Regulation of P-glycoprotein and Glucocorticoid Receptor Expression in the Rat Intestine

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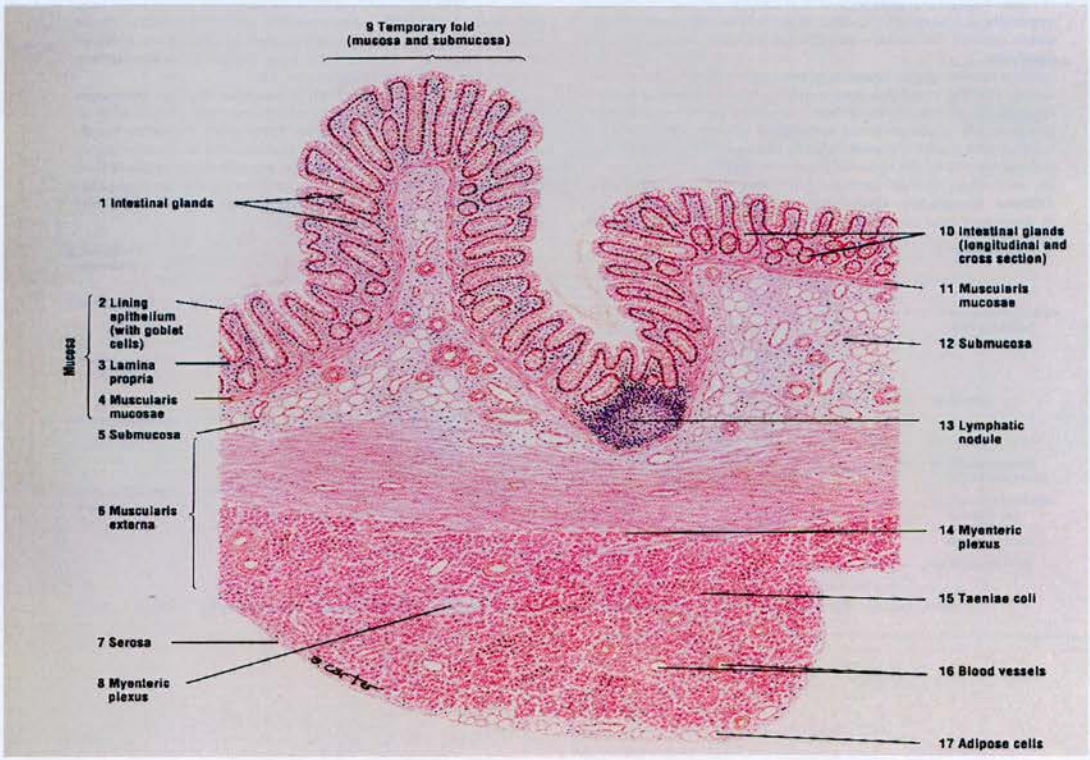

Figure 1.1: Diagram illustrating composition of the intestinal mucosa. The three important layers include the thick basal muscle layer (composed of both circular and longitudinal muscle), submucosa and mucosal sections. The mucosa is composed of a thin muscle layer separating the mucosa and submucosa, lamina propria and the epithelial lining. Intestinal glands (crypts) extend through the lamina propria to the thin muscle layer. Within these crypts, epithelial cells differentiate and migrate from the base of the crypt towards the tip. These cells are involved in the absorption/excretion of compounds (Keshav 2003).

found dexamethasone increased P-gp expression, an effect initiated through GR, as well as increase the levels of cytoplasmic NF-κB.

A number of observations made were novel, and collectively these data suggest a role for dexamethasone treatment as well as bacteria in the regulation of genes determining steroid sensitivity in the healthy and diseased rodent intestinal epithelium. The complex interactions between P-gp and GR expression in response to bacteria have implications for potential mechanisms by which inflammation is induced in the colon.

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Declaration

I declare that in this thesis was written by me and that the data presented within it is a result of my own work, except where specifically acknowledged in the text.

I declare that this work has not been submitted for any other degree.

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Publications

Abstracts

Moodie FM, Noble J, Satsangi J, Seckl JR. Glucocorticoid access and action in the rat colon: expression and regulation of multidrug resistance gene 1a (mdr1a), glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). *Gut* 2003; 52(suppl 1):A54. Presented at the British Society of Gastroenterology meeting, March 2003.

Moodie FM, Lyons V, Satsangi J, Seckl JR. Effects of glucocorticoids on expression of p-glycoprotein and glucocorticoid receptor in the intestinal epithelium. *Gut* 2004; 53(suppl 3):A65. Presented at the British Society of Gastroenterology meeting March 2004, and Digestive Disease Week, New Orleans, May 2004.

Moodie FM, Quin B-F, Dieleman LA, Seckl JR, Sartor RB, Satsangi J. P-glycoprotein and glucocorticoid receptor in HLA-B27 transgenic rats. Presented at the Scottish Society for Experimental Medicine, November 2004.

Moodie FM, Quin B-F, Dieleman LA, Seckl JR, Sartor RB, Satsangi J. P-glycoprotein and glucocorticoid receptor in HLA-B27 transgenic rat colon. Presented at the British Society of Gastroenterology meeting 2005.

Papers

Moodie FM, Noble J, Satsangi J, Seckl JR. Effect of glucocorticoid manipulations on genes determining tissue sensitivity to glucocorticosteroids along the rat colon. *(Manuscript in preparation).*

Moodie FM, Quin B-F, Dieleman LA, Seckl JR, Sartor RB, Satsangi J. P-glycoprotein and glucocorticoid receptor in HLA-B27 transgenic rat colon. *(Manuscript in preparation).*

Reviews

Ho G-T, Moodie FM, Satsangi J. Multidrug resistance gene 1 (P-glycoprotein): an important determinant of gastrointestinal disease? *Gut* 2003;11:1-5.

Abbreviations

ACTH	Adrenocorticotrophic
ADX	Adrenalectomised
ADX-D	Dexamethasone treated ADX animals
ADX-V	Vehicle treated ADX animals
ANOVA	Analysis of Variance
AP-1	Activating Protein-1
APC	Antigen presenting cell
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
bp	Base pairs
CD	Crohn's Disease
BSA	Bovine serum albumin
cDNA	Complementary deoxyribose nucleic acid
CARD	Caspase recruitment domain
CBG	Corticosterone binding globulin
cort	Corticosterone

cpm	Counts per minute
CRH	Corticotrophin releasing hormone
Cyto	Cytosolic
CYP450	Cytochrome P450
dATP	deoxyadenosine triphosphate
DBD	DND binding domain
DMEM	Dulbecco's Minimal Essential Medium
DEPC	Diethylpyrocarbonate
dCTP	deoxyguanosine diphosphate
dH_2O	deionised water
DNA	Deoxyribose Nucleic Acid
DSS	Dextran Sodium Sulphate
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra-Acetate
ER	Endoplasmic reticulum
GALT	Gut-associated lymphoid tissue

GC	Glucocorticoids
GF	Germ-free
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Responsive Element
GTP	Guanosine Triphosphate
HEPES	(N-[2-Hydroxyethyl]piperazine N'-[2-ethanesulfonic acid
HPA	Hypothalamic-Pituitary-Axis
HSD	Hydroxysteroid dehydrogenase
HSP	Heat shock protein
IBD	Inflammatory Bowel Disease
LPS	Lipopolysaccharide
IEC	Intestinal Epithelial Cell
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IκB-α	IKappaB-alpha
IL	Interleukin

IKK-α	IκB kinase-α
IRAK	IL receptor associated kinase
kb	kilobases
kDa	kiloDaltons
LB	Luria-Bertoni / liquid broth
M cells	Microfold cells
MDP	Muramyl dipeptide
MDR	Multi Drug Resistance gene (human)
mdr1a	multidrug resistance 1a (rodent)
mdr1b	multidrug resistance 1b (rodent)
MHC	Major Histocompatibility Complex
MAPK	Mitogen-activated kinase
MOPS	(3-[N-Morpholino])propanesulphonic acid
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NF-κB	Nuclear Factor-Kappa B

NIK	NF-κB inducing kinase
Nod	Nucleotide oligomerisation domain
NT	Non-Transgenic
NTGF	Non-Transgenic in Germ-Free
NTSPF	Non-Transgenic in Specific-Pathogen Free
Nuc	Nuclear
PGN	Peptidoglycan
PG-PS	Peptidoglycan-polysaccharide
PMSF	Phenylmethanesulfonyl fluoride
POMC	Pro-opimelanocortin
RICK	RIP-like interacting CLARP kinase
RNA	Ribose Nucleic Acid
rpm	revolutions per minute
SCID	Severe combined immune deficiency
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
Sham	Sham-operation

Sham-D	Dexamethasone treated sham-operated animals
Sham-V	Vehicle treated Sham-operated animals
SPF	Specific-Pathogen Free
SSC	Saline Sodium Citrate
TCR	T-cell receptor
TEMED	N,N,N',N'-Tetramethylethyenediamine
TG	Transgenic
TGGF	Transgenic in Germ-Free
TGSPF	Transgenic in Specific-Pathogen Free
Th1	T-cell helper immune response 1
Th2	T-cell helper immune response 2
Th3	T-cell helper immune response 3
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulphonic acid
TNF-α	Tumour necrosis factor-alpha
TNFR	Tumour necrosis factor receptor

TRADD	TNF receptor associated death domain	
TRAF	TNF receptor associated factor	
Tris	Tris[hydroxymethyl]-aminomethane	
UC	Ulcerative Colitis	
UTP	Uridine triphosphate	
UV	Ultraviolet	light

Chapter 1

Introduction

1.1 BACKGROUND

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory bowel diseases (IBD) which now represent common cause of morbidity in young people. The combined prevalence of these two diseases in the United Kingdom is 400 per 100,000 (Watts & Satsangi 2002). Both diseases commonly first present in young adults (Jewell 1999). Ulcerative colitis is characterised by chronic inflammation limited to the colon, whereas in Crohn's disease inflammation may present in any region of the gastrointestinal tract, in particular the ileo-caecal region (Jewell 1999; Satsangi *et al.* 1997).

The mucosa of UC and CD patients is characterised by an infiltration of acute and chronic pro-inflammatory cells. In UC, inflammation is limited to the mucosal layer of the intestine, whereas in CD the entire wall is involved. In UC inflammation is known to initiate abscesses within the crypt, whereas in CD inflammation causes deep ulcers within the mucosa, which may penetrate the intestinal wall to initiate abscesses or fistulae (Hall 2002). This can lead to a variety of symptoms including bloody diarrhoea, passage of mucus as well as weight loss, pain, lethargy and anaemia.

The aetiology of these diseases is uncertain. However there has been recent progress made in identifying immunological, environmental and genetic factors involved in the pathogenesis of these diseases. Both environmental factors such as smoking, and genetic factors are known to contribute to the pathogenesis and outcome of IBD (Roussomoustakaki *et al.* 1998; Satsangi *et al.* 1996b; Satsangi *et al.* 1997). Concordance rates in siblings and twins have provided strong evidence that UC and CD are related polygenic diseases (Hampe *et al.* 1999). More importantly for this thesis, the development of transgenic animal models, such as the HLA-B27 transgenic rat and the mdr1a knockout mouse, which develops intestinal inflammation by 12 weeks after birth, has emphasised the importance of immune dysfunction and gut flora contributing to the disease states (Breban 1998; Panwala *et al.* 1998).

Although new therapeutic drugs are emerging, corticosteroid therapy remain the drug treatment of choice for active disease. However between 10-20% of patients are steroid insensitive, not responding to therapy, and a similar proportion will become steroid-dependent, unable to discontinue treatment (Faubion *et al.* 2001; Honda *et al.* 2000). Alterations in the intestinal expression P-glycoprotein (P-gp) and glucocorticoid receptor (GR), both of which are involved in determining steroid sensitivity, have been implicated in disease (Farrell *et al.* 2000; Langmann *et al.* 2004; Rogler *et al.* 1999) although the molecular mechanism behind the regulation of these genes remains elusive.

This introductory chapter reviews the structure and function of the intestinal epithelium, and mechanisms by which it protects itself against potential inflammatory mediators as well as immune regulation within the intestine. Factors involved in initiating disease, in both man and animals will be discussed, with particular emphasis on the role of bacteria and disease in animal models of inflammation. The role of glucocorticoids in the treatment of disease will be reviewed, as well as the actions of glucocorticosteroids in regulating genes determining intestinal steroid sensitivity. Finally the aims of the experiments described in chapter 3-7 of this thesis are listed.

1.2 INTESTINAL EPITHELIUM

The intestinal epithelium functions as a barrier, separating the contents of the lumen from the epithelial lining, and plays a pivotal role in maintaining intestinal homeostasis despite the persistent presence of potentially pro-inflammatory substances. To prevent these substances from breaching the barrier and initiating disease, a number of structural and functional barriers are present and will be outline below.

1.2.1 Structure and Function of the Epithelium

1.2.1.1 Basic Structure

The epithelial lining forms a continual layer of cells throughout the gastrointestinal tract, and regulates the entry of luminal contents. It is made up of three main layers: mucosal, submucosal and muscle layers (see figure 1.1). The mucosal layer is of great importance as it is involved in the absorption and excretion of substances from the body as the first cellular barrier separating the epithelium from the lumen contents (Meddings *et al.* 2003).

A thin muscle layer separates the mucosa from the submucosal and large muscle layers (Keljo & Squires 2000). The mucosal layer is composed of the lamina propria and the epithelial lining. In the colon, the colonic mucosal layer is indented with long tubular extensions called colonic crypts, which extend through the lamina propria to the thin muscle layer (see figure 1.1). In the small bowel, the epithelial lining is characterised by inward folds of the inner lining (plicae) and villi, each with a covering of hair-like extensions called brush-border (Hall 2002), and this aids in the absorptive function of this area of the intestine (see 1.2.1.2).

As the gut is a major site of immune regulation the intestinal mucosa is heavily laden with macrophages, lymphocytes and other cells involved in the immune system in lamina propria or between epithelium cells (intraepithelial cell lymphocytes) (Makala *et al.* 2004). It is not surprising therefore that around 25% of intestinal mucosa is comprised of lymphoid tissue. The gut recognises potential pathogenic bacteria in Peyers patches located in the mucosa of the small intestine (Makala *et al.* 2004).

These are specialised lymph nodes, part of the gut-associated lymphoid tissue (GALT), and are located in the mucosal layer of the intestine extending into the submucosal layer (MacDonald 2003a).

Epithelial cells lining the mucosa are constantly renewed every 2-3 days, and this aids in maintaining a protective barrier in response to stress and injury (MacDonald 2003b). Cells within the crypt migrate and differentiate from the base, where they reside as stem cells, up towards the tip of the crypt where they become integrated into the surface epithelium. As cells differentiate, they develop characteristics of intestinal epithelial cells which include maintaining water and electrolyte homeostasis; mucosal defence; and epithelial efflux mechanisms. Stem cells are located at the base of the crypt as the environment of the crypt lumen of the crypt is less hostile; bacterial concentrations are low (Meddings *et al.* 2003). This ensures the protection of the stem cell from a potentially lethal environment and ensures the viability of the epithelium (Meddings *et al.* 2003).

1.2.1.2 Epithelial Function

The epithelial cells lining this layer are tightly packed and form a lipid bilayer, which allows the absorbance of lipid soluble molecules but offers a strong barrier against water soluble compounds (Meddings *et al.* 2003). In the small intestine the digestion is the most active process occurring in this area, and it is here where the most nutrients are absorbed, aided by the high epithelial surface layer produce by the villi. No digestion occurs in the colon. Epithelial cells also play a pivotal role in maintaining salt and electrolyte balance, nutrient absorption as well as immune homeostasis in the intestine (Keljo & Squires 2000). These cells are also involved in actively transporting potentially toxic substances out of cells and also produce substances involved in mucosal defence (both of which will be discussed in detail in section 1.2.2) (Ho *et al.* 2003; Meddings *et al.* 2003; Meyer & Schmidit 1994).

1.2.1.2.1 Water and Electrolyte Balance

The colonic epithelium, especially the distal colon, is a key tissue in maintaining electrolyte balance (Sellin 2000). The cells of the colonic epithelium express the mineralocorticoid receptor (MR). This receptor is activated by the mineralocorticoid aldosterone, which is produced by the adrenal gland. Activation of MR by aldosterone inhibits sodium chloride electrogenic absorption while inducing electrogenic sodium absorption (Meyer & Schmidit 1994). Other glucocorticoids, such as dexamethasone, activate MR although these agonists induce the opposite electrogenic changes noted by aldosterone (Meyer & Schmidit 1994). To maintain an electrolyte balance during times of increased glucocorticoid concentrations, such as during stress or steroid treatment, an enzyme called 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is co-localised with MR (Ronald & Funder 1996). This enzyme inactivates physiological glucocorticoids (cortisol, corticosterone), enabling aldosterone selectively to bind MR and initiates its effects.

Recent studies in Wistar rats have shown regional differences in water and electrolyte absorption along the rat colon (Nishinaka & Matsuura 2004). The main action of water transport across the epithelium occurs by diffusion, and is dependent on the presence of the osmotic gradient. Nishinaka and Matsuura noted that perfused distilled water was not absorbed in the small intestine, unlike the colon of healthy rats. However after the intestine was perfused with isotonic solutions, potassium was seen to be mostly absorbed in the small intestine, whereas net chloride and sodium absorption occurred in the colon. This absorption pattern in the colon is most likely due to the presence of MR which activates sodium and chloride absorption. This suggests that these receptors are not as abundant in the small intestine and therefore may explain this regional variation in electrolyte absorption.

1.2.1.2.2 Immune Homeostasis

The epithelium is in contact with a flora of bacteria, consisting of around 500 microbial species (Gilmore & Ferretti 2003). This leads to an infiltration of mononuclear cells, including T-cells involved in activating the immune response into the mucosa. In fact, in the 'normal' lamina propria, T-cells constitute one third of all cells in the lamina propria (Pallone *et al.* 2003). T-cells are activated by foreign substances, and are known to activate the immune system and be involved in cell-mediated immunity and antibody production (discussed in section 1.2.2). As the epithelium is always in contact with bacteria, a 'controlled' inflammation must be constantly present to maintain a healthy epithelial barrier function. Although the infiltration of 'foreign' compounds must be able to activate this system and produce an 'uncontrolled' inflammatory response (Pallone *et al.* 2003). Therefore the epithelium functions as an 'immuno-modulatory' organ, maintaining gut homeostasis. The mechanisms behind this will be discussed further in the following section

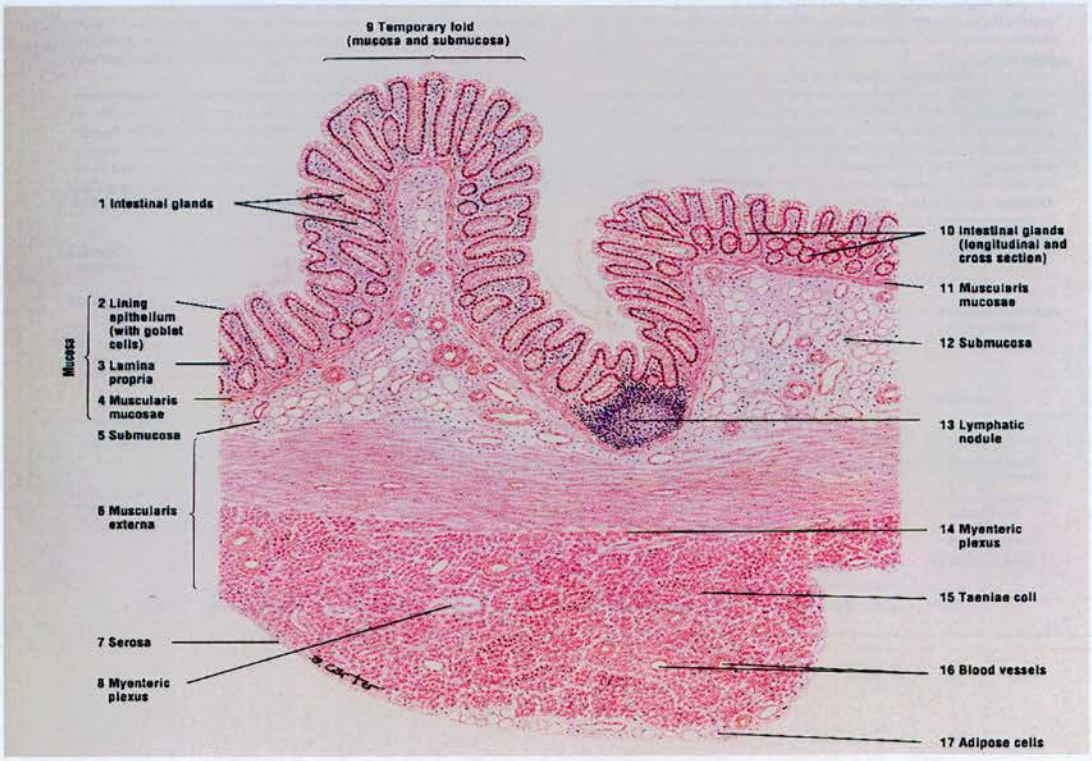


Figure 1.1: Diagram illustrating composition of the intestinal mucosa. The three important layers include the thick basal muscle layer (composed of both circular and longitudinal muscle), submucosa and mucosal sections. The mucosa is composed of a thin muscle layer separating the mucosa and submucosa, lamina propria and the epithelial lining. Intestinal glands (crypts) extend through the lamina propria to the thin muscle layer. Within these crypts, epithelial cells differentiate and migrate from the base of the crypt towards the tip. These cells are involved in the absorption/excretion of compounds (Keshav 2003).

1.2.2 Regulation of the immune system in the intestinal epithelium

One of the main functions of the intestinal epithelium is to prevent the 'uncontrolled' inflammation which may occur when foreign substances (xenobiotics), or even micro-organisms, breach the epithelial lining and enter the mucosa. The mucosal immune system is comprised of Peyer's patches, lamina propria lymphocytes, intraepithelial lymphocytes and mesenteric lymph nodes (Makala *et al.* 2004). This system maintains gut homeostasis, despite the continual exposure to potentially pro-inflammatory compounds. The mechanisms behind this will be discussed further in this section.

1.2.2.1 Acquired Immunity

1.2.2.1.1 Antigen recognition in the intestinal mucosa

Antigen presenting cells (APC) in the intestinal mucosa these are mainly composed of macrophages, dendritic cells, and B-cells. These have the ability to process antigens and present their peptide fragment on the cell surface (Makala *et al.* 2004). The proteins of the major histocompatibility complex (MHC) are critical in immune activation. Depending on the class of MHC and the other co-stimulatory molecules expressed on the cell surface, specific subset of T-cells recognise the MHC-antigen complex and the immune system is activated. MHC class II activate CD4+ cells, whereas CD8+ cells recognises MHC class I (Hershberg & Mayer 2000c).

