of plasma renin activity, plasma aldosterone and plasma corticosterone in rats. Int. J. Chronobiol. 1976; 3: 213-229.

Holman JM, Rikkers LF. Biliary obstruction and host defense failure. J. Surg. Res. 1982; 32: 208-213.

Hubbard RC, Ogushi F, Fells GA, Cantin AM, Jallat S, Courtney M, Crystal RG. Oxidants spontaneously released by alveolar macrophages of cigarette smokers can inactivate the active site of alpha-1 antitrypsin, rendering it ineffective as an inhibitor of neutrophil elastase. J. Clin. Invest.. 1987; 80: 1289-1295.

Huey R, Hugli TE. Characterization of a C5a receptor on human polymorphonuclear leukocytes (PMN). J. Immunol. 1985; 135: 2063-2068.

Johnson KJ, Varani J, Smolen JE. Neutrophil activation and function in health and disease. In: Ganulocyte response to cytokines-Basic and clinical research. Coffey RG, ed. Immunology series 57. Marcel Dekker, Inc., New York. 1992. pp. 1-46.

Jutila MA, Rott L, Berg EL, Butcher EC. Function and regulation of the neutrophil MEL-14 antigen *in vivo*: comparison with LFA-1 and Mac-1. J. Immunol. 1989; 143: 3318-3324.

Kanner J, Kinsella JE. Lipid peroxidation by H_2O_2-halide system. Lipids. 1983; 18:204-210.

Kaplan EL, Laxdal T, Quie PG. Studies of polymorphonuclear leukocytes from patients with chronic granulomatous disease of childhood: bactericidal capacity for streptococci. Pediatrics. 1968; 41:591-599.

Katz S, Grosfeld JL, Gross K, Plager DA, Ross D, Rosenthal RS, Hull M, Weber TR. Impaired bacterial clearance and trapping in obstructive jaundice. Ann. Surg. 1984; 199: 14-20.

Kim SJ, Han D, Moon KD, Rhee JS. Measurement of superoxide dismutase-like activity of natural antioxidants. Biosci. Biotechnol. Biochem. 1995; 59(5):822-826.

Kishimoto TK, Anderson DC. The role of integrins in inflammation. In Inflammation: Basic principles and clinical correlates, 2nd ed. Gallin JI, Goldstein IM and Snyderman R. eds. Raven Press, New York. 1992. pp. 353-406.

Klebanoff SJ. Oxygen metabolites from phagocytes. In: Inflammation: Basic Principles and Clinical Correlates. 2nd. ed. Gallin JI, Goldstein M, Snyderman R, eds. Raven Press, New York. 1992. pp. 541-588.

Kountouras J, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. Br. J. Exp. Pathol. 1984; 65: 305-312.

Lang ME, Jourd'heuil D, Meddings JB, Swain MG. Increased opioid binding to peripheral white blood cells in a rat model of acute cholestasis. Gastroenterology. 1995; 108: 1479-1486.

Laue L, Kawai S, Brandon DD, Brightwell D, Barnes K, Knazek RA, Loriaux DL, Chrousos GP. Receptor-mediated effects of glucocorticoids on inflammation: enhancement of the inflammatory response with a glucocorticoid antagonist. J. Steroid. Biochem. 1988; 29: 591-598.

Levy R, Schlaeffer F, Keynan A, Nagauker O, Yaari A, Sikuler E. Increased neutrophil function induced by bile duct ligation in a rat model. Hepatology. 1993; 17: 908-914.

Luscinskas FW, Cybulsky MI, Kiely JM, Peckins CS, Davis VM, Gimbrone MA. Cytokine-activated human endothelial monolayers support enhanced neutrophil transmigration via a mechanism involving both endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1. J. Immunol. 1991; 146: 1617-1625.

Marasco WA, Fantone JC, Freer RJ, Ward PA. Characterization of the rat neutrophil formyl peptide chemotaxis receptor. Am. J. Pathol. 1983; 111: 273-281.

Maridonneau-Parini I, Tringale SM, Tauber AI. Identification of distinct activation pathways of the human neutrophil NADPH-oxidase. J. Immunol. 1986; 137(9): 2925-2929.

Marlin SD, Springer TA. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell. 1987; 51: 813-819.