1.2.2.1.2 Lymphocyte Production and Activation

There are two types of lymphocytes and these are called B-cells and T-cells. B- and T-cells are produced in the bone marrow; however B-cells mature in the bone marrow before entering the circulation, whereas T-cells mature in the thymus. Each B- and T-cell is specific for each antigen.

1.2.2.1.2.1 T-cells

T-cells recognise antigens through T-cell receptors (TCR) located in the surface. Most of the T-cells in the body can be divided into two groups: CD4+ and CD8+. These are glycoproteins which determine what type cell T-cells can bind. CD4+ bind cells expressing the MHC class II, whereas CD8+ cells can bind those expressing the MHC class I (Hershberg & Mayer 2000b). Most cells in the body express class I molecules, whereas only specific APCs express class II. These cells include dendritic cells, macrophages and B-cells (Hershberg & Mayer 2000a).

CD8+ T-cells are best known as cytotoxic T-cells. These cells are capable of monitoring all the cells in the body; ready to destroy any cell which presents an antigenic peptide on a MHC class I molecule. CD4+ T-cells are important in the initiation and maintenance of cellular immune response, as they bind antigens presented by dendritic cells and macrophages. After binding IL-12 is produced from the APC which causes the T-cell to produce other cytokines including tumour necrosis factor-alpha (TNF-α) and interferon-gamma (INF-γ) (Acheson & Luccioli 2004c). This type of immune response is called Th1, characterised by the types of cytokines released by the T-cells, and results in inflammation. CD4+ T-cells are also required in the production of antibodies, where they bind B-cells to stimulate antibody production. Some cells in the gut mucosa, such as dendritic cells, macrophages and B-cells, can contain both classes of MHC; capable of activating both subsets of T-cells, and therefore are known as 'true professional APC' (Makala *et al.* 2004).

The intestinal mucosa has evolved effector mechanisms to prevent over-excessive immune reactivity to avoid inflammation and tissue injury. Firstly the reactivity of lamina propria T-cells with the T-cell receptor (TCR) is reduced, and this has been associated with the local production of immunosuppressive molecules including IL-10 and TGF-β (Pallone *et al.* 2003). Moreover, a high number of these T-cells express the Fas antigen which triggers suicidal death (apoptosis), and lamina propria T-cells have been shown to have an increased susceptibility to Fas-induced apoptosis (Pallone *et al.* 2003).

Previously it was thought that the Th1 and Th2 responses mutually suppress each other by the secretion of IFN-γ and IL-4 respectively, and this may prevent over activation of the immune system (Mills & McGuirk 2004). However, the discovery of another type of regulatory T-cell (Tr) which secretes the cytokines IL-10 and/or TGF-β, has also been shown to repress both Th1 and Th2 responses and is known as the Th3 immune response. Tr cells are produced in response to antigens, and this Th3 response damps the Th1 and Th2 responses to these antigens, allowing resolution of infection with minimal collateral damage to host tissues (Mills & McGuirk 2004). It is now thought the balance between these molecules-Th1, Th2 and Th3, is important in preventing an inflammatory reaction to bacterial flora, as well as the initiation of allergies and autoimmune diseases.

1.2.2.1.2.2 B-cells

The primary role of the B cell is antibody production. Helper T-cells bind the B-cell receptor and T-cells secrete lymphokines which drive B-cells to proliferate and produce antibodies (Acheson & Luccioli 2004). The type of helper T-cell (Th) involved in this type of immunity is called Th2. This is classified by the type of lymphokines secreted by the helper cell and these include interleukin (IL)-4, IL-5, IL-10 and IL-13 (Acheson & Luccioli 2004). B-cells recognise antigens using B-cell receptors. The antigen is engulfed, digested and a peptide fragment is displayed on the cell surface by MHC protein. There B-cells are acting as an APC. Therefore T-cells bind the MHC protein and active the immune system.

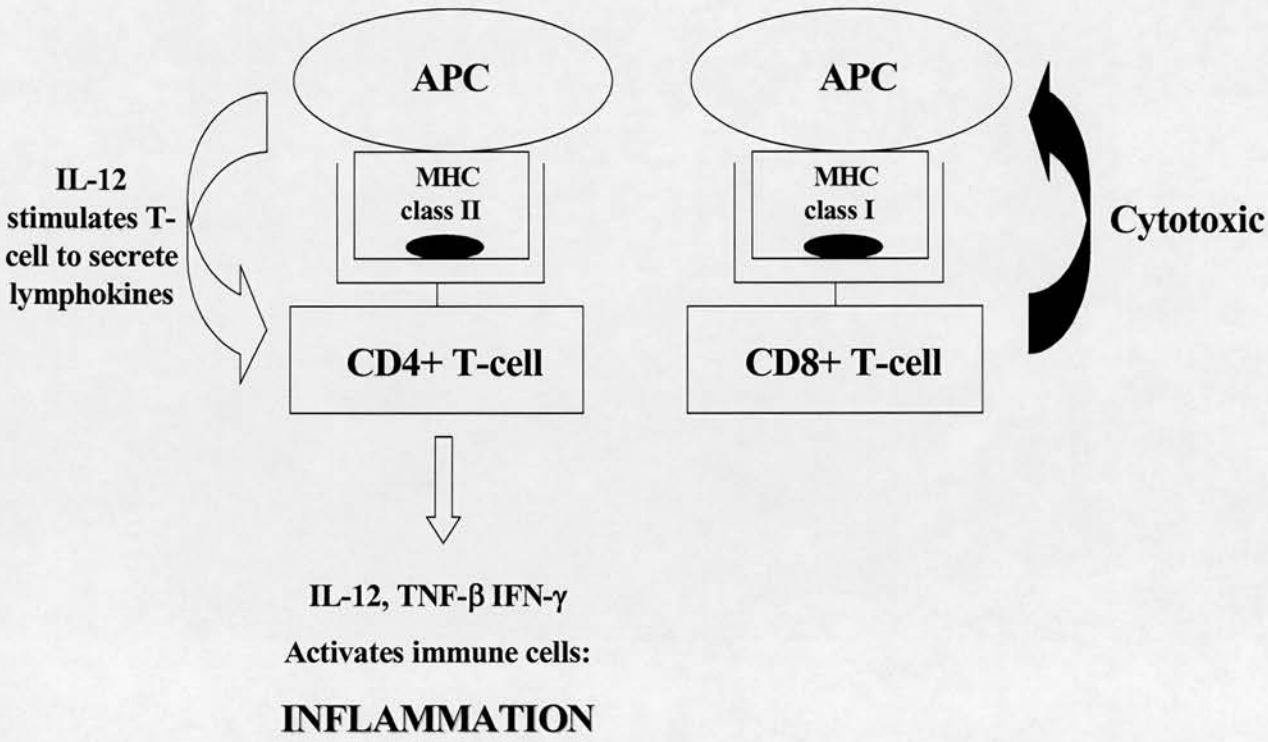


Figure 1.2: Antigen presenting cells (APCs) engulf, digest and present a peptide fragment on major histocompatibility molecules (MHC) on its surface. Depending on the class of MHC presenting the antigenic fragment, specific subsets of T-cells, CD4+ or CD8+ cells bind MHC through its surface T-cell receptor. CD4+ T-cells recognise MHC class II molecules, and after binding are stimulated by IL-12 produced by APC to secrete lymphokines. These lymphokines recruit more T-cells and other immune cells to the site of activation and initiating inflammation. This is also known as Th-1 immune response. CD8+ T-cells recognise MHC class I molecules, and after binding release cytotoxic cytokines which kill the cell presenting the antigenic fragment. (Figure adapted from (Sartor 2004b).

1.2.2.1.3 Bacterial Recognition by intestinal epithelial cells-'M cells'

There are specialised cellular mechanisms present in both man and rodents which are involved in the recognition of bacteria and in turn activate the mucosal immune system. One type of cell involved in the recognition is the microfold or 'M' cell. This appears to be a specific cell type in the intestinal epithelium over lymphoid follicles that endocytose a variety of protein and peptide antigens. This tissue is called the gut-associated lymphoid tissue (GALT). M cells transport bacterial antigens into the underlying tissue, where they are taken up by local dendritic cells and macrophages, whereby the immune system is activated after these cells present the antigen on its surface in association with the MHC molecular complex (MacDonald 2003d). Another unique feature of these lymphoid tissues is the ability to produce IgA. Production of this immunoglobulin typically requires T cell help: specific interactions between the CD4+ T-cells and dendritic cells within the area surrounding these follicles lead to secretion of transforming growth factor-β (TGF-β) which in turn favours B-cells switching to IgA secretion. B cells then migrate out of the follicles into the surrounding mucosa and release secretary IgA into the gut lumen (Acheson & Luccioli 2004).

1.2.2.2 Innate Immunity

1.2.2.2.1 Toll-like Receptors

Another mechanism whereby the body recognises bacterial products involve the family of Toll-like receptor (TLR). There have been ten TLR genes cloned and characterised, where the structure is similar to the IL-1 receptor (Abreu *et al.* 2002). In the intestine, TLR4 is involved as the sole sensor for lipopolysaccharide (LPS-a component in the wall of gram negative bacteria), whereas TLR2 involves the recognition of peptidoglycans and lipoprotein present in gram-positive bacteria (Abreu *et al.* 2001b).

TLR4 is thought to act as a transmembrane co-receptor with CD14 recognising LPS. LPS binding to the receptor recruits intracellular adapter proteins, such as MyD88 (Neish 2004). This adapter protein subsequently activates a phosphorylation cascade of mitogen-activated kinases (MAPK). The first of this includes the IL-1 receptor associated kinase (IRAK). Binding of IRAK to Myd88 induces the phosphorylation of IRAK, which subsequently causes it to disassociate from Myd88 and phosphorylates tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Moynagh 2003). This ultimately leads to the phosphorylation and activation of IκB kinase kinase (IKK) complex, where IKK phosphorylates I-κB-α (an inhibitor of NF-κB); inducing its degradation; allowing the pro-inflammatory transcription factor Nuclear Factor Kappa B (NF-κB) to translocate to the nucleus, inducing an inflammatory response (Cario *et al.* 2000). See figure 1.3.

In the intestinal epithelium in health, TLR4 protein expression has been shown to be expressed at low levels, a mechanism thought to have been evolved to prevent the over-activation of the immune system in the bacterial flora (Abreu *et al.* 2001a). However in patients with inflammatory bowel disease, colonic biopsies have shown increased TLR4 expression (Abreu *et al.* 2002). Pro-inflammatory cytokines have been shown to increase TLR4 expression (Abreu *et al.* 2002), and may facilitate in the perpetuation of inflammation due to the increased responsiveness to bacterial flora.

1.2.2.2.2 Caspase recruitment domains (CARD)/nucleotide-binding oligomerisation domain (Nod)

These are intracellular proteins, thought to be involved in the recognition of intracellular LPS, peptidoglycan (PGN) and/or invasive bacteria (Hisamatsu *et al.* 2003). CARD15/Nod2 and CARD4/Nod1 represent regulatory proteins with a nucleotide-binding oligomerisation domain (Nod) and N-terminal caspase recruitment domains (CARD) and are involved in programmed cell-death and immune responses (Rosenstiel *et al.* 2003). Leucine rich repeats in the Nods confer recognition of microbial molecules such LPS and PGN, where binding leads to activation of NF-κB through a RICK-dependent domain (Hisamatsu *et al.* 2003).

The dipeptide D-Glu-*meso*-DAP is the minimal PGN structure required for sensing by CARD4/Nod1, whereas the muramyl dipeptide (MDP) is the minimum essential structure of PGN that is recognised by CARD15/Nod2 (Philpott & Girardin 2004). Nod 1 is known to detect gram-negative bacteria, whereas Nod 2 recognises a broader range of bacteria, including both gram-negative and gram-positive bacterial products (Philpott & Girardin 2004b). Bacterial clearance is accelerated in cells expressing CARD15/Nod2 (Jobin 2003), and disruption of CARD15/Nod2 gene has been identified as a susceptibility locus for Crohn's disease (IBD1); mutated CARD15/Nod2 may lead to persistent immune activation due to the loss of bacterial clearance (Hisamatsu *et al.* 2003; Jobin 2003). Recent data has also suggested that CARD15/Nod 2 may 'dampen' the pro-inflammatory effect of activated TLR, and this may explain why a mutation in this gene is associated with both an increase in NF-κB activation and susceptibility for Crohn's disease (O'Neill 2004).

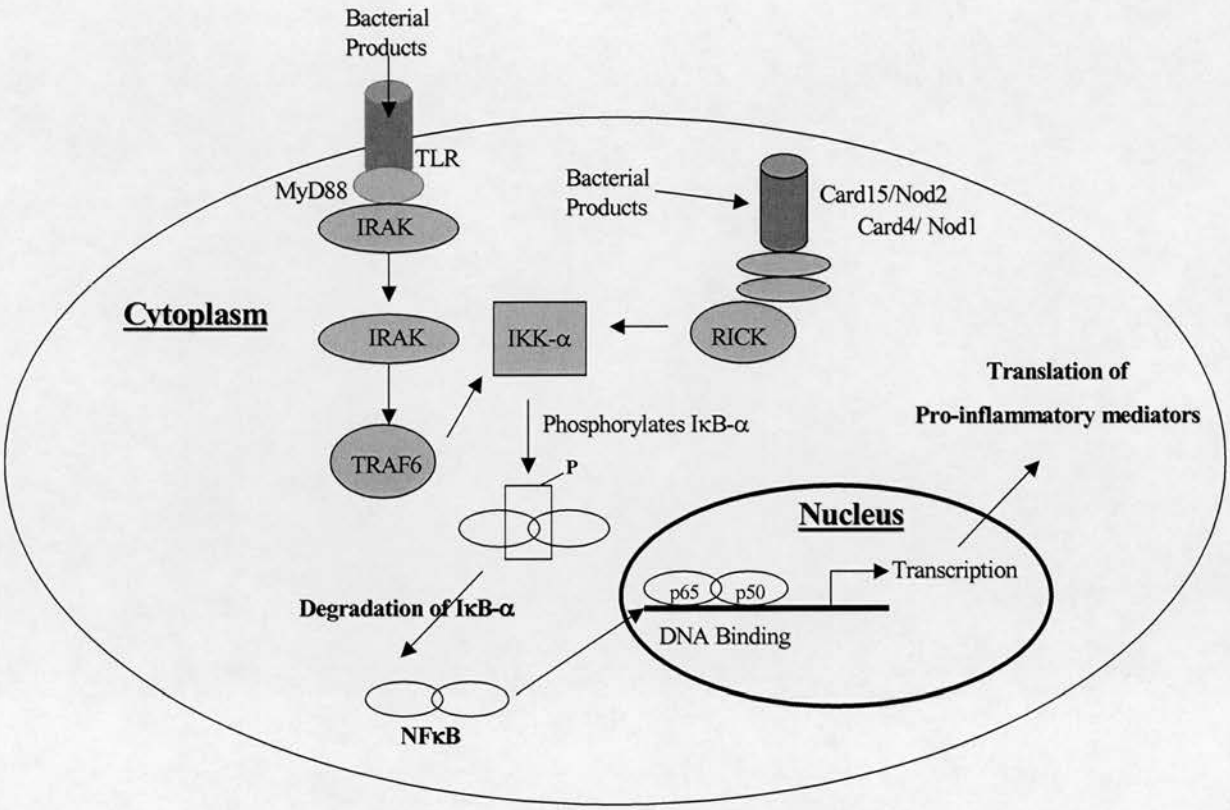


Figure 1.3: Toll-like receptors on the cell surface and intracellular Nod 1 and Nod 2 recognise bacterial products. Binding of these products leads to a phosphorylation cascade which ultimately leads to the activation of the pro-inflammatory cytokine NF-κB. TLR, such as TLR4, recruit adaptor proteins (MyD88 as one example), which bind IL-1 receptor associated kinase (IRAK). Binding causes the phosphorylation of IRAK, where is dissociates and phosphorylates tumor factor necrosis factor 6 (TRAF6). Intracellular bacterial products are recognised by Nod 1 and Nod 2. Binding activates the RICK-dependent signalling pathway. TRAF6 and RICK induce the phosphorylation of IκB kinase kinase (IKK), which subsequently phosphorylates IκB-α, inducing its degradation and this releases NF-κB, where it translocates to the nucleus and causes the transcription of pro-inflammatory molecules. Figure adapted from (Neurath *et al.* 1998)

1.2.3 Secretary mucosal defense proteins

The epithelium not only acts as a physical barrier but is also involved in the secretion of molecules which reduce the access of luminal molecules and micro-organisms into the epithelium (Meddings *et al.* 2003). There are three main types of secretory molecules known to be involved in mucosal defence, and these include antimicrobial peptides (defensins), mucus and trefoils

1.2.3.1 Defensins

Defensins (also known as cryptidins in mice) are known to have a broad antimicrobial activity and act by disrupting microbial cell walls (Cunliffe & Mahida 2004). α-defensins are secreted by Paneth cells and predominantly located in small intestine, whereas β-defensins are produced in the colon (Meddings *et al.* 2003). It has been shown that bacterial products activate the secretion of α-defensins, and mucosal inflammation is associated with increased expression of α-defensins (Meddings *et al.* 2003). Although the role of defensins have not been established, it is thought they play a role in protecting epithelial cells from microbial populations in the crypt, and prevent bacterial invasion (Ayabe *et al.* 2004).

1.2.3.2 Mucus

A layer of mucus forms a gel between the luminal contents and the epithelium. It has visco-elastic properties which function as a cushion as well as a permeable barrier (Meddings *et al.* 2003). In the colon, the mucus barrier increases from distal to proximal colon (Meddings *et al.* 2003). Mucus is composed of glycoprotein called mucins. A variety of different mucins exist leading to differences in their physical properties. Sulphation of mucins is likely to be an adaptive response to bacteria and confers resistance to bacterial degradation (Shirazi *et al.* 2000b). Sulphated mucins are located in areas of the GI tract where there are large number of bacteria, such as the colon (Meddings *et al.* 2003).

Mucus is produced by globlet cells, and is broken down by bacteria (Shirazi *et al.* 2000f). There is evidence that in patients with UC the mucus barrier is disrupted (Meddings *et al.* 2003). Reduced secretion and production of mucus as well as increased mucus degradation by bacterial enzymes (Meddings *et al.* 2003) have been implicated. A putative susceptibility locus, identified by genome-wide scanning, maps close to the intestinal mucin 3 gene (MUC3) on chromosome 7q22, and has led to investigation of the contribution of MUC3 polymorphisms to disease susceptibility. Mucus production is also seen to be impaired in animals models of inflammation, further supporting a dysregulated barrier in disease (Renes *et al.* 2002)

1.2.3.3 Trefoils

These peptides are small proteins produced by globlet cells. So far there are three known-TFF1, TFF2 and TFF3 (Shirazi *et al.* 2000). As they are produced by the same cells as mucus, trefoils are ubiquitously expressed in the mucus throughout the GI tract (Shirazi *et al.* 2000). The main functions include mucosal protection and restitution (cell migration from an edge of an injury site to cover an epithelial defect) (Meddings *et al.* 2003). The protective effect of trefoils is likely to be due to the enhanced protective role of mucus, as an increase in mucus viscosity has been reported when trefoils are mixed with mucin glycoproteins (Shirazi *et al.* 2000). Animals deficient for the gene encoding the trefoil seen in intestine and colon (TFF3) show increased mucosal damage, whereas over expression of the TFF1 gene protects the epithelium (Shirazi *et al.* 2000). A recent study has shown trefoil pretreatment ameliorated colitis in dextran sodium sulphate induced inflammation in mice (Soriano-Izquierdo *et al.* 2004) These supports the theory that trefoils aid in the biophysical barrier properties of mucus and protection of the intestinal epithelium.

1.2.4 Epithelial Efflux Transporters

As molecules are able to pass into the intestinal mucosa, there is a system in place which inactivates these compounds and transports them back across the epithelial lining in to the lumen of the GI tract, where they are excreted from the body. This process involves transporter pumps (known as P-glycoprotein) and metabolising enzymes. These will be discussed further in this section.

1.2.4.1 P-glycoprotein

1.2.4.1.1 Structure

P-glycoprotein is 170kDa transmembrane protein, which belongs to the adenosinetriphosphate (ATP) binding cassette superfamily of transporters encoded by the multidrug resistance (MDR) genes (Ho *et al.* 2003). P-glycoprotein is 1280 amino acids long, and consists of two homologous halves joined by a flexible linker. Each half contains a hydrophobic N-terminal domain consisting of six transmembrane domains which form the channel (pore), followed by a hydrophilic C-terminal domain containing the nucleotide binding site (Endicott & Ling 1989). Energy required for the transport through the pore is derived from ATP hydrolysis utilizing ATP bound to the nucleotide-binding site. There are two MDR genes in humans-MDR1 and MDR3, and three in rodents-mdr1a, mdr1b and mdr2 (Endicott & Ling 1989). MDR1 and mdr1a in humans and rodents respectively, have been implicated in steroid resistance (Kramer *et al.* 1993; Silverman *et al.* 1991), whereas the MDR3 and murine mdr2 genes are involved in the secretion of phosphatidylcholine into the bile (Fardel *et al.* 2001).

P-glycoprotein pumps are located on the apical surfaces columnar epithelial cells the colon and distal small bowel, as well as epithelial cells located in bilary ductules, proximal ductules of the kidney pancreas, and also adrenal gland (Borst *et al.* 1993; Ho *et al.* 2003). P-glycoprotein is expressed in the epithelium of the choroids plexus of the brain, and has been shown to be involved in the transport of drug across the blood-brain barrier (Mercier *et al.* 2004). Other cells have also been shown to

express P-glycoprotein at varying levels, these include macrophages, dendritic cells as well as T- and B-cell lymphocytes (Klimecki *et al.* 1994).

1.2.4.1.2 Role of Intestinal P-glycoprotein

P-glycoprotein is thought to be involved in many different functions from cell volume regulation to the efflux of drugs such as glucocorticosteroids and anti-cancer drugs as well as xenobiotics from cells (Benet & Cummins 2001; Borst *et al.* 1993; Lange & Gartzke 2003; Valverde *et al.* 1996). In the intestinal epithelium expression of these pumps in the luminal surface of the epithelial cells suggest a role in the secretion of endogenous and exogenous toxin substances (Ho *et al.* 2003; Patel *et al.* 2002). The development of the *mdr1a* knockout mice has shown the importance of P-glycoprotein in maintaining a protective barrier; knockout mice develop colitis in a specific-pathogen free environment whereas non-transgenic mice remain healthy (Panwala *et al.* 1998).

The role of P-glycoprotein in the detoxification process has been of recent interest. Molecules that penetrate the plasma membrane, including drugs and other foreign (xenobiotics) compounds undergo a process of detoxification and biotransformation to less toxic substances, which are then excreted via cellular efflux mechanisms (P-glycoprotein pumps). Nuclear pregnane X receptor (PXR) is a transcription factor known to be involved in xenobiotic induction of the cytochrome P450 (CYP450) enzymes (Quattrochi & Guzelian 2001). These enzymes lead to the oxidation of compounds and as they mostly catalyse the first step of biotransformation, the function is called phase I. Phase II metabolism is mediated by several different enzymes; including UDP glucuronosyltransferases, where this enzyme conjugates compounds to glucuronic acid, glutathione-S-transferases, sulphotransferases and acetyltransferases (Dietrich *et al.* 2003). These changes make the compounds more suitable for excretion into the bile or urine. Phase III metabolism is the elimination of these products from cells by transporter pumps (Dietrich *et al.* 2003).

Studies have shown the enzyme CYP450 is co-induced with MDR1 (Geick *et al.* 2001), and PXR has been shown to induce CYP450 enzymes (Cai *et al.* 2002; Mei *et al.* 2004). It is thought PXR recognises xenobiotics compounds, and this induction

activates CYP450, where it metabolises the compounds into less toxic substances. The subsequent induction of MDR (P-glycoprotein) transports the compounds out of the cell and into the lumen to be excreted and therefore suggests a protective role for P-glycoprotein in the intestine (see figure 1.4). The protective role of P-glycoprotein in the intestine is further supported by data from mdr1a knockout mice which develop inflammation in the presence of bacteria; data from UC patients where reduced colonic PXR and MDR1 was noted (Langmann *et al.* 2004; Panwala *et al.* 1998); and finally indirectly by the findings that polymorphisms in the MDR1 is associated with a reduction in P-glycoprotein expression in UC (Ho *et al.* 2005; Schwab *et al.* 2002). These data imply a dysregulation in xenobiotic metabolism/excretion, in particular P-glycoprotein, may contribute to intestinal inflammation.

1.2.4.1.3 Pharmacological Regulation of P-glycoprotein

The high concentration of P-glycoprotein in the intestinal epithelium makes it an important membrane involved in the bio-availability of drugs in the body. Studies in man have shown P-glycoprotein is stimulated by the antihypertensive drug prazosin and the steroid hormone progesterone (Shapiro *et al.* 1999). P-glycoprotein in the intestinal epithelium has also been shown to actively transport steroid hormones dexamethasone, cortisol and aldosterone as well as anti-fungal, and anti-microbials out of cells into the lumen (Ueda *et al.* 1992; Van Asperen *et al.* 1998)

The influence of corticosteroids on the expression of P-glycoprotein expression has been of considerable interest, especially since increased expression of P-gp may be involved in steroid resistance not only in inflammatory bowel disease (Farrell *et al.* 2000; Farrell & Kelleher 2003) but other inflammatory disease including asthma (Adcock & Lane 2003). Nonetheless, at present, the literature is incomplete and inconsistent.

Studies *in vitro* have demonstrated differences in dexamethasone regulation of P-glycoprotein expression, with increases in mdr1a noted in rat and mouse hepatoma cells but not in non-hepatoma cells (Schuetz *et al.* 1995; Zhao *et al.* 1993). Rat hepatocytes treated with dexamethasone were seen to have decreased P-gp expression and increased accumulation of doxorubicin, showing this pump is involved in the

transport of this drug out of cells (Fardel *et al.* 1993). This suggests divergent regulation of P-glycoprotein by steroids in normal versus tumoral cells.

Data from *in vivo* studies have shown a tissue specific regulation of P-gp by dexamethasone; increased mdr1a was noted in liver and lung but not kidney (Demeule *et al.* 1999). Other *in vivo* studies have also shown the importance of endogenous steroids (corticosterone) in regulating activity of P-glycoprotein (Murakami *et al.* 2002). Plasma extracted from inflammation-induced rats showed an inhibitory effect on P-gp expression in Caco-2 cells compared to plasma from healthy rats, suggesting corticosterone may be involved in the in vivo regulation of P-glycoprotein (Murakami *et al.* 2002).

Collectively these data suggest a tissue- and species-specific regulation of P-glycoprotein. In addition, disease states per se may also induce differential regulation of this gene.

1.2.4.2 Other transmembrane pumps

Other transporter pumps which have been shown to be expressed on the surface of epithelial cells include multidrug resistance protein 2 (MRP2) and the breast cancer related protein (BCRP) (Courtois *et al.* 1999; Taipalensuu *et al.* 2004). Similar to MDR1, these are also members of the ATP binding cassette family which utilise the energy from ATP to efflux substances out of cells (Dietrich *et al.* 2003). MDR1, MRP2 and BCRP are also known as ABCB1, ABCC2 and ABCG2 respectively (Dietrich *et al.* 2003). All are located throughout the intestine at varying levels, are thought to be involved in the bioavailability of drugs as well as protecting the intestinal mucosa through the efflux of xenobiotics.

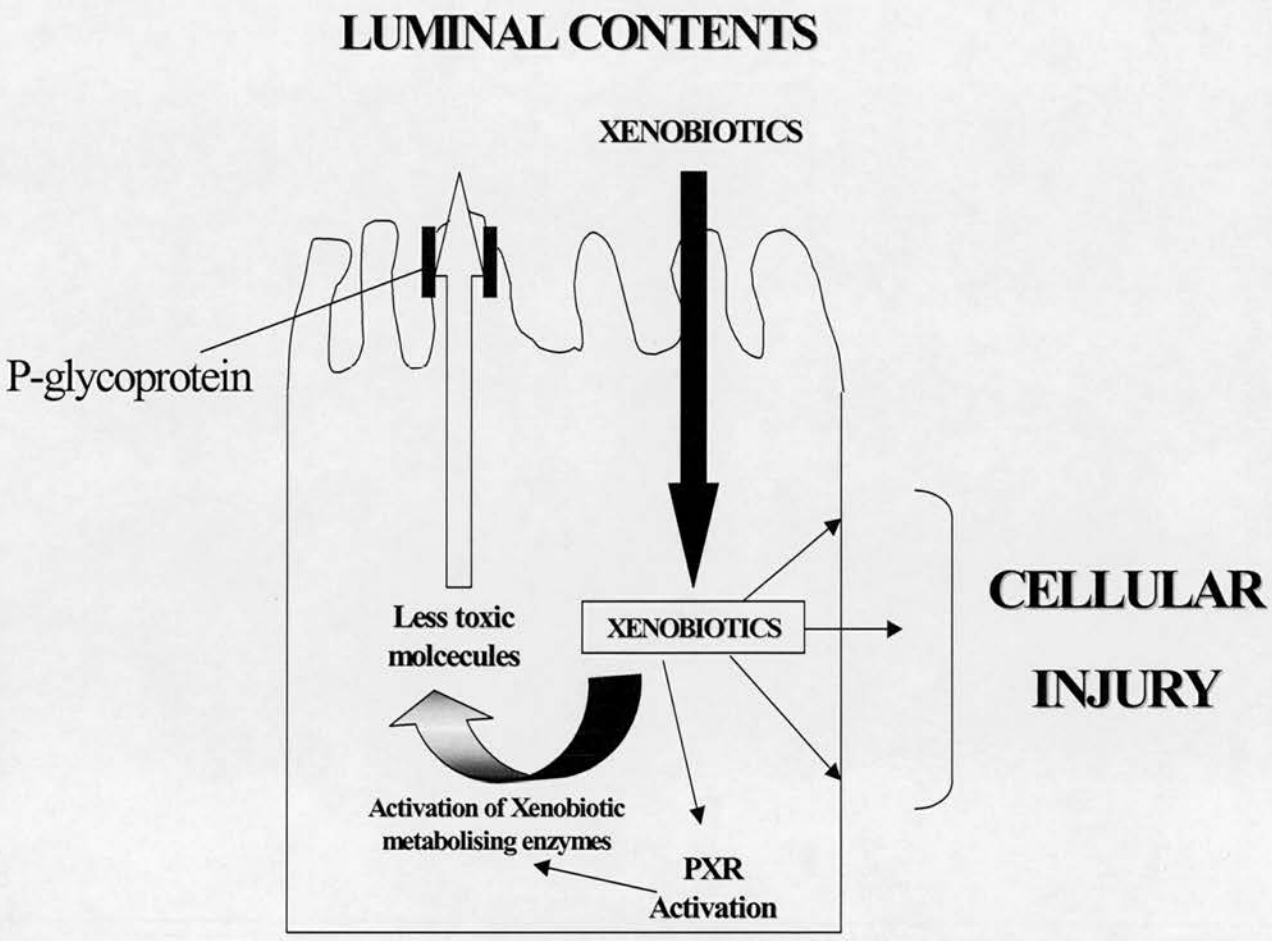


Figure 1.4: Diagram illustrating the possible role of P-glycoprotein in the detoxification of xenobiotics in intestinal epithelial cells. Xenobiotics compounds present in the lumen of the intestine enter the cell, and the pregnane X receptor (PXR) is activated. PXR is known to activate the transcription of enzymes which are involved in the metabolism of xenobiotics to less toxic molecules. Transporter pumps, including P-glycoprotein, actively transport these molecules out of the cell into the luminal contents, where they are excreted. Abnormalities in this system have been implicated in inflammatory bowel disease, where it is has been suggested that a decrease in PXR and P-gp expression may perpetuate inflammation due to the increased concentrations of xenobiotics and cause further cellular injury.

1.3 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) encompasses two bowel disorders, Ulcerative colitis (UC) and Crohn's disease (CD). Ulcerative colitis is characterised by inflammation limited to any part of the colon, whereas in Crohn's disease inflammation may present in any region of the gastrointestinal tract, in particular the ileo-caecal region (Jewell 1999; Satsangi *et al.* 1997). Histologically in UC, the inflammation is limited to the mucosal and spares the deeper layers of the bowel wall, unlike CD where it can be seen through all the layers and as a result the wall is thickened. Inflammation in ulcerative colitis is maximal in the rectum and extends up the colon in a continuous manner however, in CD, diseased tissue is interspaced between normal areas of tissue (Hall 2002). Both UC and colonic CD present in a similar manner with bloody diarrhoea, passages of mucus and other symptoms including weight loss and lethargy.

These symptoms can also be accompanied by complications associated with IBD. These complications include haemorrhaging, dilatation, perianal lesions and intestinal cancer (Jewell 1999; Picco & Bayless 2000). Stricturing can also occur which may cause intestinal obstruction, and growth retardation has been noted in children diagnosed with IBD (Picco & Bayless 2000). The most dangerous complication is perforation of the intestine, which has been associated with a mortality rate of 16% (Picco & Bayless 2000). Despite this, the improvements in the management of IBD over the years has reduced the mortality rate for both diseases, and patients with IBD now have a life expectancy similar to that of the general public (Hall 2002).

1.3.1 Factors contributing to IBD

Although the aetiology of these diseases remain uncertain, recent progress has in identified genetic, environmental and immunological factors involved in the pathogenesis of these diseases (Jewell 1999; Ronald & Funder 1996; Watts & Satsangi 2002). It is believed that IBD results from an interaction between genetic, immune and environmental factors. The marked increase in these diseases in the last half a century cannot be solely due to alterations in genetics, and clearly reflect an as yet unidentified environmental influence (Danese *et al.* 2004). These factors will be outline below:

1.3.1.1 Genetic

Strong epidemiological data have implicated genetic background as having a pivotal role in the susceptibility to inflammatory bowel disease. Concordance rates in twin studies, multiply affected families, as well as ethnic differences in susceptibility provided the driving force behind detailed genetic investigations in patients with inflammatory bowel disease. European studies involving twin pairs have shown the concordance rates to be 36% and 4% for Crohn's disease patients and 16% and 4% in ulcerative colitis patients, for monozygotic and dizygotic twin pairs respectively (Orholm *et al.* 2000; Thompson *et al.* 1996). Familial studies have shown between 6-35% of IBD patients have an affected family member (Russell *et al.* 2004). Other studies have shown that the prevalence rates for IBD is increased in the Jewish population in contrast to Afro-American populations, who have the lowest reported rates of IBD than any other population studied (Russell *et al.* 2004; Satsangi *et al.* 1997; Watts & Satsangi 2002). Environmental factors are also thought to underlie differences within and between ethnic groups, illustrated by Probert and colleagues, who reported Indian immigrants to Britain had an increased incidence of ulcerative colitis compared to the indigenous Indian population (Probert *et al.* 1992).

Genome wide scanning has identified several regions of linkage with susceptibility to UC and CD. IBD1 on chromosome 16, is now confirmed as a susceptibility locus for Crohn's disease, and recent data has provided strong evidence that the CARD/Nod-2 gene is the critical determinant involved in a defect of the innate immune response

(Brent & Shugart 2004; Roussomoustakaki *et al.* 1998). IBD2 on chromosome 12q13, has shown linkage most strongly with ulcerative colitis; IBD3 on chromosome 6p23, involves the major Histocompatibility complex and is implicated in both UC and CD and in determining disease phenotype; IBD4 on chromosome 14q11-12 has been implicated in Crohn's disease and IBD5 on chromosome 5 is also a susceptibility loci for early onset Crohn's disease (Brent & Shugart 2004; Hampe *et al.* 1999; Rioux *et al.* 1998; Roussomoustakaki *et al.* 1998; Russell *et al.* 2004; Satsangi *et al.* 1996a; Satsangi *et al.* 1997; Stokkers *et al.* 1999; Watts & Satsangi 2002).

1.3.1.2 Environmental

1.3.1.2.1 Diet

Although genes have been shown conclusively to be involved in the susceptibility to inflammatory bowel disease, the increase in IBD patients in the 20th century, especially in young people, suggest that other factors must be involved in disease susceptibility. It is thought environmental factors impact on the susceptibility and progression of disease due to the genetic background of the susceptible individual. There are many different environmental factors that may have an influence in IBD. These include diet, contraceptive drugs and most importantly smoking. Dietary factors including the intake of fat and refined sugar, have been implicated as a risk factor whereas increasing fibre intake in the diet in the form of fruit and vegetables, seems to decrease the risk of IBD (Danese *et al.* 2004). However no consensus has emerged and this represents an area of ongoing research.

1.3.1.2.2 Smoking

Smoking has been shown to have definite effects on disease pathogenesis. In Crohn's disease, smoking not only contributes to disease susceptibility but also affects progression of disease and the need for surgery (Danese *et al.* 2004). In contrast, smoking in UC patients may attenuate the disease process (Jewell 1999). Although the precise chemical involved in the attenuation of UC is unknown, transdermal nicotine has been shown to have beneficial effects in the induction of remission in

patients with ulcerative colitis (McGrath *et al.* 2004). Moreover the increased incidence of early-onset disease in children has emphasised a role for passive smoking, as children rarely smoke at the time of diagnosis (Russell *et al.* 2004). Further large scale studies are required to evaluate the importance of the effect of various environmental factors, particularly passive smoking, in the initiation and progression of inflammatory bowel disease.

1.3.1.2.3 Bacteria

Over the past ten years there has been substantial work carried out on the role of bacteria in IBD, and evidence now suggests 'normal' commensal enteric bacteria play a key role in the development of chronic intestinal inflammation. Animal models of inflammation, which are reviewed in detail in section 1.4, have provided vital information governing the effect of the immune system, particularly bacteria, in IBD. These models have also shown that over-expression/deletion of immuno-regulatory mediators, T-cells, cytokine receptors and intracellular signalling molecules induce colitis (Sartor 2004b). This suggests a variety of different pathways are involved in the regulation of homeostasis in response to bacteria. However for the purpose of this thesis, the role of commensal bacteria in man in health and disease will be reviewed.

1.3.1.2.3.1 Role of bacteria in health

It is well known that bacteria are involved in the metabolism of food substances, nutrient and vitamin production, detoxification of dietary carcinogens, differentiation of epithelial cells as well as the development of the mucosal immune system (Sartor 2004b; Shanahan 2004). Intestinal colonisation with enteric bacteria in man is relatively stable throughout their lifetime, and prevents colonisation with pathogenic bacteria by competing for substrates and mucosal binding sites and therefore prevents binding by other pathogens. However after antibiotic treatment the intestinal balance is disrupted and therefore makes the host more susceptible to recolonisation with potentially pathogenic bacteria which may induce an inflammatory response. Bacteria are also involved in the maturation of the immune system. Animal models have shown the importance of bacteria in immune regulation as animals in germ-free environments have underdeveloped gut-associated lymphoid tissue (GALT, see

section 1.2.2.3.1), as well as an underactive T- and B-cell response (Sartor 2004b). These observations suggest bacteria are involved in 'priming' immune cells.

As discussed in section 1.2.2.3.1, lymphoid tissue is involved in the production of IgA antibodies. M-cells transport bacterial antigens into the underlying tissue, where they are taken up by local dendritic cells and macrophages, whereby the immune system is activated after these cells present the antigen on its surface together with the major Histocompatibility complex (MacDonald 2003). B-cells, with the help of T-cells, secrete this immunoglobulin. IgA antibodies for commensal bacteria are thought to exist and prevent binding to the epithelium and initiating disease. Therefore these antibodies are involved in maintaining homeostasis in an environment where there are potentially pathogenic organisms. In normal hosts, regulatory T-cells (Tr) and APCs also inhibit pathogenic immune responses to commensal bacterial by several pathways which include IL-10 (Sartor 2004b). IL-10 is a cytokines which stimulates T-regulatory (Tr) cells to secrete IL-10 and TGF-β. IL-10 and/or TGF-β feeds back to the APC, and inhibits the secretion of further cytokines and therefore prevents an inflammatory response (Mills & McGuirk 2004c). Tr cells prevent the over-activation of immune responses to antigens by dampening Th1 responses and therefore inhibit an immune response to commensal pathogens.

Mechanisms by which cells recognise pathogens are mediated through cell-surface and cytoplasmic receptor called toll-like receptor and CARD/Nod2 domain. TLR recognise the bacterial products including lipopolysacchride, flagellin as well as peptidoglycan, whereas CARD/Nod2 recognises peptidoglycan (Sartor 2004b). These receptors activate NF-κB activating the immune system. In a healthy individual this response will be resolved quickly, preventing further damage and therefore disease. It has also been suggested that the P-glycoprotein pump, encoded by the MDR1, may be involved in the efflux of bacterial products out of cells, and therefore may play a role in protecting the cell from potentially harmful substances. Moreover inherited and/or mutations in TLR4, CARD15/Nod2, MDR1 (all discussed in detail in sections 1.2.2.3 and 1.2.4) as well as other altered expression of genes involved in mucosal defence (defensins, trefoils and mucin reviewed in section 1.2.3), have been implicated in IBD.

This homeostatic environment characteristic of a healthy individual does not appear to hold true in patients with IBD. A combination of genetic mutations or triggers from the environment may disrupt the mucosal defence system. These disruptions may include a defect in the epithelial barrier, immune system or bacterial clearance. This mucosal defect may subsequently result in an increased uptake of bacteria into the mucosa, an over-activated immune system and therefore inflammatory bowel disease.

1.3.1.2.3.2 Role of bacteria in disease

Clinical studies have shown the importance of bacteria in disease, as the use of antibiotics, probiotics and faecal diversion have all been shown to aid in maintaining the remission and resolution of disease (Shanahan 2004; Swidsinski A *et al.* 2002). Bacteria within epithelial cells has been shown to be increased in patients with IBD compared to healthy controls, as well as the balance between beneficial to pathogenic bacteria (Danese *et al.* 2004; Sartor 2004b; Sartor 2004c). However investigation of the role specific bacterial pathogens in the inflammatory process is limited in man unlike animal models, where numerous mono-association studies have implicated specific bacterial species in disease initiation.

Nevertheless, a number of bacteria, particularly anaerobic bacteria, have been shown to be increased in IBD and linked to the recurrence of disease (Sartor 2004d). *Bacteroides* species has been seen to be increased in faecal samples from Crohn's disease patients (Giaffer *et al.* 1991), and a decreased faecal concentrations of *Bacteroides vulgatus* correlated with response to the antibiotic metronidazole (Sartor 2004b), as well as aiding post-operative remission (Rutgeerts *et al.* 1995). In CD patients where disease reoccurs after ileocolonic resection, an increase in mucosal *E. coli*, *Bacteroides* species and *Fusobacterium* species has been observed, as well as a decrease beneficial bacteria, *Lacobacillus* species and *Bifidobacterium* species (Neut *et al.* 2002). Other studies have shown a decrease in the diversity of colonic associated bacteria in patients with Crohn's disease, and have shown a reduction in anaerobic bacteria including *Bacteroides* and *Lactobacillus* species in patients with Crohn's disease (Ott *et al.* 2004)

Although changes in the composition of commensal flora have been associated with IBD, the metabolic function of faecal bacteria has also been shown to be altered during disease. *E. coli* have been shown to have an increased adherent phenotype to epithelial cells in IBD as well as producing cytotoxins whereas *B. vulgatus* has been shown to produce mucin degrading enzymes which therefore disrupts the barrier function of the epithelium (Darfeuille-Michaud *et al.* 2004; Ruseler-van Embden JG *et al.* 1989; Sartor 2004b; van Nuenen MH *et al.* 2005). The production of cytokines have been shown to increase the proliferation of *E .coli*, and these mediators could therefore have similar stimulatory effects on epithelial adherence and production of cytotoxins, which in turn would exacerbate inflammation and mucosal injury (Sartor 2004b; van Nuenen MH *et al.* 2005).

Therefore changes in the composition of bacterial flora, alterations in their metabolic function as well as changes in the barrier function and uptake/clearance of bacteria, may all be involved in the initiation, perpetuation and exacerbation of IBD.

1.4 ANIMAL MODELS OF INFLAMMATION

Animal models of IBD have not just provided a mechanism to explore the *in vivo* effects of potentially therapeutic drugs, but have provided important information on the pathogenesis of disease. Since the first description of animal colitis in 1964 (Kraft *et al.* 1964) many more animal models have emerged. These have been categorised into four groups, depending on the induction method of inflammation, and will be discussed further in section 1.4.1 (Hoffmeyer *et al.* 2000). The importance of bacteria the initiation of disease in animal models is worthy of note; animals in germ-free conditions either have reduced or absent intestinal inflammation compared to conventionally raised animals.