Martins MA, e Silva PMR, Castro Faria Neto HC, Bozza PT, Dias PMFL, Lima MCR, Cordeiro RSB, Vargaftig BB. Pharmacological modulation of Paf-induced rat pleurisy and its role in inflammation by zymosan. Br. J. Pharmacol. 1989; 96: 363-371.

McEver RP, Beckstead JH, Moore KL, Marshall-Carlson L, Bainton DF. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Wiebel-Palade bodies. J. Clin. Invest. 1989; 84: 92-99.

Michaelsson M. Bilirubin determination in serum and urine. Scand. J. Clin. Lab. Invest. 1961. 13(Suppl. 56): 1.

Motyka B, Griebel PJ, Reynolds JD. Agents that activate protein kinase C rescue sheep ileal Peyer's patch B cells from apoptosis. Eur. J. Immunol. 1993; 23: 1315.

Ohshio G, Miyachi Y, Kudo H, Niwa Y, Manabe T, Tobe T. Effects of sera from patients with obstructive jaundice on the generation of oxygen intermediates by normal polymorphonuclear leukocytes. Liver. 1988; 8:366-371.

Pain JA, Cahill CJ, Bailey ME. Perioperative complications in obstructive jaundice: therapeutic considerations. Br. J. Surg. 1985; 72:942-945.

Patel KD, Zimmerman GA, Prescott SM, McEver RP, McIntyre TM. Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. J. Cell. Biol. 1991; 112: 749-759.

Peers SH, Moon D, Flower RJ. Reversal of the anti-inflammatory effects of dexamethasone by the glucocorticoid antagonist RU38486. Biochem. Pharmacol. 1988; 37(3): 556-557.

Perretti M, Flower RJ. Cytokines, glucocorticoids and lipocortins in the control of neutrophil migration. Pharmacol. Res. 1994; 30(1): 53-59.

Peskar BM, Lange K, Hopper U, Peskar BA. Ethanol stimulates formation of leukotriene C4 in rat gastric mucosa. Prostaglandins. 1986; 31: 283-293.

Pitt HA, Cameron JL, Postier RG, Gadacz TR. Factors affecting mortality in biliary tract surgery. Am. J. Surg. 1981; 141: 66-72.

Pober JS, Gimbrone MA, Lapierre LA, Mendrick DL, Fiers W, Rothlein R, Springer TA. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor and immune interferon. J. Immunol. 1986; 137:1893-1896.

Reddy VY, Pizzo SV, Weiss SJ. Functional inactivation and structural disruption of human α2-macroglobulin by neutrophils and eosinophils. J. Biol. Chem. 1989; 264: 13801-13809.

Rollins TE, Springer MS. Identification of the polymorphonuclear leukocytes C5a receptor. J. Biol. Chem. 1985; 260: 7157-7160.

Roos D, Weening RS, Voetman AA, Vanschaik MLJ, Bot AAM, Meerhof LJ, Loos JA. Protection of phagocytic leukocytes by endogenous glutathione. Studies in a family with glutathione reductase deficiency. Blood. 1979; 53: 851-866.

Roos D, Weening RS, Wyss SR, Aebi HE. Protection of human neutrophils by endogenous catalase. Studies with cells from catalase-deficient individuals. J. Clin. Invest. 1980; 65: 1515-1522.

Root RK, Cohen MS. The microbicidal mechanisms of human neutrophils and eosinophils. Rev. Infect. Dis. 1981; 3(3):565-598.

Roughneen PT, Didlake R, Kumar SC, Kahan BD, Rowlands BJ. The protective effects of hepatic dysfunction on vascularized allograft survival. J. Surg. Res. 1987; 42: 447-453.

Roughneen PT, Gouma DJ, Kulkarni AD, Fanslow WF, Rowlands BJ. Impaired specific cell-mediated immunity in experimental biliary obstruction and its reversibility by internal biliary drainage. J. Surg. Res. 1986; 41: 113-125

Roughneen PTM, Drath DB, Kulkarni AD, Kumar SC, Andrassy RJ, Rowlands BJ. Inflammatory cell function in young rodents with experimental cholestasis: investigations of functional deficits, their etiology, and their reversibility. J. Pediatr. Surg. 1989; 24: 668-673.

Rubens F, Brash J, Weitz J, Kinlough-Rathbone J. Interactions of thermally denatured fibrinogen on polyethylene with plasma proteins and platelets. J Biomed. Mater. Res. 1992; 26: 1651-1658.