1.4.1 Categories of animal models of inflammation

1.4.1.1 Chemically induced models

Inflammation can be induced in all rodents, including rats, mice, guinea-pigs and rabbits. These models are cheap to produce, and have been primarily used in pre-clinical drug-discovery trials where large numbers of animals can be included (Sartor 2004a). The most commonly used models include indomethacin-, dextran sodium sulphate (DSS) - , trinitrobenzene sulphonic acid (TNBS)/alcohol- and peptidoglycan-polysaccharide (PG-PS)-induced models.

1.4.1.1.1 Indomethacin-induced

Subcutaneous administration of indomethacin leads to mucosal ulcers mostly in the small intestine, within three days. This type of induced inflammation model has been predominately studied in rodents, and duration has been shown to be strain specific; Lewis rats have been shown to have inflammation 77 days after injection, whereas some Fischer rats resolve inflammation by 14 days (Sartor 2004a). Moreover, bacterial colonisation is also important in disease as germ-free animals have reduced inflammation and antibiotic treatment reduces inflammation (Robert & Asano 1977;

Sartor 2004b). Cyclooxygenase enzymes inhibitors exacerbate disease (Sartor 2004a). There is no evidence to support a role of T-cell in this model of inflammation, and a defective epithelial barrier is most likely to be the cause of inflammation.

1.4.1.1.2 DSS-induced

DSS administrated in the drinking water of rodents including, rats, mice and hamsters induces a pancolitis with symptoms mimicking ulcerative colitis including bloody diarrhoea, weight loss. Histology shows colonic ulceration and an infiltration of mononuclear cells into the mucosa (Tahara *et al.* 2003). A role for bacteria in disease has been demonstrated as broad-spectrum antibiotics attenuates disease (Hans *et al.* 2000). Evidence suggests that inflammation in this model may be more due to epithelial injury rather than T-lymphocyte immune activation as inflammation also occurs in mice devoid of T-cells (immuno-deficient; SCID mice) (Axelsson *et al.* 1996), which makes this useful model to study epithelial injury and repair.

1.4.1.1.3 TNBS/alcohol-induced

An enema of TNBS/alcohol induces distal colitis in rats, mice and rabbits, however the severity of inflammation has been shown to be determined by genetic background and strain (Sartor 2004a). An over aggressive immune system to TNBS/alcohol has been suggested where the immune response is Th-1 dominated. Antibiotic treatment has been shown to prevent inflammation (Videla *et al.* 1994). The disadvantage of this model is the variable severity of inflammation, and the potential toxicity of this chemical.

1.4.1.2 Spontaneous-induced inflammation models

There are three examples of animals which develop spontaneous inflammation: Cotton-top tamarins, C3H/Hej Bir mice, SAMP-1/Yit mice (Hoffmann *et al.* 2003).

1.4.1.2.1 Cotton-top tamarins

Inflammation in tamarins, which are marmosets, shows a similar disease progression as that noted in UC. Inflammation persists throughout life and in episodic flares (Hoffmann *et al.* 2003). Bacteria play a primary role in inflammation, in particular *E. coli,* and in the absence of bacteria no disease is present, although it has been suggested that a genetic mutation in the MHC class I gene is involved in the susceptibility of disease (Hoffmann *et al.* 2003). As these animals are endangered species further investigations are limited.

1.4.1.2.2 C3H/Hej Bir and SAMP-1/Yit mice

C3H/Hej Bir mice are unresponsive to LPS due to a point mutation in toll-like receptor-4, and develop colitis by 4 weeks of age (Hoffmann *et al.* 2003). Bacteria play a pivotal role in disease, as mice in germ-free conditions do not develop inflammation (Elson & Cong 2002). In this model, inflammation is transdermal and is characterised an over-reactivity of T-cell to bacterial antigens (Elson & Cong 2002). SAMP-1/Yit mice develop ileal inflammation by 20 weeks of age with less severe inflammation noted in colon (Sartor 2004a). Disease can be transferred to severe combined immune deficient (SCID) mice (immuno-deficient) showing a T-cell driven mechanism (Hoffmann *et al.* 2003; Strober *et al.* 2001). Bacteria also play a role, as germ-free animals remain healthy and antibiotic treatment attenuates disease (Cong *et al.* 2002b). Studies in these mice are limited due to the restricted availability of these animals.

1.4.1.3 T-cell transfer

This involves using animals which are immuno-deficient (lack immune cells), which include RAG-1 and 2 knockout mice as well as SCID mice. A T-cell surface marker, CD45RB, is highly expressed in naïve T-cells but expression decreases upon activation. Transfer of $CD45RB^{high}$ CD4+ T-cells from normal mice into SCID mice induce pancolonic inflammation by 8 weeks after transfer (Hoffmann *et al.* 2003). The inflammation noted is similar to that seen in UC, even though there is a Th1 driven immune response (Hoffmann *et al.* 2003). T-cell trafficking is dependent on bacteria as pathogenic T-cells responsive to bacterial antigens caused disease when transferred to SCID mice, whereas T-cell from 'healthy' mice caused no inflammation (Cong *et al.* 2002a).

Another model includes a CD8+ T-cell specific for heat shock protein 60 (HSP60). Transfer induces small bowel inflammation even in a germ-free environment. This is the only model which uses CD8+ T-cells and one of the few models which still develop inflammation in a germ-free environment.

1.4.1.4 Genetically-induced models

There are a number of genetically induced models of inflammation. All but one model are mice models, the exception being the HLA-B27-B_2 transgenic rat. Genetic models can be further subdivided into the type of immune response-Th1 and Th2. As discussed in section 1.2.2.2, and this is defined by the types of cytokines released by the T-cells. Noteworthy, most of the genetically-induced models of inflammation are Th1 mediated, except the T-cell receptor (TCR) α knockout model (Sartor 2004a).

For the purpose of this thesis, the HLA-B27 transgenic rat model, mdr1a and IL-10 knockout mice (-/-) model will be discussed.

1.4.1.4.1 IL-10 -/- mouse

These mice develop progressive pancolitis by 2 months of age when in specific pathogen free (SPF), but not in the absence of bacteria (Madsen KL. *et al.* 2000). Inflammation is associated with crypt elongation, mononuclear cell infiltration, ulceration, with transmural inflammation occurring in the later stage, (Sartor 2004a).

IL-10 is an anti-inflammatory cytokine characterised by its inhibitory actions on pro-inflammatory cytokine production by macrophages, and also down-regulating the expression of MHC class II molecules on APC and IL-12 production; in turn suppressing Th1 and Th2 immune responses (Sartor 2004a). These mice have been shown to have increased levels of the Th1 cytokines, IFN-γ and TNF-α (Bhan *et al.* 1999; Song *et al.* 1999). Double knock out studies have shown CD4+ T- but not B-cells are required for in the development of inflammation (Hoffmann *et al.* 2003)

Moreover, gnotobiotic studies have shown exposure to bacteria increase intestinal CD4+ T-cell secretion of IFN-γ and TNF-α in IL-10 knockout animals, in contrast to lymphocytes from wild-type animals which have negligible levels (Sartor 2004a). These studies have also shown a role for specific subsets of bacteria responsible for disease severity. A group of 6 bacterial species; *Bacteroides vulgatus, Streptococcus faecium, E. coli, Peptostreptococcus productus, Eubacterium contartum*, and *Streptococcus avium* was shown to produce minimal inflammation (Sellon *et al.* 1998) However, monoassociation studies with *E. faecalis* induced distal colitis (Balish & Warner 2002). Importantly, metronidazole (an antibiotic effective against anaerobic bacteria) reduced established colitis, where ciprofloxacin (effective against aerobic bacteria) was ineffective (Madsen KL. *et al.* 2000). Probiotic treatment with *Lactobacillus* and *Bifidobacterium* attenuates colitis in these animals, but colitis is prevented when animals are given a combination of four *Lactobacillus*, three *Bifidobacterium* and one *Streptococcus* species (Sartor 2004b). These data support a primary role for bacteria in disease, and suggests a role for specific subsets of bacteria in initiating inflammation, and imply specific subsets of bacteria perpetuate inflammation once disease is established.

1.4.1.4.2 *mdr 1a* -/- mouse

As described earlier in section 1.2.4.1.2, the *mdr1a* gene, MDR1 in humans, encodes a P-glycoprotein transmembrane pump which is thought to play a protective role in the intestinal epithelium, and may be involved in the xenobiotic detoxification process (Langmann *et al.* 2004; Li *et al.* 1999). It is also known to be involved in the active transport of drugs out of the cell and therefore minimises cellular exposure to potentially toxic substances (Ambudkar *et al.* 1999).

mdr1a -/- models develop colitis only in specific-pathogen free (SPF) but not germ-free (GF) conditions, providing further support for a protective role for P-glycoprotein in the small and large intestine (Panwala *et al.* 1998). Colitis develops by 20 weeks of age. Histologically, the intestinal inflammation involves elongated crypts, crypt abscesses and an infiltration of mononuclear cells. Diarrhoea is also associated with disease. As with most genetically engineered models of inflammation, a Th1 immune response predominates in the colon, with high expression of IFN-γ, TNF-α and IL-12 (Sartor 2004a). It has been shown that this disease is mediated by alterations in the epithelium as bone marrow transplant experiments show these reciprocate mice do not develop colitis (Sartor 2004a), implying this disease does not originate in bone marrow-derived cells.

Studies have also shown inflammation can be prevented and resolved using oral antibiotics (Panwala *et al.* 1998). Importantly, as with many of the other genetically engineered models, specific bacteria have been shown to potentiate disease. *Helicobacter* species have been shown to have varying effects on inflammation in these animals; *H. bilis* potentiated inflammation, whereas *H. hepaticus* delayed the onset of disease (Burich *et al.* 2001; Maggio-Price *et al.* 2002). This clearly further supports a role for specific bacteria initiating inflammation.

1.4.1.4.3 HLA-B27 transgenic rat

Expression of the human HLA-27/β_2 microglobulin gene in Fischer 344 or Lewis rats, but not mice, leads to the development of colitis, duodenal inflammation, peripheral and axial arthritis as well as skin and nail lesions (Sartor 2000). By 10 weeks of age colitis is developed when rats are housed in an SPF environment, but absent in bacterial free conditions. Onset is different in adult rats, where if animals re-housed from a bacterial free into an SPF environment, colitis develops after four weeks of bacterial colonisation, with predominately caecal inflammation (Sartor 2004a). The inflammation is characterised by elongated crypts, mucosal thickening as well as infiltration of mononuclear cells (Sartor 2000).

Mucosal expression of IFN-γ, TNF-α and IL-12 was increased in diseased animals (DIELEMAN *et al.* 2004), consistent with a Th-1 immune response. Moreover, colitis can be induced in healthy HLA-B27 null rats through bone marrow engraftment (Breban et al. 1993; Breban et al. 1996) or by transferring CD4+ and CD8+ T-cells. Engraftment of bone marrow from non-transgenic into HLA-B27 transgenic rats, can eradicate inflammation (Rath 2003). This implies disease is mediated through bone marrow derived cells. This contrasts with the *mdr1a* -/- mouse model, in which the lack of epithelial P-glycoprotein expression appears to be critical in disease pathogenesis.

Germ-free transgenic animals have no intestinal inflammation, although skin and nail lesions are still present (Sartor 2004a). Gnotobiotic studies in these transgenic rats have shown, as with other models of inflammation, specific bacteria induce disease. Initial studies showed the importance of *Bacteroides* species in the initiation of disease in these transgenic animals (Rath et al. 1996). Further studies reveal that *Bacteroides vulgatus* induced a more severe colitis than that seen when animals were colonised with 5 different types of bacteria without *B. vulgatus*. Monoassociation studies showed colonisation with *E. coli* did not induce inflammation in these transgenic rats (Rath et al. 1999). Similar to mdr1a -/- mice, differences in the severity of inflammation induced by bacteria from the same *Bacteroides* genus occur; *B. vulgatus* and *B. thetaiomicron* induce disease, but *B. distasonis* colonisation does not. These data support a species-specific role of bacteria in initiating inflammation.

Treatment with narrow spectrum antibiotics (ciprofloxacin and metronidazole) attenuate colitis, however treatment with broad-spectrum antibiotics (vancomycin/imipenum) reverse established colitis (Sartor 2004a; Sartor 2004b). Probiotic treatment using Lactobacillus prevented reoccurrence of inflammation after antibiotic treatment, but was ineffective at reversing active, established disease (Sartor 2004b).

1.4.2 Conclusions from animal studies

Animal model studies of intestinal inflammation have demonstrated four main factors involved in disease. The studies have emphasised the important of bacterial flora in the initiation of intestinal inflammation, as well as the strong genetic influence which governs the severity of disease. Moreover, a dysregulated immune system and a defect in the intestinal barrier have been implicated as an inducer of inflammation. Studies using the models of inflammation have also shown inflammation is T-cell mediated.

It is clear these studies have enabled detailed investigations into possible factors involved in inflammation, and have provided vital information into immune dysregulation and factors involved in initiating disease, especially the role of specific bacteria. These studies provide a platform to study disease factors in man, with the potential for new therapeutic routes in the treatment of IBD.

1.5 GLUCOCORTICOIDS

Glucocorticoids, cortisol in man and corticosterone in rodents, are essential for survival and exert an influence on most systems of the body including fluid and electrolyte homeostasis, physiological response to stress and more importantly for this thesis, the immune system. This section will review glucocorticoid synthesis, metabolism and secretion as well as the actions of glucocorticoids in the body, with particular emphasis on their role in inflammation.

1.5.1 Synthesis, release and metabolism

Glucocorticoids are part of a family of steroid hormones that have a common precursor cholesterol, where each steroid is given its unique properties by the substitution of chemical groups at various positions on the backbone molecule.
They are synthesised from cholesterol in the mitochondria and endoplasmic reticulum (ER) of cells in the zona fasciculata and zona reticularis in the adrenal cortex. Steroid biosynthesis is catalysed by a series of enzymes of the cytochrome (CYP) P450 (CYPP450) family, and by isozymes of 3beta-hydroxysteroid dehydrogenase. This process is further detailed in figure 1.5.

1.5.2 Hypothalamic-Pituitary Axis (HPA axis)

The synthesis and release of glucocorticoids from the adrenal cortex involves an array of hormonal interactions with the hypothalamus, pituitary and adrenal. Neuronal and other stimuli, such as stress, initiate the release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus into the hypothalamic-hypophyseal portal system which leads into the anterior pituitary. This leads to the release of adrenocorticotrophic (ACTH) from the anterior pituitary. This is formed from cleavage from the polypeptide pro-opimelanocortin (POMC). ACTH binds receptors on the adrenal cortex cell surface where it stimulates steroidogenesis and the release of adrenocortical steroids including glucocorticoids. Glucocorticoids have an inhibitory effect on AVP and CRH synthesis as well as POMC processing and ACTH release (Kretz *et al.* 1999). This provides a negative feedback mechanism which helps maintain physiological plasma glucocorticoid

concentrations. See figure 1.6. This feedback system is mediated through glucocorticoid receptors (Kitchener *et al.* 2004b). CRH, AVP and ACTH are released in a pulsatile manner, with a circadian rhythm. This leads to alterations in plasma glucocorticoid levels throughout the day. In man, levels are highest before waking and decline throughout the day, whereas in rats, levels peak in the evening (Friess E. *et al.* 1995; Kitchener *et al.* 2004).

Circulating glucocorticoids are predominantly bound to a protein called corticosteroid binding globulin (CBG) and albumin, with about 5-10% circulating as free steroid (Hammond 1990; Yudt & Cidlowski 2001b). The lipophilic nature of these hormones allows them to pass freely through the cell membrane. It is only when the steroid is free that it can diffuse across cell membranes and bind intracellular glucocorticoid receptor.

Pre-receptor metabolism of glucocorticoids plays an important role in modulating local glucocorticoid action by regulating hormone access to the receptor. This involves the 11β-hydroxysteroid dehydrogenase (HSD) enzymes of which there are two types. These enzymes catalyse the interconversion of active corticosterone and cortisol to their respective inactive 11-keto forms (11-dehydrocorticosterone, cortisone). The reaction directions in vivo of the isozymes differ such that 11β-HSD1 re-activates whereas 11β-HSD2 inactivates glucocorticoids. 11β-HSD2 has been shown to be of great importance in protecting the foetus from high concentration of maternal glucocorticosteroids, as well as being localised in kidney tubules, where it protects MR from over-activation by glucocorticoids (Li *et al.* 1996; Rabbitt *et al.* 2002; Shimojo *et al.* 1997; Whorwood *et al.* 1994). The intestine is another mineralocorticoid target tissue (see section 1.2.1.2.1) and 11β-HSD2, but not 11β-HSD1, has been shown to be highly expressed in the mucosa (Whorwood *et al.* 1994).

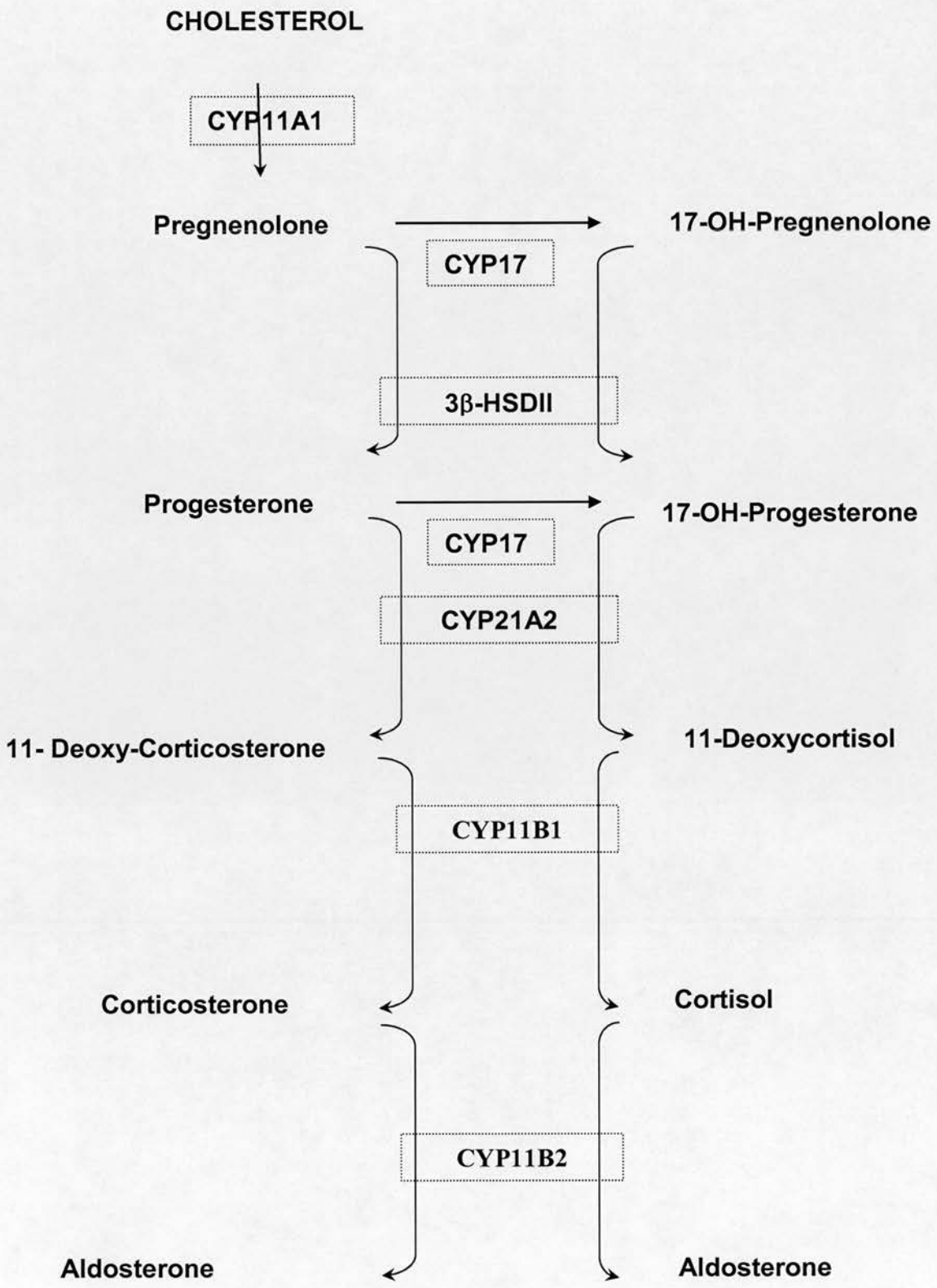


Figure 1.5: Adrenocortical steroid biosynthesis. This illustrates the cytochrome (CYP) enzymes involved in the biosynthesis of adrenocortical steroids from their precursor cholesterol, in the rodent (left) and in man (right).

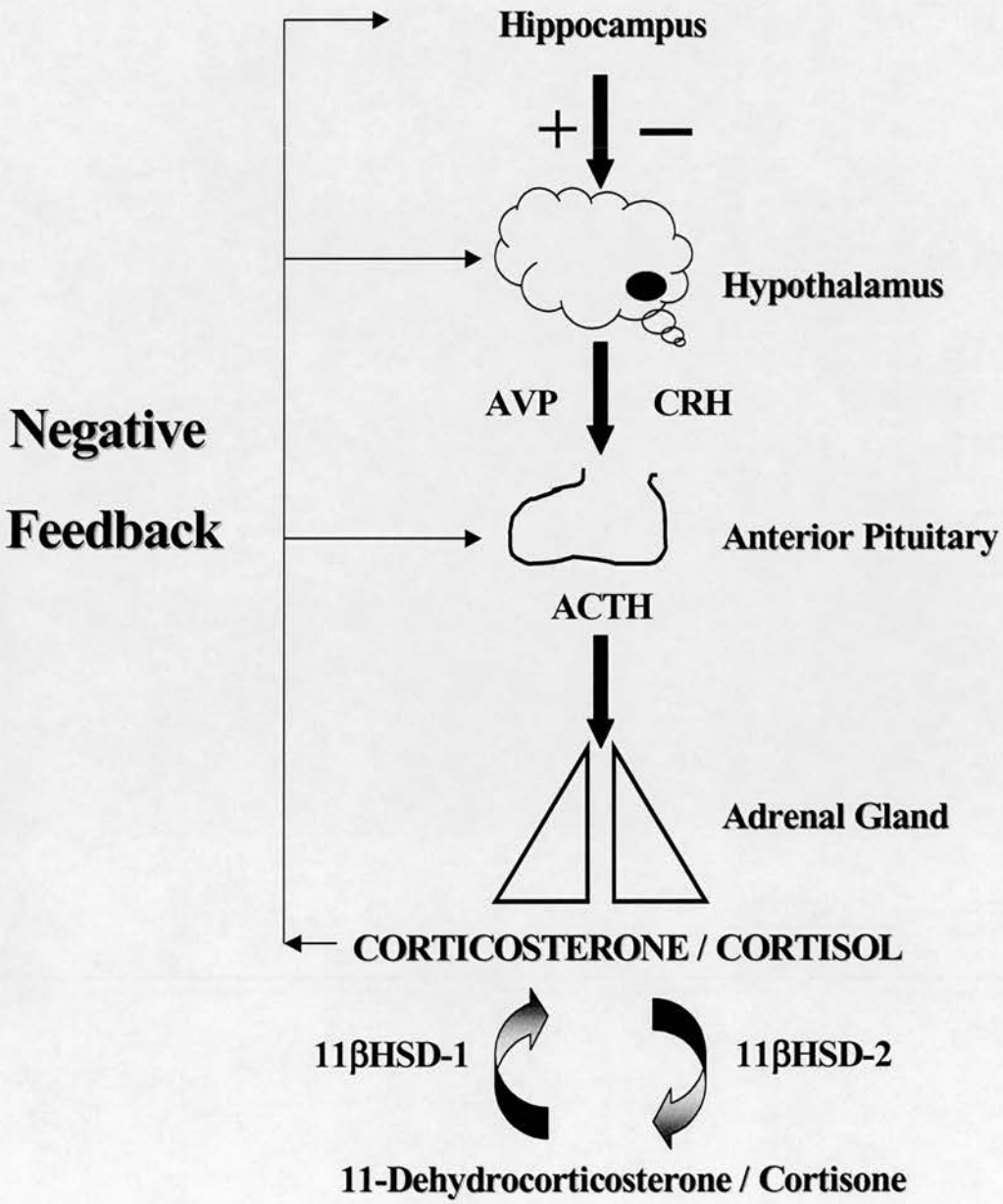


Figure 1.6: The HPA axis. Corticosterone or cortisol (in rodents and man respectively) is released from the adrenal gland in response to ACTH (adrenocorticotrophic hormone) secretion from the anterior pituitary, secretion of which was stimulated by AVP (arginine vasopressin) and CRH (corticotrophin releasing hormone) from the PVN (periventricular nucleus) in the hypothalamus. A negative feedback system exists where corticosterone/cortisol inhibits release of AVP and CRH as well as ACTH release from the hypothalamus and anterior pituitary respectively. It also influences neuronal projections to the hypothalamus from the hippocampus which can either increase or decrease AVP and CRH secretion.

1.5.3 Glucocorticoid Receptor

1.5.3.1 Structure

The glucocorticoid receptor is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors, and mediates transactivation of target genes by binding sequence specific recognition elements in their promoter region. Nuclear hormone receptors are characterised by comprising of a variable amino-terminal domain (N-terminal), DNA binding domain, and a carboxy-terminal (C-terminal) ligand binding domain. The DNA binding domain (DBD) is composed of two highly conserved zinc finger regions and is the most conserved region among nuclear receptors. These are responsible for target site recognition by hormones containing a glucocorticoid responsive element (GRE), stabilizes protein-DNA interactions and required for dimerisation (Necela & Cidlowski 2004; Oakley *et al.* 1996). The central DBD is also required for the repression of other transcription factors such as nuclear factor-κB and AP-1 (Necela & Cidlowski 2004). The C-terminal contains the ligand binding site for hormones as well as sequences which interact with chaperones, receptor dimerisation and transactivation (Oakley *et al.* 1996).

The human GR receptor contains 9 exons. Exon 1, of which there are multiple alternatives, consists solely of 5'-untranslated sequence, and exon 2 encodes the amino-terminal portion of the receptor. The two putative zinc fingers are separately encoded by two exons, and a total of five exons combine to form the cortisol-binding domain (Encio & Detera-Wadleigh 1991). There have been two classes of glucocorticoid receptor found in human- α andβ. These are formed by alternative splicing of exon 9. Until this exon each receptor contains the same 727 amino acids, but diverge beyond this point with GR-α having an additional 50 amino acids, and GR-β having an additional 15 non-homologous amino acids (Hecht *et al.* 1997; Oakley *et al.* 1996; Vottero & Chrousos 1999). GR-α is further categorised into GR-A and GR-B, as two forms produced by alternative translation of the same gene (Yudt & Cidlowski 2001).

1.5.3.2 Receptor activation

In the absence of ligand GR is localised in the cytoplasm of cells. Here it forms a large complex with proteins including HSP90, HSP70 and HSP56, where HSP90 prevents DNA binding (Hecht *et al.* 1997; Vottero *et al.* 2002). GR also interact with transcription factors; more importantly for this thesis, GR interact directly with NF-κB and AP-1 in the cytoplasm of cells and prevents their DNA binding and subsequent transcription (Oakley & Cidlowski 1993). After ligand binding, GR-α dissociates from the complex, dimerises, and translocates to the nucleus where it binds to glucocorticoid responsive elements (GRE) in the gene promoter. GR-β is generally thought not to bind ligand, however some studies have shown it binds GREs, and therefore it is thought to have a dominant negative effect on transcription (Vottero & Chrousos 1999). There are positive and negative GREs noted in the promoters of gene; GR-α binding negative GRE prevents transcription, whereas binding to a positive GR-α induces transcription (Burnstein *et al.* 1990) (see figure 1.7). The transcriptional activity of GR-α is dependent on various co-activators and co-repressors present in the complex, which facilitate in the recruitment of basal transcriptional machinery (Vottero *et al.* 2002). This complex leads to chromatin remodelling, which enables GR to bind DNA and therefore increase/decrease the transcription of target genes (Jenkins *et al.* 2001).

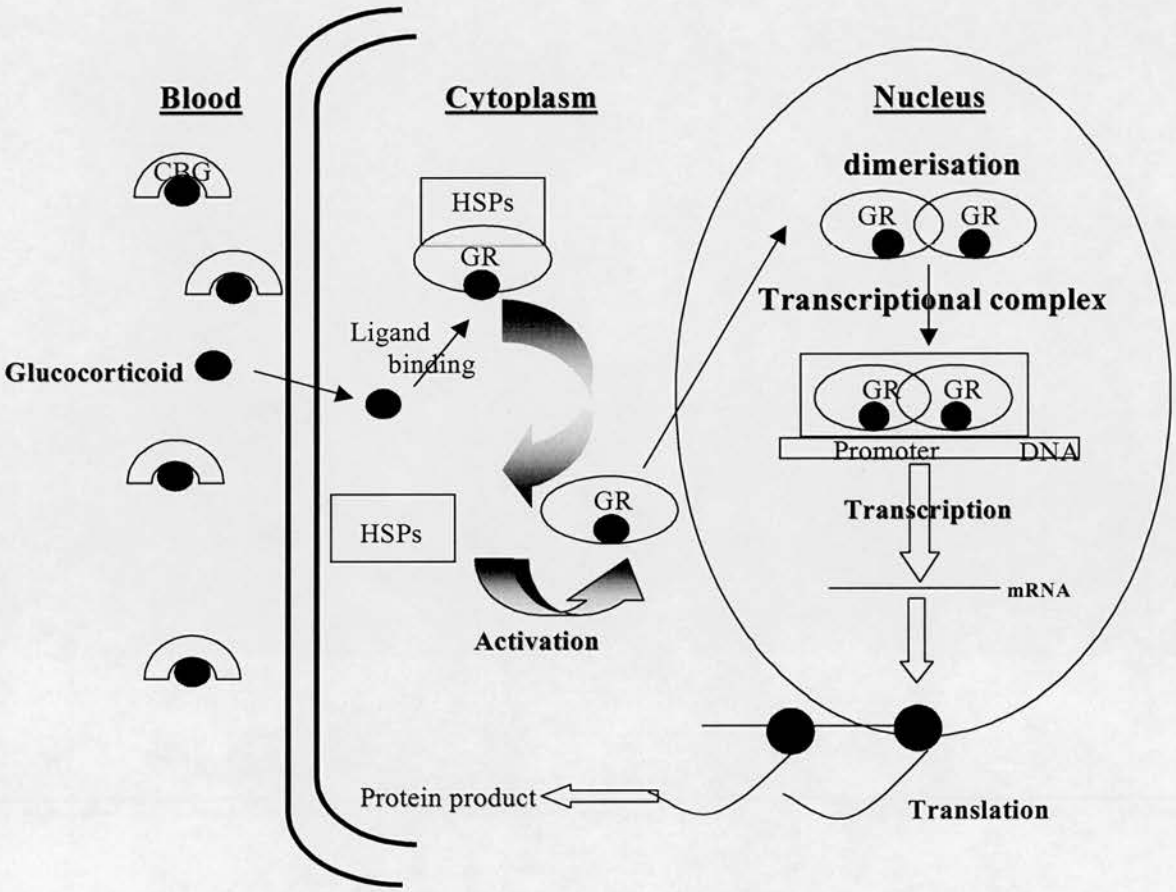


Figure 1.7: Glucocorticoid transcriptional activation. Corticosterone/cortisol dissociates from its binding globulin (CBG) and passes across the cell membrane. Free glucocorticoids in the blood, for example dexamethasone can also pass across the cell membrane and into the cytoplasm of the cell. In the cytoplasm, ligands bind GR which is bound to heat shock protein (HSPs) complex. Binding causes HSP to dissociate and GR dimerises before translocating into the nucleus. In the nucleus, a transcriptional complex binds glucocorticoid recognition elements in the DNA to increase/decrease transcription of target genes. Transcription leads to the subsequent translation of a protein product.

1.5.3.3 Receptor regulation

As glucocorticoids regulate numerous distinct physiological processes, glucocorticoid receptors are expressed in most cells, although the sensitivity to glucocorticoids varies between individuals, as well as tissues and cell types (Lim-Tio *et al.* 1997; Vottero & Chrousos 1999). The level of expression of GR is critical for cell function where transgenic mice with a reduction in tissue levels of GR have immunological, metabolic and neuroendocrine abnormalities (Pepin *et al.* 1992).

Binding sites for a number of transcription factors have been shown to be present in the GR promoter, and this may be important in tissue- and cell-specific GR regulation. Post-translational modification may also regulate GR. Multiple translation initiation sites have been identified in exon 1 in rat and humans, and these give rise to a number of GR mRNAs (Breslin *et al.* 2001; McCormick *et al.* 2000a). In rats, it has been shown different exons are tissue-specific, and this shows the tissue specific variation in the control of GR (McCormick *et al.* 2000b) Glucocorticoids themselves regulate GR expression, where an over-activation of the receptor, such as during stress where there is an increase in the concentration of corticosterone/cortisol, and this subsequently causes the down-regulation of GR to help maintain 'physiological' activation levels (Freeman *et al.* 2004). Alterations in tissue levels of GR, or even levels of the inhibitory isoform-GR-β, may be involved in glucocorticoid resistance (Rogler 2000).

1.5.4 Mineralocorticoid Receptor

The mineralocorticoid receptor belongs to the same nuclear hormone receptor superfamily as the glucocorticoid receptor family, and therefore both types of receptors share the structural and structural homology (Funder 1997). The physiological mineralocorticoid, aldosterone, acts via MR to regulate water and electrolyte absorption. Glucocorticoids also act through this receptor and regulate ion fluxes. As corticosterone/cortisol circulates at a much higher concentration than aldosterone, the enzyme 11-beta Hydroxysteroid dehydrogenase type 2 (11βHSD2, which inactivates corticosterone/cortisol) co-localises with MR, and therefore enables aldosterone to bind MR and regulate water and salt absorption (Sheppard *et al.* 1999).

MR encodes for a 107kDa protein consisting of 984 amino acids . The gene is composed of 10 exons. In man, the first 2 exons are known as exon 1α and 1β and are untranslated, whereas the other 8 exons are encode the entire protein. Experiments in rodents have shown 3 untranslated areas of the gene known as 1α, 1β and 1γ (Pascual-Le Tallec & Lombes 2005). As with the glucocorticoid receptor, exon 2 encodes for the N-terminal domain, exons 3 and 4 encode for the two zinc fingers of the DNA binding domain, and the last five exons encode for the ligand binding domain of the receptor. Alterative transcription of the untranslated regions generates two mRNA isoforms known as MR-α and MR-β, however the exact function of these transcripts are unknown (Pascual-Le Tallec & Lombes 2005).

There is an abundance of the mineralocorticoid receptor and 11βHSD2 in the distal nephron and colon, and this renders these areas key mineralocorticoid tissues. However, MR has also been shown to be expressed in other tissues including the hippocampus and hypothalamus, but as 11βHSD2 is absent in these tissue, MR is primarily activated by corticosterone/cortisol (Rogerson *et al.* 2003). Activation of MR by aldosterone, and the subsequent dimerisation and translocation of MR from the cytoplasm of cells into the nucleus (as seen with GR) induces the transcription of aldosterone induced proteins involved in altering sodium transport across the epithelium. This ultimately alters blood pressure, which is involved in cardiac hypertrophy and fibrosis (Funder 1997).

1.5.5 Glucocorticoid effects

1.5.5.1 Glucocorticoid effects on metabolism

Glucocorticoids regulate the metabolism of various substances including carbohydrates, protein and fat mainly in the muscle and adipose tissue in the periphery and in the liver, with the subsequent effect of increasing blood glucose concentrations. Glucocorticoids increase glycogen synthesis by stimulating glycogen synthase and inhibiting glycogen breakdown. Moreover, glucocorticoids increase hepatic gluconeogenesis by stimulating two key enzymes: phosphoenolpyruvate carboxykinase and glucose-6-phosphate. Glucocorticoids also mobilise substrates for hepatic gluconeogenesis, by stimulating release of amino acids from skeletal muscle and fatty acids and glycerol from adipose tissue (Roden & Bernroider 2003). Site-specific alterations in fat metabolism in situations of glucocorticoid excess, leads to redistribution favouring visceral fat as noted in Cushing's disease.

1.5.5.2 Glucocorticoid effects on blood pressure

There are a number of mechanisms which glucocorticoid effect blood pressure. These include increasing vascular sensitivity to the effects of catecholamines, in particular noradrenaline and angiotensin II, as well as decreasing the production of the vasodilators nitric oxide and prostacyclin (Yang & Zhang 2004). Glucocorticoids also act on the distal nephron to regulate salt and water reabsorption, inturn altering fluid volume and therefore blood pressure.

1.5.5.3 Glucocorticoid effects on mood and behaviour

Glucocorticoids exhibit a range of neuropsychiatric and behavioural effects, with sleep patterns, mood and receptor of sensory signals all effected by these hormones (de Kloet *et al.* 1998; McEwen *et al.* 1986). During glucocorticoid excess rapid eye movement, sleep disturbances and depression can all occur, whereas insufficient

glucocorticoid levels can lead to depression and apathy. It is known that glucocorticoids have neurotoxic effects (Sapolsky 1999).

1.5.5.4 Glucocorticoid effects on growth and development.

An excess of glucocorticoids inhibit the linear skeletal growth, probably due to its direct effects on bone, muscle and connective tissue metabolism (as mentioned earlier). It is also involved in cell-differentiation, where administered to pre-term babies encourages lung development. However, glucocorticoids given during pregnancy, in man and rodents, lead to reduced birth weight and possible 'programming effects' of the HPA axis, which leads to the development of cardiovascular and metabolic problems in later life (Bloom *et al.* 2001; Nyrienda & Seckl 1998).

1.5.6 Glucocorticoids and Inflammation

Glucocorticoids have profound inhibitory effects on inflammation and have therefore been exploited clinically in the treatment of inflammation and autoimmune disease and prevention of rejection of transplanted organs. The anti-inflammatory effects of the glucocorticoids are due to actions on blood vessels, inflammatory cells and inflammatory mediators (Barnes 1998), and these will be outline below.

1.5.6.1 Inflammatory mediators

Glucocorticoids increase the transcription of anti-inflammatory cytokines, as well as decrease transcription of pro-inflammatory mediators. Lipocortin synthesis is increased which inhibits the enzyme phospholipase A_2 and in turn prevents the production of lipid mediators (Barnes 1998). The interleukin (IL)-1 receptor antagonist is also increased and this prevents binding by its ligand IL-1, and reduces inflammation. IL-10 secretion from macrophages has been shown to be increased after glucocorticoid treatment. This cytokine prevents the transcription of many pro-inflammatory mediators, possibly through its interaction with the pro-inflammatory

transcription factor NF-κB (Barnes 1998). Glucocorticoids bind directly with NF-κB and AP-1 preventing transcription of target genes. Moreover, glucocorticoids increase the transcription of IκB which is an inhibitor of the pro-inflammatory transcription factor NF-κB in monocytes and lymphocytes, but not endothelial cells (Brostjan *et al.* 1996).

1.5.6.2 Inflammatory transcription factors

NF-κB is a pro-inflammatory transcription factor formed from a heterodimer typically consisting of p65 and p50 monomeric protein. It is characterised by a Rel homology domain important in DNA and IκB binding (de Bosscher *et al.* 2003). It is localised in the cytoplasm where it is bound to its inhibitory protein IκB. IκB is a tripartite molecule, consisting of an N-terminal domain for proteolytic cleavage, central domain for interaction with NF-κB, and a C-terminal domain which is essential for sequestration in the cytoplasm (Neurath *et al.* 1998). There are numerous isoforms, of which IκB-α has been shown to be important in NF-κB activation.

During inflammation, cell surface receptors become activated by pro-inflammatory mediators, such as IL-1 or TNF, and this initiates a phosphorylation cascade. This cascade involves the activation of the NF-κB inducing kinase (NIK). TNF activates NIK via the TNF receptor associated death domain (TRADD) and TNF receptor associated factor (TRAF), whereas IL-1 and IL-18 activates NIK via IRAK and TRAF (Neurath *et al.* 1998). NIK phosphorylates IκB kinase (IKK), which in turn phosphorylates IκB-α on serine residues. IκB-α thereafter becomes ubiquitinated, and this causes degradation via the ubiquitin/proteasome pathway (Neurath *et al.* 1998). Degradation of IκB-α enables NF-κB to translocate from the cytoplasm into the nucleus, where it increases the transcription of pro-inflammatory cytokines. See figure 1.8. There is a negative feedback loop by which NF-κB increases the transcription of IκB to prevent over-activation (de Bosscher *et al.* 2003).

Studies in knockout animals have shown the p65 subunit essential for embryonic development, and animals with an overactivation of NF-κB had small spleens and

thymus. This shows NF-κB is essential at regulating immune-cell function (Neurath *et al.* 1998). An increase in the degradation of IκB-α and an increase in NF-κB expression have been shown to be present in epithelial cells from IBD patients, however after glucocorticoid treatment, NF-κB is localised in the cytoplasm opposed to untreated cells where its localised in the nucleus (Thiele *et al.* 1999).

Activating protein-1 (AP-1) is a pro-inflammatory transcription factor encoded by oncogenes and composed of homo- or hetero-dimers of the fos, jun and activating protein families. The heterodimer fos/jun has been shown to be involved inflammation where it increases the transcription pro-inflammatory genes, including IL-2. Glucocorticoids bind directly with AP- 1 preventing its pro-inflammatory actions (Barnes 1998).

1.5.6.3 Immune cell function

Glucocorticoids also have important effects of the cells of the immune system T- and B-cells, neutrophils, and monocytes. T-cells play a pivotal role in initiating chronic inflammation (see section 1.2.2) and glucocorticoids have been shown to inhibit the activation, proliferation and induce T-cell apoptosis, and this indirectly inhibits B-cell and macrophage activation (Barnes 1998). This in turn blocks the release of pro-inflammatory mediators, and therefore aiding in the resolution of inflammation. Glucocorticoid treatment also decreases dendritic cell expression (which are important antigen-presenting cells (APC) known to activate the immune response), as well as increasing the survival of neutrophils, although the mechanisms behind these opposing effects are of yet unknown (Barnes 1998). Other anti-inflammatory actions include inhibiting the release of pro-inflammatory cytokines from macrophages (another APC) (see section 1.2.2).

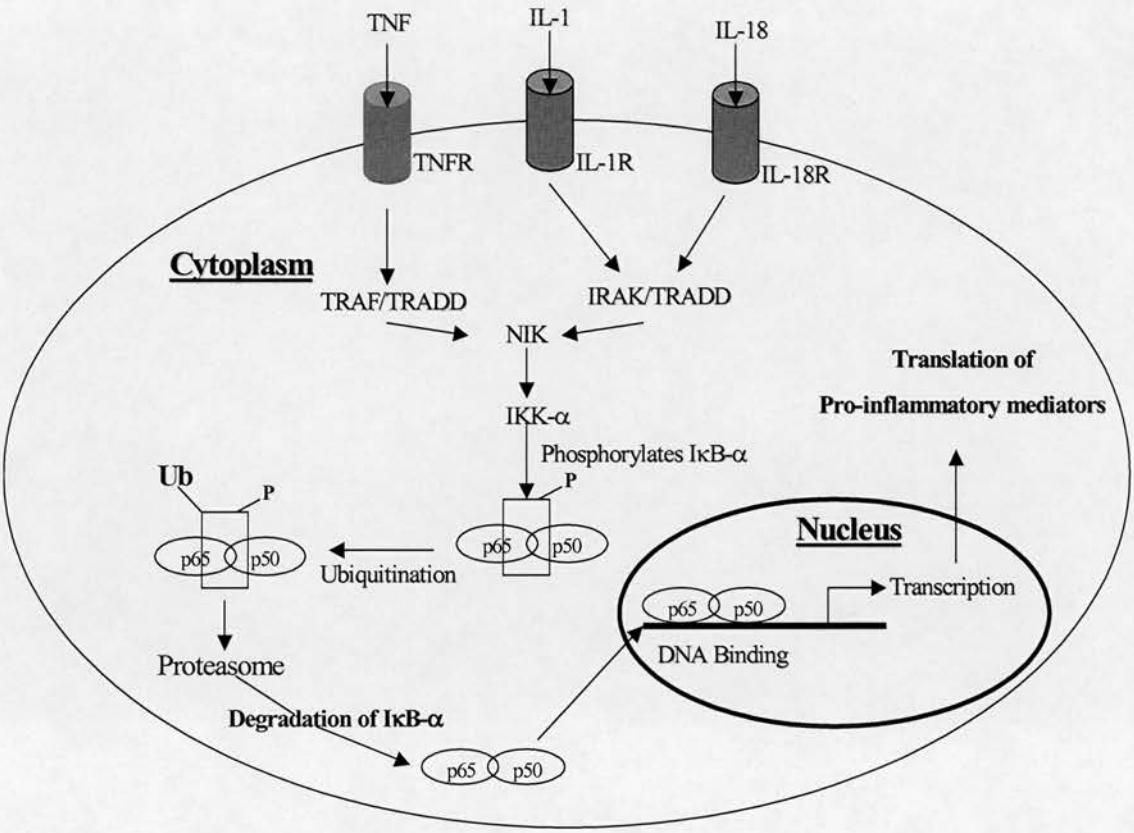


Figure 1.8: Mechanism of NF-κB activation by pro-inflammatory cytokines IL (interleukin)-1, IL-18 and TNF (tumour necrosis factor). Binding of TNF to its receptor (TNFR) activates a TNF receptor associated factor (TRAF) via a receptor associated adaptor protein called TNF receptor associated death domain (TRADD). Binding of IL-1 and IL-18 to their receptors IL-1R and IL-18R respectively, causes the activation of TRAF and the IL receptor associated kinase (IRAK). These complexes active NIK (NF-κB inducing kinase), which inturn activates IKK-α (IκB kinase kinase-α). This causes the phosphorylation of IκB-α, which induces the ubiquitination of IκB-α and this targets IκB-α for degradation by a proteasome. This enables NF-κB to translocate into the nucleus where it binds DNA to increase the transcription of target genes, and subsequent translation of pro-inflammatory mediators. Figure adapted from (Neurath *et al.* 1998)

1.5.7 Glucocorticoid resistance

Although glucocorticoids are effective in the treatment of inflammatory disease, including asthma and inflammatory bowel disease, a proportion of patients are steroid resistant. The pathophysiology for steroid resistance has been well studied for diseases such as asthma and rheumatoid arthritis, but not for IBD (Farrell & Kelleher 2003).