Ruch W, Cooper PH, Baggiolini M. Assay of H2O2 production by macrophages and neutrophils with homovanillic acid and horse-radish peroxidase. J. Immunol. Methods. 1983; 63: 347-357.

Russell J, von Ritter C, Tso P, Granger DN. Effects of ursodeoxycholate on leukocyte-endothelial cell adhesion induced by lipid mediators. Gastroenterology. 1994; 104(4, part 2): A982.

Salvemini D, Manning PT, Zweifel BS, Seibert K, Connor J, Currie MG, Needleman P, Masferrer JL. Dual inhibition of nitric oxide and prostaglandin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors. J. Clin. Invest. 1995; 96: 301-308.

Scriven MW, Horrobin DF, Puntis MCA. Study of plasma and red cell phospholipid fatty acids in extrahepatic cholestatic jaundice. Gut. 1994; 35:987-990.

Segal AW, Abo A. The biochemical basis of NADPH oxidase in phagocytes. Trends Biochem. Sci. 1993; 18:43-47.

Segal AW, Peters TJ. Analytical subcellular fractionation of neutrophils from patients with chronic granulomatous disease. Q. J. Med. 1978; 47:213-220.

Selsted ME. Nonoxidative killing by neutrophils. p. 129-132. In: Lehrer RI. moderator. Neutrophils and host defense. Ann. Intern. Med. 1988; 109:127-142.

Shimizu K, Amagaya S, Ogihara Y. Analysis of corticosterone in the serum of mice and rats, using high performance liquid chromatography. J. Chromatog. 1983; 272:170.

Smolen JE, Boxer LA. Functions of neutrophils. In: Hematology. 5th. Edition. Beutler E, Lichtman MA, Coller BS, Kipps TJ. eds. McGraw-Hill, Inc., New York. 1995. pp. 779-798.

Spielberg SP, Boxer LA, Oliver JM, Allen JM, Schulman JD. Oxidative damage to neutrophils in glutathione synthetase deficiency. Br. J. Haematol. 1979; 42: 215-223.

Springer TA, Lasky LA. Sticky sugars for selectins. Nature. 1991; 349: 196-197.

Springer TA, Thompson WS, Miller LJ, Schmalstieg FC, Anderson DC. Inherited defiency of the Mac-1, LFA-1, p150,95 glycoprotein family and its molecular basis. J. Exp. Med. 1984; 160:1901-1918.

Springer TA. Adhesion receptors of the immune system. Nature. 1990; 346: 425-434.

Springer TA. Traffic signals for lymphocytes recirculation and leukocyte emigration: the multistep paradigm. Cell. 1994; 76: 301-314.

Staunton DE, Dustin ML, Springer TA. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature. 1989; 339: 61-64.

Sternberg EM, Hill JM, Chrousos GP, Kamilaris T, Listwak SJ, Gold PW, Wilder RL. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. Proc. Natl. Acad. Sci. USA. 1989; 86: 2374-2378.

Sternberg, VI, Bouley MG, Katz BM, Lee KJ, Parmar SS. Negative endocrine control system for inflammation in rats. Agents Actions. 1990; 29(3/4): 189-195.

Stryer L. Biochemistry. Third edition. W.H. Freeman and Company, New York. 1988. p. 297.

Swain MG, Maric M. Prevention of immune-mediated arthritis in cholestatic rats: involvement of endogenous glucocorticoids. Gastroenterology. 1994; 107: 1469-1474.

Swain, MG, Tjandra K, Kanwar S, Kubes P. Neutrophil adhesion is impaired in a rat model of cholestasis. Gastroenterology. 1995; 109: 923-932.

Swain, MG, Vergalla J, Bergasa N, Jones EA. Sympathetic nerves, but not the adrenal gland, contribute to elevated plasma levels of met-enkephalin in rats with acute cholestatic hepatitis. Regul. Pept. 1993; 46: 535-542.

Thomas EL, Grisham MB, Jefferson MM. Myeoloperoxidase-dependent effect of amines on functions of isolated neutrophils. J. Clin. Invest. 1983; 72: 441-454.

Thomas EL. Myeloperoxidase-hydrogen peroxidase-chloride antimicrobial system: effect of exogenous amines on antibacterial action against *Escherichia coli*. Infect. Immun. 1979; 25: 110-116.