Studies have shown that between 16-20% of patients with IBD are steroid resistant (Faubion *et al.* 2001; Munkholm *et al.* 1994). Munkholm and colleagues showed that in patients diagnosed with CD between the years 1979-1987, 20% were steroid resistant and only 55% of the responders remained in prolonged response after treatment had finished (30days). A more recent study revealed 16% of UC and CD patients were unresponsive to steroid therapy and the 1 year outcomes showed only 32% and 48% of CD and UC patients were corticosteroid free and in remission (Faubion *et al.* 2001). These studies emphasis the major problem physicians encounter when treating patients with IBD in the clinical setting, and warrants further studies to investigate the molecular mechanisms underlying steroid insensitivity in these patients.

For the purpose of this study current understanding of the molecular mechanisms behind steroid resistance in IBD will be reviewed. Research has highlighted three main molecular mechanisms behind steroid resistance in IBD:

1.5.7.1 Cytoplasmic concentration of glucocorticoids

The multidrug resistance gene encodes for a P-glycoprotein transporter pump (Ho *et al.* 2003; Silverman *et al.* 1991). This pump is known to transport various drugs out of cells, reducing their efficacy, and has been implicated in steroid resistance (see section 1.2.4.1) (Farrell *et al.* 2000). An increase in glucocorticoid metabolism by cytochrome P450 enzymes has also been implicated in steroid resistant asthma (Barnes 1998). Moreover, an increase in expression of MDR1 has been shown in peripheral blood lymphocytes and epithelial cells in IBD patients who required surgery due to failed medical therapy (Farrell *et al.* 2000). It has been suggested that

expression of MDR1 in patients requiring corticosteroid therapy may be a predictive factor for determining steroid sensitivity. Treatment with inhibitors of P-glycoprotein may reverse glucocorticoid resistance by facilitating in the uptake of glucocorticoids into cells (Meijer *et al.* 2003).

1.5.7.2 Glucocorticoid receptor

T-lymphocytes are thought to play an important role in steroid-insensitivity in the setting of IBD. Studies have shown that glucocorticoid treatment inhibited T-cell proliferation from samples taken from steroid responders, although did not inhibit T-cell proliferation in samples from non-responders (Farrell & Kelleher 2003), and a reduced binding affinity of GR in T-cells has been implicated. Of note, no difference in GR mRNA expression has been found between steroid responders and non-responders in UC, suggesting the density of GR mRNA is not important in determining steroid-sensitivity in IBD (Flood *et al.* 2001). Therefore reduced affinity of steroids for GR would reduce the potential efficacy of steroid in the resolution of inflammation

Increased expression of the inhibitory glucocorticoid receptor-beta (GR-β) has been implicated in steroid resistance, as it does not bind ligand but does bind the DNA preventing activation of target genes (Barnes 1998). An increase in expression of this isoform has been noted in lymphocytes from steroid resistant UC patients compared steroid responders, and this phenomenon has been mirrored in steroid resistant asthma and rheumatoid arthritis (Chikanza *et al.* 2003; Farrell & Kelleher 2003; Honda *et al.* 2000). As the expression levels of GR-β compared to GR-α within cells is far less, it seems unlikely that these levels of GR-β can produce a dominant-inhibitory effect over GR-α. Further studies are required to investigate possible inducers of GR-β expression, as inflammatory cytokines may increase expression and therefore may aid in the perpetuation of inflammation (Farrell & Kelleher 2003).

1.5.7.3 Interactions between GR and transcription factors

Another possible mechanism which is involved in steroid resistance is abnormalities in the interaction of GR with pro-inflammatory transcription factors. Studies have shown epithelial expression of NF-κB in steroid resistant Crohn's disease (CD) patients, whereas in steroid-sensitive patients, localisation of NF-κB was predominantly in lamina propria (Bantel *et al.* 2002). Further work also revealed that AP-1 and upstream kinases were active in epithelial cells from steroid resistance CD epithelial cells, opposed to steroid-sensitive patients where expression was localised to the lamina propria (Bantel *et al.* 2002). This suggests that steroid resistance in CD is associated with epithelial activation of these pro-inflammatory mediators. The mechanisms remains elusive although a possible reason could be due constitutive expression of these transcription factors in epithelial cells, which 'over-run' the cell and buffer the anti-inflammatory effects of the limited number of GR (Barnes 1998; Farrell & Kelleher 2003).

1.6 THESIS AIMS

The aim of this thesis was to investigate the underlying molecular mechanisms involved in determining steroid sensitivity in the rat intestine, both in health and inflammation. The work has concentrated on the role of P-glycoprotein and glucocorticoid receptor. The following specific aims were addressed:

1. To study the regional distribution of genes (mdr1a, GR, MR and 11βHSD-2) involved in steroid access and efficacy in the healthy rat colon, and the effect of systemic glucocorticoid treatment on expression.

2. To investigate the effect of antibiotic induced alterations in colonic flora on the expression of P-glycoprotein and GR in the healthy rat colon.

3. To study the relationship between intestinal inflammation and expression of P-glycoprotein and glucocorticoid receptor in the HLA-B27 transgenic rat colon. Gene expression was compared between animals raised in specific-pathogen free (SPF) and germ free (GF) conditions.

4. To investigate the effect of systemic dexamethasone treatment on expression of these genes in the HLA-B27 transgenic diseased model of colitis.

5. The molecular mechanisms underlying the regulation of P-glycoprotein and glucocorticoid receptor by dexamethasone were investigated *in vitro* using the rat small intestinal crypt cell line (IEC-6).

Chapter Two

Methods and Materials

2.1 MATERIALS

Unless otherwise stated all chemicals, reagents and drugs were purchased from Sigma, Poole, UK. All enzymes for molecular biology were purchased from Promega, Southampton, UK. All radioactively labelled steroids, radioactive isotopes and secondary horseradish-peroxidase linked antibodies were purchased from Amersham, Little Chalfont, UK. Sources other than these are indicated.

2.1.1 Buffers and Solutions

Alkaline SDS solution: 0.2M NaOH, 1% w/v SDS

Blocking solution: 25g milk (Bio-rad), 500µl Tween, 50mls 10xTBS (see below) made up to 500mls with distilled water.

Borate Buffer: 8.25g boric acid, 2.7g NaOH, 3.5ml conc. HCl (10M) and 5g BSA made up to 1litre with distilled water, pH 7.4. Stored at -20°C and thawed at room temperature immediately before use.

Box Buffer: 20ml 20xSSC buffer, 50ml deionised formamide made up to 100ml in DEPC-treated water (see below).

Buffer A: 10mM HEPES, 10mM KCL, 2mM $MgCl_2$, 1mM DTT, 0.1mM EDTA, 0.2mM NaF, 1µg/ml Leupeptin, 0.4mM PMSF, 1x stock protease cocktail inhibitors (Complete, Roche Diagnostics, East Sussex, UK.

Buffer B: 10% Nonidet P40 in distilled water

Buffer C: 50mM HEPES, 50mM KCL, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 10% Glycerol, 0.2mM NaF, 0.1mM PMSF, 1x stock protease cocktail inhibitors.

Caesium Chloride/TE solution: 100g CsCl dissolved in 100ml TE buffer (see below).

Citrate Buffer:1.8ml 1M Citrate Acid (105.07g dissolved in 500ml distilled water) and 8.2ml 1M Sodium Citrate (147.05g dissolved in 500ml distilled water), made up to 1l with distilled water.

DEPC-treated water: Distilled water mixed with diethylpyrocarbonate (DEPC; 300µl/ 100 ml), shaken and left for 1-24 hours prior to autoclaving.

Deionised formamide: 150ml Formamide mixed with 15g Amberlite ion exchange resin (MB-6113) (BDH, Lutterworth, UK) for 1 hour, filtered twice to remove Amberlite and stored at -20°C.

1Kb DNA ladder: 20µg 1Kb ladder (Life Technologies, Paisley, UK), in 200µl distilled water with 10% (v/v) loading buffer.

250mM EDTA (pH 8.0): 80mls water was added to 9.3g $Na_2EDTA.2H_2O$. pH was adjusted to 8.0 with NaOH and the volume adjusted to 100mls.

GTE: 50mM glucose, 25mM Tris, 10mM EDTA, pH 8.0

Homogenisation Buffer: 50mM Tris pH 7.5, 0.25M sucrose, 5mM EDTA, 20mM sodium molybdate, 1x stock cocktail protease inhibitors (Complete, Roche), 1µg/ml aprotinin, 1µg/ml leupeptin, 1mM PMSF, made up in distilled water.

2xHybridisation buffer: 1.2M NaCl, 20mM Tris-HCl, 2x Denhardts, 2mM K_2-EDTA, 0.2mg salmon sperm DNA, 0.2mg yeast tRNA and 2g dextran sulphate made up to 10ml in DEPC-treated water, stored at -20°C.

4x Laemmli buffer: 4% SDS, 20% glycerol, 2mM DTT, 125mM Tris pH 6.8, 16% β-mercaptoethanol, bromophenol blue.

LB agar: Luria-Bertoni broth with 15g agar per litre broth added before autoclaving.

Loading buffer: 40% sucrose w/ v, 0.25% bromophenol blue (w/v) in distilled water.

Luria-Bertoni broth: 10g bactotryptone, 5g bacto yeast extract, 5g NaCl made up to 1 litre with distilled water and autoclaved immediately.

Lysis buffer: 1xPBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1x stock protease cocktail inhibitors (Complete, Roche Diagnostics, East Sussex, UK), 1μg/ml aprotinin, 1μg/ml leupeptin, 1mM PMSF.

4% Paraformaldehyde in 0.1M phosphate buffer: 20mM NaH_2PO_4, 80mM Na_2HPO_4 in 1l DEPC-treated water, heated to 80°C prior to addition of 40g paraformaldehyde. Stirred for 1 hour to dissolve and stored at 4°C.

Phosphate Buffer: 0.2M NaH_2PO_4 0.6M Na_2HPO_4, 5mM EDTA. Autoclaved before use.

Phosphate buffered Saline (PBS): 0.1M phosphate buffer with 137mM NaCl, 2.7mM KCl in distilled water, pH 7.4, autoclaved before use.

5M Potassium Acetate: 60ml of 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml water.

2xPre-hybridisation buffer: 1.2M NaCl, 20mM Tris-HCl, 2x Denhardt's, 2mM K_2-EDTA, 10mg salmon sperm DNA, 0.2mg yeast tRNA made up to 10ml in DEPC-treated water, stored at -20°C.

RNase A buffer: 25mg Rnase A dissolved in 1mM Tris, 15mM NaCl made up to 2.5ml with distilled water (10mg/ml). Heated to 100°C for 15 minutes then cooled to room temperature and stored at -20°C. RNase A (10mg/ml) added to RNase box buffer (3μl/ml).

RNase Box buffer: 0.5M NaCl, 10mM Tris, 1mM EDTA in distilled water.

Running buffer: 50mM TRIZMA base, 0.38M Glycine, 1% SDS made in distilled water.

20x Saline Sodium Citrate buffer (SSC): 3M NaCl, 0.3M Na citrate in 1l DEPC-treated water, pH 7.0, autoclaved before use.

5M Sodium Chloride: 29.55g added to 100ml DEPC-water

Stripping buffer: 1M Glycine pH 2, 0.1% SDS, 0.1% Tween made in distilled water

10xTBE buffer: 0.9M TRIZMA base, 0.9M Boric acid, 20mM K_2-EDTA in distilled water.

10xTBS buffer (Tris buffered-saline): 0.2M TRIZMA base, 1.4M NaCl in distilled water, pH 7.6.

TE buffer: 10mM Tris-HCl, 1mM EDTA, pH 7.5, autoclaved before use.

5xTranscription optimised buffer: 200mM Tris-HCl, 50mM NaCl, 30mM $MgCl_2$, and 10mM spermidine (ready mixed from Promega).

Transfer Buffer: 50mM TRIZMA base, 0.38M Glycine, 20% Methanol in distilled water, cooled to 4°C.

0.1M Triethanolamine: 13.3ml Triethanolamine dissolved in 800ml DEPC-water pH 8, volume adjust to 1l (using sterile glassware to avoid autoclaving)

2.1.2 Equipment

GeneQuant RNA/DNA Calculator Hyper-processor	*Amersham Pharmacia Biotech, Little Chalfont, UK*
Beckman J2-MC Centrifuge	*Beckman Instuments, High Wycombe, Buckinghamshire, UK.*
Electophoresis Power Pac 300 Western Equipment	*Bio-Rad Laboratories Ltd., Hamel Hampstead, UK.*
EL 312e Bio-Kinetics Microplate Reader	*Bio-Tek Instruments Inc., Winooski, Vermont, USA.*
Labofuge 400R Centrifuge (Used in Cell Culture)	*Heraeus, Brentwood, Essex, UK.*
Ultra-Turrax T8 auto-homogeniser	*Ika, Labortechnik, Staufen, Germany.*
Leica Cryostat	*Leica Microsystems, UK.*
1450 Microbeta Plus Liquid Scintillation Counter	*Wallac Oy.*

2.1.3 Software

MCID-M4 Image Analysis V.3.0 Rev 1.5	*Imaging Research*
Zeiss KS3000	*Imaging Associates, UK.*
Fujifilm Fluorescent Image Analyser FLA-200 V.1.0	
Aida 2.0 Auto Image Data Analyser	*Raytest Scientific Ltd., Sheffield, UK.*
Graphpad Prism 3.03	*Graphpad Software Inc, USA.*
Statisica v.5.0	*Statsoft, Tulsa, Oklahoma, USA.*
Multicalc Advanced v2.0	*Wallac Oy.*

2.2 ANIMAL MAINTENANCE

Male Wistar rats were obtained from Charles River, Kent, UK at 6 weeks of age. Animals were maintained under controlled conditions of lights (lights on 0800 h-2000 h) and temperature (21-22°C), with ad lib access to drinking water and standard rat chow (Special Diet Sevices, Witham, UK). Rats were acclimatised to their environment for a period of at least a week before surgery/treatment. After surgery and during treatment periods the animals were under the primary care of Mrs June Noble of the Molecular Medicine Centre, assisted by the animal technicians of the Biomedical Research Facility, Western General Hospital. Rats were housed six per cage, except during antibiotic treatment where they were housed three per cage.

2.2.1 Surgery

Mrs June Noble of the Molecular Medicine Centre, Western General Hospital, carried out all surgical procedures under the terms of the UK Home Office Animals (Scientific Procedures) Act, 1986. 8 week old rats were anaesthetised with 4% halothane and either bilaterally adrenalectomised (ADX) or sham-operated (Sham) through dorsal incisions and the incisions closed with staples. After regaining consciousness rats were re-caged and monitored closely. All ADX rats were maintained on 0.9% saline as drinking water to maintain their electrolyte balance.

2.2.2 Killing and harvesting of tissues

After treatment rats were killed by CO_2 asphyxiation and subsequent decapitation. Unless otherwise stated, colons were removed by dissection, longitudinally cut so the colon was in an open structure, and sectioned into ten equal lengths. Each section was then quickly placed and held on powdered dry ice until frozen.

For the colonic samples from the HLAB27 transgenic and non-transgenic rats, which were a gift from Professor Sartor, colons were dissection, sections from the caecum, proximal colon and rectum were frozen directly in OCT medium.

All tissue samples were stored at minus 80°C.

2.3 CELL LINES

2.3.1 Maintenance of cell lines

IEC-6 cells (derived from a rat small intestinal crypt epithelial cell, Quaroni et al. 1979) were maintained at 37°C with 5% CO_2, 95% O_2 in Dulbecco's minimal essential medium (DMEM (Gibco, Paisley, UK)) supplemented with 5% heat-inactivated foetal calf serum, insulin (0.1 IU/ml), penicillin/streptomycin (100μg/ml) and L-glutamine (2mM). Cells were routinely split 1:4 when confluent. To harvest and split cells, they were washed with serum-free DMEM (10ml), then treated with trypsin/EDTA in HBSS (1.5ml) for 1-2min to release the cells from the flask surface, then resuspended in DMEM (8.5ml) with serum. Cells were then diluted as appropriate in DMEM with serum.

2.3.2 Seeding cells

For all cell line experiments cells were harvested 48hr before treatment by centrifugation at 1000 x g for 5min and resuspended in medium (30ml). Cells were counted using an Improved Neubauer haemocytometer (Hawksley) and if necessary diluted further to give a cell count of 5 x 10^6 cells/ml. 1ml of cell suspension was added to each 60mm dish containing 3ml of medium (3 dishes per treatment group) and incubated at 37°C with 5% CO_2. 24hr before treatment the DMEM containing serum was replaced with DMEM containing serum stripped of steroids. All treatments were added to plated cells in DMEM containing stripped serum.

Serum was stripped of corticosteroids by adding 5g of activated charcoal to 500ml serum and stirring overnight at 4°C. Serum was centrifuged at 1000 x g for 5min to pellet the steroid-bound charcoal, and the supernatant sterile filtered through a series of filters (Sartorius, Goettingen, Germany) with decreasing pore size (1.20μM, 0.4μM, and 0.2μM).

2.4 ASSAYS

2.4.1 Protein Estimation

The protein concentrations of tissue homogenates and cell lysate preparations were determined colorimetrically using a Bio-Rad protein assay kit. A range of protein standards (0.05–0.5mg/ml) was prepared in duplicate in distilled water from the provided protein standard (BSA, bovine serum albumin). Protein assay dye reagent was diluted 1:5 in distilled water. Diluted protein assay dye reagent (200µl) was added to protein standard (10µl) or appropriately diluted tissue homogenate/lysate in a 96-well plate, mixed and left at room temperature for 20min to allow colour development. Absorbance of samples at λ570nm was measured using an ELISA plate reader, and the concentration of protein in each sample was estimated from the standard curve. An example of a standard curve is shown in Figure 2.1.

2.4.2 Corticosterone radioimmunoassay

Plasma corticosterone levels were measured in aliquots of thawed plasma by radioimmunoassay. This assay was developed by Dr CJ Kenyon. Plasma samples were diluted 1 in 10 in borate buffer and denatured at 65° C for 30min to dissociate corticosterone (B) from corticosterone-binding globulin. A range of concentrations of B were prepared (0.6-320nM) to allow construction of a standard curve. Samples and standards were incubated with a mixture of $[^3H]_4$-B (10,000cpm per sample) and B antibody (1 in 10,000 dilution, produced by Dr CJ Kenyon, Molecular Medicine Centre, Western General Hospital) in borate buffer in a total volume of 70µl for 1hr. Scintillation proximity assay beads (SPA; Amersham, Bucks, UK) were then added to each sample and the samples were incubated overnight. The SPA beads bind to the primary antibody and if the primary antibody is bound to $[^3H]_4$-B the SPA beads cause scintillation of the radioactive signal. As the concentration of unlabelled B increases there is competition between binding of unlabelled and labelled B to the primary antibody, and the radioactive signal decreases. Samples were counted on a Wallac Microbeta Plus liquid scintillation counter using the Multicalc programme. The concentration of B in each sample was estimated from the standard curve. The inter- and intra-assay coefficients of variation were <10%

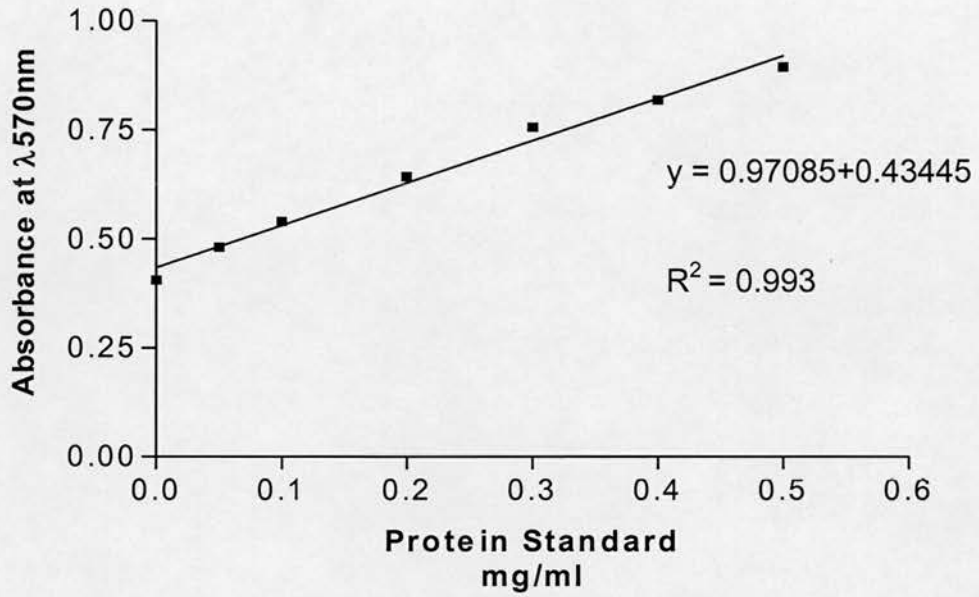


Figure 2.1: Protein Assay Standard Curve

A Bio-Rad protein assay kit was used to colorimetrically determine the protein concentration of tissue homogenates. Absorbance of protein standards of known concentrations (0.0–0.5 mg/ml) at λ560nm was measured using an ELISA plate reader. A standard curve was produced allowing subsequent estimation of protein concentrations in each tissue homogenate. Samples were prepared in a dilution allowing estimation in the middle range of the curve.

2.5 PREPARATION OF cDNA PLASMIDS

2.5.1 Bacterial Transformation

Escherichia coli HB101 cells were grown in 100ml of Luria-Bertoni (LB) broth at 37°C in a shaking incubator until they reached mid-log phase (A_{600} = 0.3-0.6). They were then centrifuged at 1000 x g for 5min at 4°C, the pellet re-suspended in cold calcium chloride (0.1M; 20ml) and left on ice for between 10min and 2hr. The centrifugation step was repeated to re-pellet the cells and the cells were re-suspended in cold calcium chloride (0.1M; 2ml). The competent cells were stored on ice in the fridge for up to 3 days before transformation.

Competent cells (200µl) were mixed with plasmid DNA (50ng) and left on ice for 20min. The cells were heat shocked at 42°C for 50s and placed back on ice. The heat shock and the calcium chloride lead to the incorporation of the plasmid DNA into the cells. The cells were spread onto LB agar plates containing ampicillin (100µg/ml), and the plates incubated overnight at 37°C. Only cells that had incorporated the plasmid DNA grew on the plates containing ampicillin, as HB101 cells do not have inherent ampicillin resistance.

2.5.2 Plasmid DNA preparation

A single transformed bacterial colony was selected from an agar plate and incubated for 6 hours in LB (2ml) containing ampicillin (100µg/ml) (Sambrook & Russell 2001). This was then added to LB (500ml) containing ampicillin (100µg/ml) and incubated overnight at 37°C. The culture was centrifuged at 3500 x g for 5min at 4°C in a Beckman J14 centrifuge, and the supernatant discarded. The cell pellet was resuspended in cold GTE buffer (12ml) and freshly prepared alkaline SDS (24ml). The mixture was shaken vigorously by hand and left on ice for 10min. Cold potassium acetate (5M; 16ml) was added and the mixture left on wet ice for 10 min before being centrifuged at 3500 x g for 5min at 4°C in a Beckman J14 centrifuge. The mixture was filtered through two layers of sterile gauze to remove the precipitate, isopropanol (32ml) was added to the filtrate and the mixture was left at room temperature for 30min to precipitate the DNA. The DNA was pelleted by centrifugation at 7800 x g for 3min at 4°C in a Beckman J20 centrifuge, and the supernatant discarded. The DNA pellet was left to dry. The DNA pellet was resuspended in TE buffer (2.2ml), CsCl (2.95g) added and dissolved and ethidium bromide (100µl, 10mg/ml) added. The mixture was transferred to Beckman Quickseal ultracentrifuge tubes, topped up with CsCl/TE solution (1g/ml) and centrifuged at 175 000 x g for 20hr at 20°C in a Beckman Optima TLX ultracentrifuge. The DNA was separated into bands that could be visualised by the pink colour of the ethidium bromide. These DNA bands were removed using a 21gauge needle and syringe, transferred to fresh ultracentrifuge tubes, topped up with CsCl/TE solution (1g/ml) and centrifuged at 356 000 x g for 4hr at 20°C. The DNA bands were collected as above and the ethidium bromide was removed by extracting repeatedly with

isopropanol until the pink colour disappeared. The DNA was transferred to dialysis tubing and dialysed against three changes of TE buffer. The concentration and purity of the DNA was assessed spectrophotometrically using a GeneQuant RNA/ DNA Calculator.

2.6 ^{35}S *In Situ* HYBRIDISATION

In situ hybridisation involves the reaction whereby a ^{35}S-UTP labelled 'antisense' RNA probe hybridises to complementary RNA in tissue sections by means of hydrogen bonding. This enables the visualisation of the exact cellular and/ or structural location of specific mRNAs (indicating transcription of the corresponding gene). To ensure the specificity of the 'antisense probe', ^{35}S-UTP labelled RNA 'sense' probes of similar length, nucleotide content and specific activity but not complimentary to the gene were included in each experiment.

To prevent degradation of target mRNA by exogenous RNases only RNase free, sterile solutions and equipment were used for *in situ* hybridisation experiments.

2.6.1 Slide Preparation

During the handling of microscope slides, gloves were worn at all times to prevent RNase contamination. Prior to use, glass twinfrost microscope slides (BDH, UK) were coated in 3-aminopropyltriethoxysilane in order to prevent section dehiscence. Slides were racked and washed in the following series of solutions; HCl (0.2M) for 3min, DEPC-treated water for 3min, 3-aminopropyltriethoxysilane in acetone (2%; filtered through $NaSO_4$) for 10s, acetone for 3min (twice), and finally DEPC-treated water for 3min. Slides were air-dried for 30-60min before baking at 50°C overnight. Dried slides were wrapped in aluminium foil and stored for up to 6 months.

2.6.2 Tissue section preparation

Frozen tissue sections were cut using a Leica cryostat. Tissues frozen at -80°C were placed in the cryostat chamber at -20°C and allowed to equilibrate for approximately 30min. Following equilibration, tissues were embedded in Cryo-m-bed embedding

compound (Brights, UK) and positioned in the correct orientation for sectioning. 10μm thick sections were thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides. Slides with tissue sections were stored at -80°C until required.

2.6.3 Fixation protocol

To preserve tissue morphology, prevent RNA degradation and allow easy penetration of probe slides were removed from the -80°C freezer and kept on dry ice until the start of the fixation procedure. Slides were fixed in ice cold paraformaldehyde (4%) in phosphate buffer (0.1M) for 10min. This maintains tissue morphology and inhibits endogenous ribonucleases. Slides were rinsed twice in 1x PBS for 5min and acetylated in triethanolamine (0.1M) with acetic anhydride (0.25%) for 10min (this reduces non-specific binding of the probe to positively charged amino groups in tissues), and rinsed in 1x PBS for 3min. Following dehydration through a series of ethanol solutions (70, 80 and 95% ethanol in DEPC-treated water) slides were air dried for 30min.

2.6.4 Probe templates for *in situ* hybridisation

Plasmids containing cDNA fragments for GR, MR and 11β-HSD2 were a gift from Mrs June Noble. The plasmid containing the mdr1a cDNA fragment was a gift from Dr O Meiger and was synthesised as described previously in 2.5. These fragments were incorporated into a plasmid which contained RNA polymerase promotors. This enables the transcription of either 'antisense' or 'sense' probes.

2.6.5 Restriction enzyme digestion of plasmids

Plasmid DNA (20μg) was digested with the appropriate restriction enzyme to linerise for the production of either 'antisense' or 'sense' probes (1U/μg, see table 2.1) in a total volume of 100μl for 1hr at 37°C. Digestion of the DNA was confirmed by electrophoresis of 1μl of the digest through a 1% agarose gel (prepared by dissolving agarose (0.5g) in 0.5 x TBE and adding ethidium bromide (1μl/100ml)). The digest

was compared with uncut plasmid and a 1kB DNA ladder containing fragments ranging from 75bp-12kb under UV light at 254nm.

The remaining digest was then purified using a DNA Purification kit (Hybaid, Ashford, Middlesex) and resuspended in DEPC-treated water (50µl). Recovery of the DNA fragment was confirmed by electrophoresis of 1µl of the DNA solution through a 1% agarose gel as described above.

2.6.6 Synthesis of ^{35}S-UTP labeled ribo-probes

For all probes, linear cDNA template (0.5-1µg) was transcribed by incubation with ATP, CTP and GTP (0.3mM each), ^{35}S-UTP (s.a. 800Ci/ mmol), DTT (10mM), RNase inhibitor (0.5µl), and appropriate the polymerase enzyme (1µl) at the optimal transcription temperature for the specific RNA polymerase (see table 2.2), in a final volume of 10µl. Following incubation, DNase 1 (RNase free) (1µl) was added and reactions incubated at 37°C for a further 15min to degrade the DNA template, after which probes were placed on ice for 1-5min and purified using NICK columns (Pharmacia Biotech, Sweden) to remove unincorporated radioactivity. The column was prepared by washing through with TE buffer (3ml). The probe mixture was then applied to the column. The column was washed with TE buffer (400µl) and the initial elutant discarded. Labelled probe was eluted in an additional TE buffer (400µl).

For each probe, the total activity was estimated by counting 1µl of probe in 1ml PicoFluor 40 scintillant fluid (Canberra Packard, UK) in duplicate in a β-counter (minimum activity required $2x10^5$ cpm/µl). The purity of each probe was determined by running 1-2µl on a urea gel (3.6g urea, 1.32ml acrylamide, 0.1% ammonium persulphate (v/v), 10µl TEMED in 1x TBE) and exposing the gel to Kodak Biomax-MR film (HA West Ltd, Edinburgh, UK), which should produce a single black band on the film when developed. Probes were stored at -20°C until required, for a maximum of 7 days.

Fragment	Plasmid	Antisense Enzyme	Sense Enzyme
Mdr1a- 889bp	BS	Not1	EcoR1
GR- 673bp	GEM3	Ava1	EcoR1
MR- 513bp	GEM4	Hind1	EcoR1
11βHSD2- 659bp	GEM-T	Not1	Nco1

Table 2.1: Plasmid and restriction enzyme summary table

cDNA fragments flanked by restriction sites were incorporated into a plasmid. The restriction sites enabled the linearisation of the plasmid using the appropriate restriction enzymes. These enzymes linerised the plasmid in the correct orientation so either the antisense or sense riboprobe was transcribed from the linear cDNA template.

2.6.7 Pre-hybridisation

Following fixation, slides were pre-hybridised with 200µl/ slide of 2x pre-hybridisation buffer diluted 1:1 with deionised formamide, at 50°C for 2hr. Dampening two layers of Whatman No.3 chromatography paper with box buffer humidified the slide boxes, hence preventing tissue sections from drying out.

PROBE	RNA POLYMERASE	OPTIMUM TRANSCRIPTION TEMPERATURE (°C)
mdr1a- as	T3	37
mdr1a- s	T7	37
GR- as	T7	37
GR- s	SP6	40
MR- as	SP6	40
MR- s	T4	37
11β-HSD2- as	T7	37
11β-HSD2- s	SP6	40

Table 2.2: RNA Polymerase conditions for ribo-probe generation

The specific RNA polymerase enzyme required to transcribed individual antisense (as) and sense (s) probes is given above with the optimal temperature at which the enzyme transcribes.

2.6.8 Hybridisation

Sense and antisense probes were thawed and added to 2x hybridisation buffer diluted 1:1 in deionised formamide to give a final probe concentration of $10x10^6$ cpm/ml. Probes were denatured at 75°C for 10min and placed on ice before addition of DTT (10mM). Pre-hybridisation buffer was drained from slides and appropriate probe (200µl) was applied to slides. Slides were hybridised in sealed, humidified boxes at 50°C for an optimum of 16 hours.

2.6.9 RNase treatment and washes

Following hybridisation, slides were washed three times in 2x SSC for 5min and carefully wiped dry around the sections with lens tissue. 200µl RNase A buffer was applied to each slide. Slides were incubated at 37°C for 1 hr in humidified boxes (1 layer of Whatman No.3 chromatography paper dampened with RNase box buffer) to remove unhybridised probe.

2.6.10 Visualisation of Hybridisation

Slides were exposed to Kodak Biomax-MR film for 3-5 days. Afterwards, slides were individually dipped in NTB-2 photographic emulsion (Kodak, UK, diluted 1:1 with DEPC-treated water at 42°C) and exposed in light-tight boxes for 3-4 weeks at 4°C. Slides were developed in D19 solution (HA West Ltd, UK) diluted 1:1 with water at 15°C, fixed in Amfix solution (HA West Ltd, UK) diluted 1:5 with water at 15°C, rinsed in water and counterstained with haematoxylin and eosin.

2.6.11 Quantification of silver grains

The number of silver grains per epithelial cell was measured using the Zeiss KS3000 program. Crypts were arbitrarily divided into three planes (basal, mid and tip), and a total of 30 or more epithelial cells randomly selected from 2 similar sections were counted within the designated areas for each probe per animal. Background measurements were made over adjacent non-expression tissue and all slides were blinded. When measuring the density of the autoradiograph films, images were captured via a digital camera and the optical density (units were given arbitrarily)of whole tissue/epithelial areas was measured, and a average density from 2 tissue sections calculated using the MCID-M4 Image Analysis V.3.0 Rev 1.5 program.

2.7 WESTERN BLOTTING

The quantity of specific protein present in a tissue was determined using Western blotting. Total protein from lysed cells or tissue homogenate was prepared as described below and the protein concentration estimated using Bio-Rad protein assay described in section 2.4.

2.7.1 Preparation of cell lysate and tissue homogenates

To prepare total cell lysate, medium was aspirated after treatment and cells washed with phosphate-buffered saline (1ml). Lysis buffer (500µl/group) was added to the dishes. Cells were scraped and pipetted into eppendorf tubes; cell debris was pelleted by centrifugation at 2000g at 4°C for 5min in a microcentrifuge. The supernatant was frozen at minus 20°C until use.

To prepare nuclear and cytosolic extracts, cells were washed as above and cells detracted by scrapping the monolayer and resuspended in 1ml PBS. Cells were centrifuged at 12,000rpm for 15sec at 4°C, and the pellet resuspended in 400µl of Buffer A and incubated on ice for 15min. Thereafter 7µl of Buffer B was added to the cell suspension and vortex for 15sec, centrifuged at 14,000rpm for 30sec at 4°C and the supernatant collected (cytosolic fraction). The nuclei were resuspended in 50µl of Buffer C and incubated for 20min (vortexing frequently). Debris was collected by centrifuging at 10,00rpm for 5min at 4°C. The supernatant contains nuclear extracts. Both fractions were then frozen at minus 20°C until use.

Tissues were roughly dissected while frozen and 0.5-1g of tissue was added to 1ml homogenising buffer and mechanically homogenised. Homogenates were centrifuged at 2000g at 4°C for 5min to allow any small amounts of unhomogenised tissue to sink to the bottom and the supernatant was removed and frozen at minus 20°C until required.

2.7.2 Separation and transfer of proteins by SDS-PAGE

1mm thick SDS-PAGE gels were prepared in the vertical electrophoresis system (Bio-Rad). 8% resolving gel containing 5.3ml distilled water, 2ml 40% acrylamide, 2.5ml 1M tris pH 8.8, 0.1% 10% SDS, 75µl 10% ammonium persulphate and 10µl TEMED was poured between glass plates to two-thirds of their height. The surface was covered with water-saturated butanol until the gel set, which was then washed out and a 4% stacking gel (7.565ml distilled water, 1.25ml 1M Tris pH 6.8, 1ml 40% acrylamide, 0.1ml 10% SDS, 75µl 10% ammonium persulphate and 10µl TEMED) poured on top, and a comb added.

40µg of protein was diluted 1:4 in 4x Laemmli buffer and denatured for 5min by heating to 95°C and immediately loaded into each well. Samples were electrophoresised in running buffer at 30mA versus molecular weight markers (Bio-rad) until the dye front reached the base of the gel. Proteins are separated according to their weight, with smaller species migrating further over the same period. The resolving gel was removed and the gel pre-soaked in cold transfer buffer, along with ECL blotting membrane, for 15min. Proteins were then transferred to the membranes by electroblotting in cold transfer buffer at 250mA for 3hr. Complete transfer was verified by the loss of marker dyes from the gel.

2.7.3 Blocking membranes & antibody preparations

Membranes were transferred to dishes containing blocking solution and left overnight at 4°C on an orbital shaker to reduced non-specific binding. Primary antibody dilutions (see table 2.3) in blocking solution were added to the membrane for 2hr, followed by 3x 5min washes in blocking solution on the orbital shaker. Secondary antibody dilutions (see table 2.3) in blocking solution were applied to the membrane for 1hr. This was followed by 4x 5min washes in 1x TBS to remove unbound antibody.

A secondary antibody application could be made to the same membrane after the existing antibody complex had been stripped. Blots were incubated for 1hr with

stripping buffer on the orbital shaker, washed 3x 5min in 1x TBS then blocked as before.

Primary Antibody & Dilution		Secondary Antibody & Dilution	
Monoclonal-C219 P-glycoprotein	1:200	Anti-mouse	1:1000
Polyclonal - Glucocorticoid Receptor	1:400	Anti-rabbit	1:1000
Monoclonal -AP-1	1:300	Anti-mouse	1:1000
Monoclonal-NF-κB (p65)	1:300	Anti-mouse	1:1000
Monoclonal IκB-α	1:300	Anti-mouse	1:1000

Table 2.3: Primary and secondary antibody combinations and dilutions

2.7.4 Protein detection and quantification

The antibody complex bound to the membrane was visualised using the ECL chemoluminescence method. Secondary antibodies are attached to a horseradish-peroxidase molecule which catalyses the oxidation reaction of luminol in the presence of hydrogen peroxide and light is emitted. The light produced exposes chemoluminescence-sensitive film in areas corresponding to the specific protein bands on the membrane. 1:1 mixtures of ECL reagent 1 and 2 (Amersham Biosciences) were applied to the membrane for 1min, drained, wrapped in transparent film, and placed under ECL film for a period of time sufficient to obtain visible bands. The film was developed using a hyper-processor. Film images were scanned and the band intensity on the resulting files analysed using AIDA or MCID-M4 Image Analysis programs.

2.8 STATISTICS

All values are expressed as mean ± standard error. Differences in mean values made on two comparable treatment groups were tested by Student's t-test, and between multiple groups by One-Way ANOVA followed by LSD or Tukeys post-hoc comparisons for parametric data. More statistical details for specific experiments are given in individual chapters.

Chapter 3

Expression of genes determining colonic glucocorticoid sensitivity in the rat colon: regional variation and effect of glucocorticoids

3.1 INTRODUCTION

As discussed in section 1.1, GC pharmacotherapy continues to be a mainstay of treatment for inflammatory bowel disorders (IBD), including Crohn's disease and ulcerative colitis (Arnott *et al.* 2003). However, physiological variations in sensitivity to GCs in healthy individuals and the phenomenon of glucocorticoid-resistance in inflammatory disorders such as asthma and IBD have highlighted the importance of tissue sensitivity to GCs in determining biological responses *in vivo*. GC insensitivity poses major problems in clinical practice with 20-40% of patients with IBD developing resistance to GC therapy (Faubion *et al.* 2001). Before we begin to address the mechanisms which underlie steroid resistance in IBD, we first need to investigate colonic expression of key genes involved in steroid sensitivity, in health, and the effect of glucocorticoids on gene expression.

The density of the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) are key determinants of tissue sensitivity, and both are expressed in the colonic epithelium (Sheppard *et al.* 1999; Whorwood *et al.* 1993). These receptors are regulated in a tissue-specific manner by their glucocorticoid ligands, but such control is highly cell and organ specific; for example glucocorticoids have been shown to down-regulate GR in B-cell lymphocytes, but up-regulate the same gene product in T-cells (Denton *et al.* 1993). Although previous studies have been reported (Meyer & Schmidit 1994; Sheppard *et al.* 1999), the fine details of GC regulation of GR and MR within specific regions of the crypts and along the length of the colon is not fully clarified, and yet this knowledge is essential to begin to comprehend its varying tissue GC sensitivity.

Recent observations have suggested that tissue responses to GCs are determined not only by the expression levels of GR and /or MR in particular cells but also by pre-receptor systems that 'gate' ligand access to receptors. Best documented are the actions of the isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD). 11ß-HSD2 is highly expressed in aldosterone-selective target organs such as the distal nephron and colon where 11β-HSD2 ensures that intrinsically non-selective MR bind aldosterone *in vivo* in the face of many-fold excess of circulating GC (Whorwood *et al.* 1993; Whorwood *et al.* 1994). Absence of 11ß-HSD2 leads to illicit occupation of

MR by GCs, which cause the syndrome of apparent mineralocorticoid excess (sodium retention, hypertension and potassium loss).

An additional control of access to specific tissues of synthetic and some physiological steroids is the multi-drug resistance (MDR) gene, which actively transports its substrates, including many GCs and xenobiotics, out of the cell (Endicott & Ling 1989; Fardel *et al.* 2001a; Ueda *et al.* 1992). In rodents mdr1a and mdr1b are involved in steroid transport (Silverman *et al.* 1991; van Tellingen 2001). The development of the transgenic knockout mouse mdr1a-/-, which suffer severe spontaneous intestinal inflammation in germ-free conditions, has provided the strongest evidence to date that mdr1a/P-gp contributes to maintaining a protective barrier in the intestine (Panwala *et al.* 1998). Recent data using a mouse model of colitis, where inflammation was induced by dextran sodium sulphate (DSS) (LIzasa *et al.* 2003), have shown a decrease in P-gp expression within the large intestine, analogous to a decrease in MDR1 mRNA expression (Langmann *et al.* 2004) in patients with UC when inflammation was established. There are also some data to suggest that MDR1 expression (Farrell & Kelleher 2003) may be affected in patients with GC-resistant IBD. Farrell and colleagues have previously shown MDR1 expression in peripheral blood lymphocytes to be elevated in IBD patients resistant to glucocorticoids (Farrell *et al.* 2000).

Although some initial reports of the regulation of each of these key gene products by GCs have been provided, little work has been done to examine their distribution by position in colonic crypts or along the length of the colon. Moreover, details of their regulation by GCs and particularly by synthetic GCs used in pharmacotherapy remain sketchy. Finally, there are no data on their co-ordinated control *in vivo*. In this chapter we address these issues, and this provides the basic platform for the studies in animal models of intestinal inflammation, as discussed in subsequent chapters of this thesis, and for cell culture studies described in chapter 7.

3.2 METHODS

3.2.1 Animals

3.2.1.1 Animals used in preliminary mapping and in glucocorticoid manipulation experiments.

Male Wistar Rats (250-350g) were housed in cages of 6. All were sacrificed by CO_2 asphyxiation and subsequent decapitation. Colons were removed, sectioned into 10 equal length sections (1-10, proximal to distal)(see figure 3.2) and snap-frozen.

For the preliminary mapping experiments a 6-month-old healthy male rat was sacrificed, colon removed and sectioned as described above.