Tonnesen MG. Neutrophil-endothelial cell interactions: mechanisms of neutrophil adherence to vascular endothelium. J. Invest. Dermatol. 1989; 93: 53S-58S.

Tschesche H, Bakowski B, Schettler A, Knäuper V, Reinke H, Krämer S. Leukodiapedesis, compartmentalisation and secretion of PMN leukocyte proteinases, and activation of PMN leukocyte procollagenase. In: New aspects of human polymorphonuclear leukocytes. Hörl WH, Schollmeyer PJ, eds. Plenum Press, New York. 1991. pp. 39-53.

Voetman AA, Roos D. Endogenous catalase protects human blood phagocytes against oxidative damage by extracellularly generated hydrogen peroxide. Blood. 1980; 56: 846-852.

von Andrian UH, Hansell P, Chambers JD, Berger EM, Filho IT, Butcher EC, Arfors K-E. L-selectin function is required for beta-2 integrin-mediated neutrophil adhesion at physiologic shear rates *in vivo*. Am. J. Physiol. 1992; 263: H1034-H1044.

Walker BAM, Seiler AJ, Owens CA, Hagenlocker BE, Ward PA. Absence of FMLP receptors on rat macrophages. J. Leukoc. Biol. 1991; 50: 600-606.

Wallace JL, Keenan CM, Gale D, Shoupe TS. Exacerbation of experimental colitis by nonsteroidal anti-inflammatory drug is not related to elevated leukotriene B_4 synthesis. Gastroenterology. 1992; 102: 18-27.

Weisbart RH, Kwan L, Golde DW, Gasson JC. Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants. Blood. 1987; 69:18-21.

Weiss SJ, Curnutte JT, Regiani S. Neutrophil-mediated solubilization of the subendothelial matrix: oxidative and nonoxidative mechanisms of proteolysis used by normal and chronic granulomatous disease phagocytes. J. Immunol. 1986; 136: 636-641.

Weiss SJ, Lampert MB, Test ST. Long-lived oxidants generated by human neutrophils: Characterization and bioactivity. Science. 1983; 222: 625-628.

Weller A, Isenmann S, Vestweber D. Cloning of the mouse endothelial selectins. Expression of both E- and P-selectin is inducible by tumor necrosis factor. J. Biol. Chem. 1992; 267: 15176-15183.

Williams LT, Snyderman R, Pike MC, Lefkowitz RJ. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proc. Natl. Acad. Sci. USA. 1977; 74: 1204-1208.

Woodman RC, Curnutte JT, Babior BM. Evidence that *de novo* protein synthesis participates in a time-dependent augmentation of the chemotactic peptide induced respiratory burst in neutrophils - Effects of recombinant human colony stimulating factors and dihydrocytochalasin B. Free Radic. Biol. Med. 1988; 5:355-361.

Woodman RC, Newburger PE, Anklesaria P, Erickson RW, Rae J, Cohen MS, Curnutte JT. A new X-linked variant of chronic granulomatous disease characterized by the existence of a normal clone of respiratory burst-component phagocytic cells. Blood. 1995; 85(1):231-241.

Woodman RC, Reinhardt PH, Kanwar S, Johnston FL, Kubes P. The effects of human neutrophil elastase (HNE) on neutrophil function in vitro and in inflamed microvessels. Blood. 1993; 82: 2188-2195.

Worthen GS, Audi N, Vukajlovich S, Tobias PS. Neutrophil adherence induced by lipopolysaccharide *in vitro*. J. Clin. Invest. 1992; 90: 2526-2535.

Worthen GS, Schwab III B, Elson EL, Downey GP. Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries. Science. 1989; 245: 183-186.

Wozniak A, Betts WH, Murphy GA, Rokicinski M. Interleukin-8 primes human neutrophils for enhanced superoxide anion production. Immunol. 1993; 79(4): 608-615.

Wright DG, Gallin JI. Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. J. Immunol. 1979; 123: 285-294.

Zuckerman SH, Shellhaas J, Butler LD. Differential regulation of lipopolysaccharide-induced interleukin 1 and tumor necrosis factor synthesis: effects of endogenous and exogenous glucocorticoids and the role of the pituitary-adrenal axis. Eur. J. Immunol. 1989; 19(2): 301-305.