In the dexamethasone treatment experiments, two groups of six animals received bilateral adrenalectomies (ADX), and two groups of six animals underwent sham-operation (Sham). All surgery was carried out under anaesthesia. One group (n=10) underwent no operation (see figure 3.1). 6 animals from each operation condition (Sham/ADX) received a daily subcutaneous injection of dexamethasone (200μg/kg) dissolved in ethanol/saline (et/OH), and 6 animals from each group (Sham/ADX) received vehicle (et/OH) for 1 week. The animals which under went no operation received vehicle. All were sacrificed on day 8. On day 5 tail blood samples were obtained from all animals.

3.2.1.2 Animals used to validate the Western blotting technique

To test the validity of the Western blotting technique 6 animals were ADX; 3 animals were subcutaneously injected with dexamethasone (200μg/kg) dissolved in ethanol/saline (et/OH), and 3 animals received vehicle (et/OH). On day 3 rats were sacrificed as above, trunk blood samples taken, hippocampus, liver and colons removed. Colons were sectioned as described above.

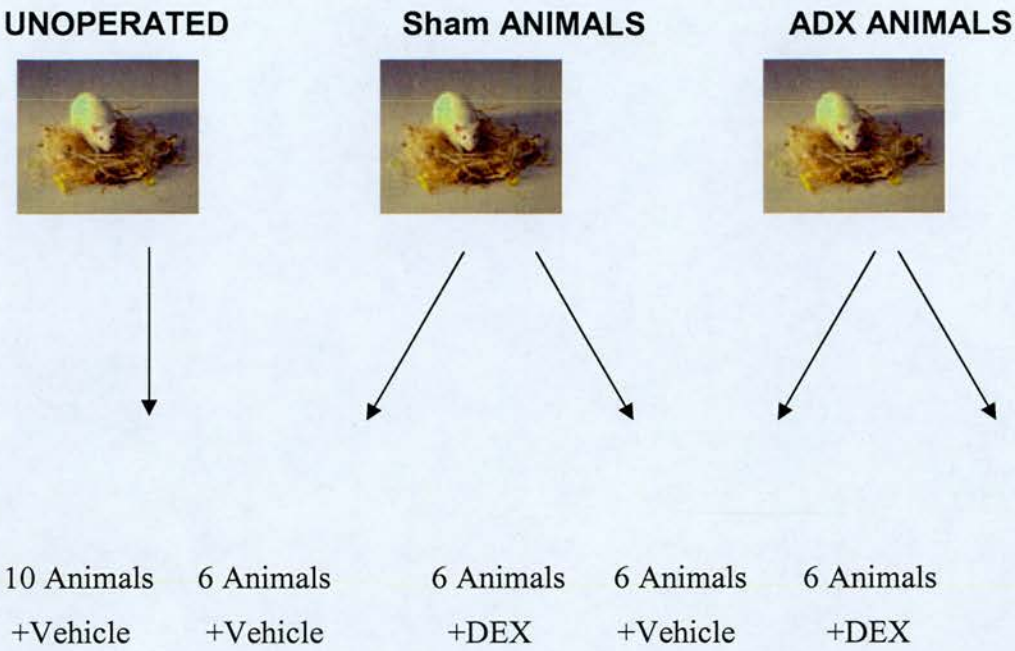


Figure 3.1: Flow Diagram illustrating drug treatments and group numbers for unoperated, Sham-operated and ADX rats.

3.2.2 Radioimmunoassay

To test the efficacy of ADX, corticosterone was measured using plasma previously collected.

3.2.3 *In situ* hybridisation

Colonic sections 1, 4, 7 and 10 (the numbers 1 to 10 represent the number of sections the colon was divided into, where 1 represents the proximal, and 10 the distal colon- see figure 3.2) were cut and mounted onto saline-coated slides and in *situ* hybridisation was performed to analyse mRNA levels using transcribed antisense ^{35}S-UTP riboprobes for GR, MR, mdr1a and 11β-HSD2 as described in section 2.6. For each probe, silver grains/epithelial cell within the axis of whole crypts (tip, mid, base) for each colonic section (1, 4, 7, and 10, proximal to distal colon) were analysed using Zeiss KS3000 program.

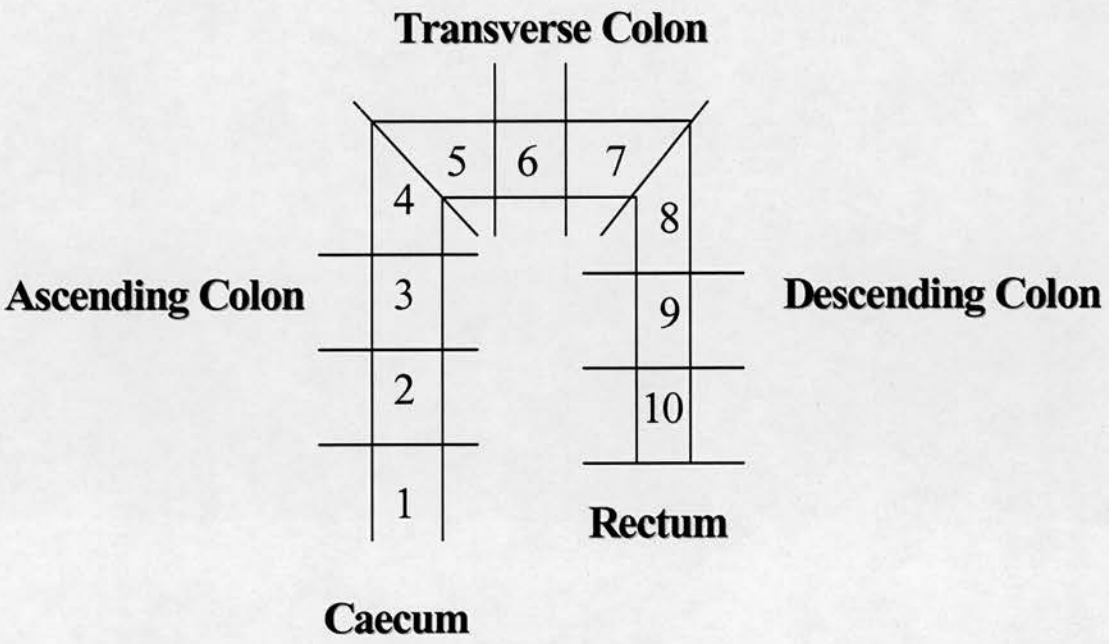


Figure 3.2: Illustration demonstrating the sectioning of the rat colon. Sections 1, 4, 7 and 10 were used to examine colonic expression of genes involved in steroid sensitivity.

3.2.4 Western Blotting

Protein levels were investigated by Western blotting. Protein homogenates from colonic sections 1, 4, 7 and 10 (where section 1 is taken from the proximal colon, and 10 the distal colon) from all animals were prepared and analysed as described in section 2.7. When testing the validity of the blotting method protein homogenates from hippocampus, liver and section 1 of the colon from ADX animals treated with/out dexamethasone were prepared as above. GR levels were investigated using 1:400 dilution of anti-rabbit polyclonal GR antibody (see table 2.3) followed by incubation of a 1:1000 dilution of an anti-rabbit horseradish-peroxidase linked secondary antibody.

Tubulin expression in each sample was measured, and used to control for discrepancies in loading. Blots were stripped in stripping buffer and reprobed with a 1:5000 dilution of a monoclonal tubulin antibody followed by a 1:1000 diluted anti-mouse horseradish-peroxidase linked secondary antibody. All bands were analysed by densitometry using the AIDA program, and results normalised against tubulin.

3.2.5 Statistics

All data are expressed as the mean ± S.E.M. Differences between values were compared using unpaired *t* test (weights) or ANOVA with post-hoc LSD comparisons.

3.3 RESULTS

3.3.1 Mapping the longitudinal and axial distribution of mdr1a, GR, MR & 11βHSD2 along the healthy rat colon

This experiment was used solely as an indicator of expression of these genes within the crypt axis. If an expression gradient was present, differences within the plane of crypt axis were analysed in subsequent experiments.

3.3.1.1 Glucocorticoid Receptor & Mineralocorticoid Receptor

Both GR and MR were widely expressed throughout the colonic epithelium. There were no differences in distribution of GR or MR mRNAs in the epithelial cells within the axis of the crypts (figure 3.3).

3.3.1.2 11β Hydroxysteroid Dehydrogenase 2

As shown in figure 3.3, 11β-HSD2 mRNA was more highly expressed in the proximal than the distal colon. 11β-HSD2 transcripts were more abundant in upper compared to the basal crypts in proximal colon. However in the distal colon (section 10) all epithelial cells within the crypt expressed similar levels of 11β-HSD2 mRNA.

3.3.1.3 Multi-Drug Resistance Gene 1a

Photomicrographs showed that mdr1a expression decreased strikingly from proximal to distal colon (figure 3.3). There was also a differential distribution of mdr1a mRNA within the axis of the crypts, with greater mdr1a mRNA expression at the tip of the crypt in the proximal colon. This differential distribution within the axis of the crypt was not present in the distal colon.

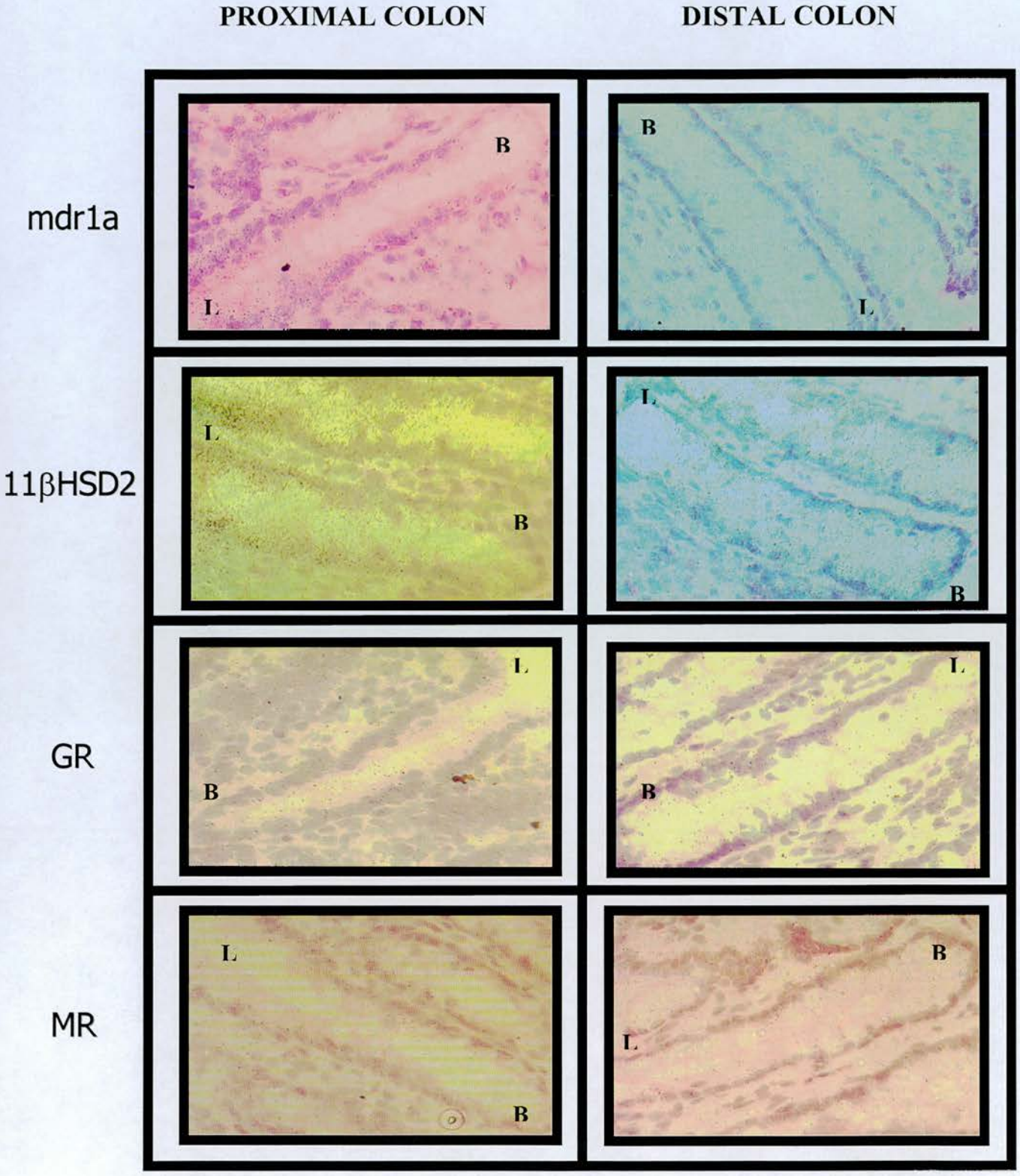


Figure 3.3: Photomicrographs illustrating hybridisation of the antisense probes within the crypt axis in the proximal (left) and distal rat colon (right). mdr1a and 11β-HSD2 had increased expression in epithelial cells at the tips compared to basal crypt in proximal colon. 11β-HSD2 was highly expressed in all epithelial cells in the distal colon unlike mdr1a, where expression was low in all areas of the distal colon. MR and GR was ubiquitously expressed in epithelial cells the proximal and distal colon.

B=Base of Crypt, L=Lumen of Crypt

3.3.2 The effects of GC manipulations on GR, MR, mdr1a & 11β-HSD2 mRNAs along the colon

In all studies of mdr1a, GR, MR and 11β-HSD2 expression, there were no differences between the vehicle treated controls (no operation) and sham-operated groups. This led to the conclusion that acute operative stress and/or anaesthesia did not affect transcription of these genes. As there had been no differences found within the crypt axis for GR and MR mRNA in the mapping experiments in the present experiment, epithelial cells were selected from all areas of the crypt and analysed together. In contrast a gradient in expression had been noted within the crypt for mdr1a and 11β-HSD2 mRNA; in view of this, epithelial cells within the tip, mid and base of the crypt were analysed separately.

3.3.2.1 Animal Weights

Sham/ADX animals treated with vehicle for 1 week had similar levels of weight gain (11% and 9%, respectively), however both Sham and ADX groups gained less compared to vehicle-treated control animals ($p<0.01$ and $p<0.001$, respectively). Sham animals treated with dexamethasone gained 33% less than the vehicle-treated Sham group, and similarly, ADX treated with dexamethasone did not gain weight (figure 3.4).

3.3.2.2 Corticosterone Levels

In unoperated control rats, mean plasma corticosterone levels were 4.4 ± 2.3 μg/dl and were similar in vehicle treated sham animals (6.3 ± 1.6 μg/dl). As expected, plasma corticosterone levels from the adrenalectomised or dexamethasone-treated animals were below the detection threshold of the assay (figure 3.5). Corticosterone measured from plasma samples from the ADX animals used to validate the western blotting method had negligible levels (results not shown).

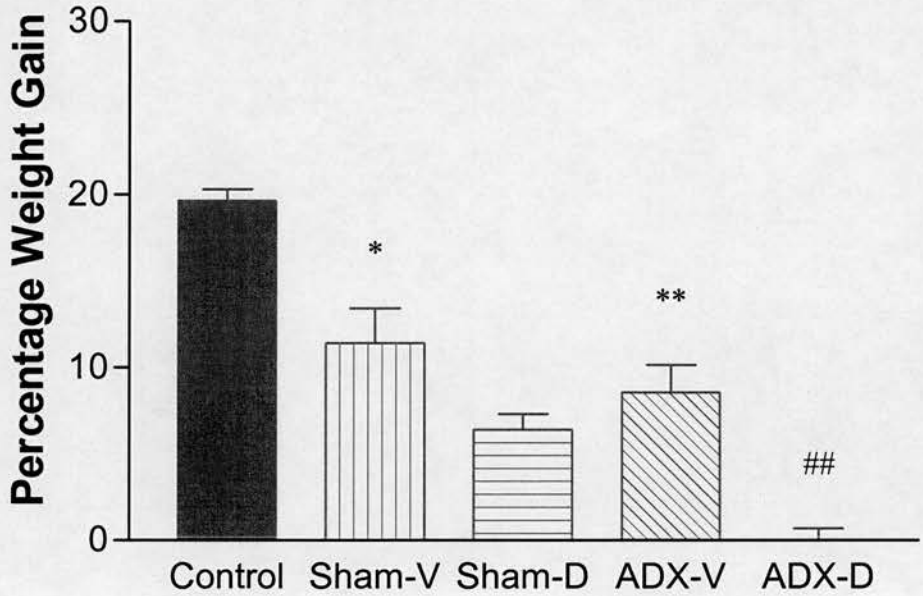


Figure 3.4: Percentage weight gain for each animal group over the treatment period. Operational stress significantly attenuated weight gain in the vehicle-treated Sham (Sham-V) and ADX animals (ADX-V) (*$p<0.01$, **$p<0.001$ respectively) compared to the unoperated control group (Control). Dexamethasone treated Sham animals (Sham-D) were seen to have a 33% reduction in weight gain compared to their vehicle treated group. ADX animals treated with dex (ADX-D) had little/no weight gain (##$p<0.001$).

* compared to control group

compared to vehicle treated ADX group

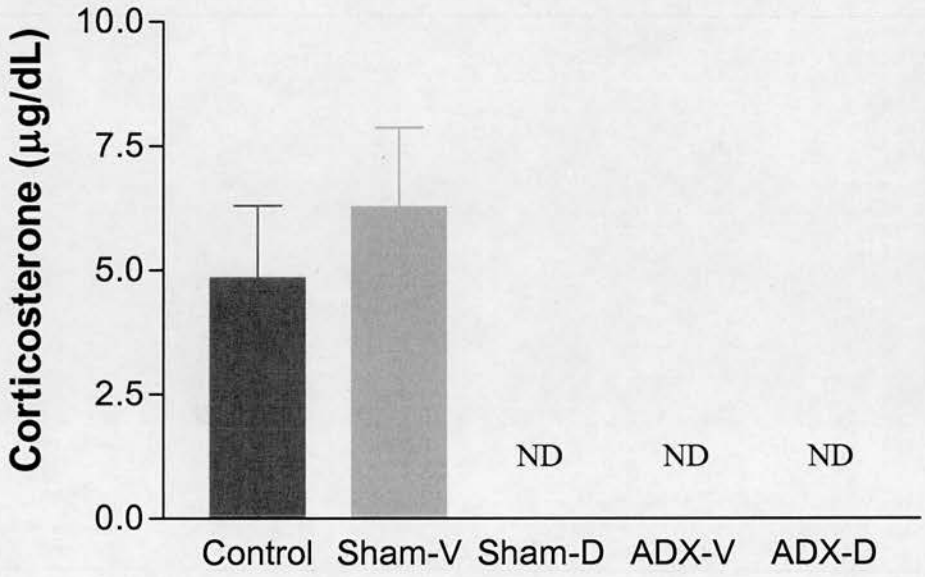


Figure 3.5: Plasma corticosterone concentrations taken on Day 5. Dexamethasone suppressed corticosterone release from the adrenals in the sham-operated animals to undetectable levels. No detectable levels (ND) of plasma corticosterone were seen in the adrenalectomised animals, unlike the vehicle treated control or sham-operated animals.

3.3.2.3 Glucocorticoid Receptor & Mineralocorticoid Receptor

Adrenalectomy increased GR and MR mRNA levels along the colon ($p<0.001$), and the extent of change was similar in all regions of the colon. Dexamethasone given to either sham-operated (Sham-D) or ADX (ADX-D) groups significantly reduced GR mRNA along the colon ($p<0.05$, $p<0.001$ respectively), compared to vehicle treated Sham or ADX animals (Sham-V, ADX-V, respectively) (figure 3.6a). In contrast, colonic MR mRNA levels were unaltered by dexamethasone in any colonic segment in either Sham or ADX groups compared to control animals, although dexamethasone did return the elevated MR mRNA after ADX to control levels (figure 3.6b). There is a slight trend towards an increase in MR/GR ratio within the distal compared to proximal colon (figure 3.7) in vehicle-treated unoperated animals, however this was not statistically significant.

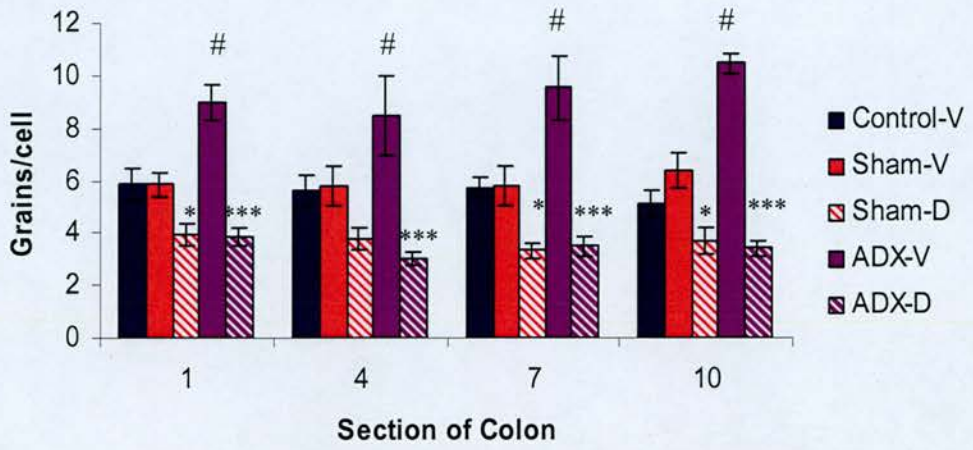
3.3.2.4 11β Hydroxysteroid Dehydrogenase 2

Adrenalectomy significantly increased 11β-HSD2 mRNA expression within all areas of the colonic crypts and all along the colon with values of $p<0.001$, except in the middle and bottom crypt areas in section 4 where the values were $p<0.01$ and $p<0.005$ respectively. The effects of ADX were returned to basal levels by dexamethasone treatment in all regions (figure 3.8).

3.3.2.5 Multi-Drug Resistance Gene 1a

Dexamethasone treatment in ADX and Sham animals (ADX-D, Sham-D respectively) reduced mdr1a mRNA in all colonic areas. ADX-D animals had significantly reduced mdr1a mRNA within the tip of the crypts in colonic section 1 and 4 ($p<0.05$) and also within the basal crypt area of section 1 of the colon. There was also significantly reduced mdr1a mRNA expression in the distal area of the colon (section10) in the tip ($p<0.005$) mid and basal areas of the crypt ($p<0.001$). A trend towards increased expression in ADX-V compared to Sham-V animals was seen in all areas of the crypt and colon, and it should be noted the most significant reductions by DEX occurred in ADX-D opposed to Sham-D animals (figure 3.9).

a)



b)

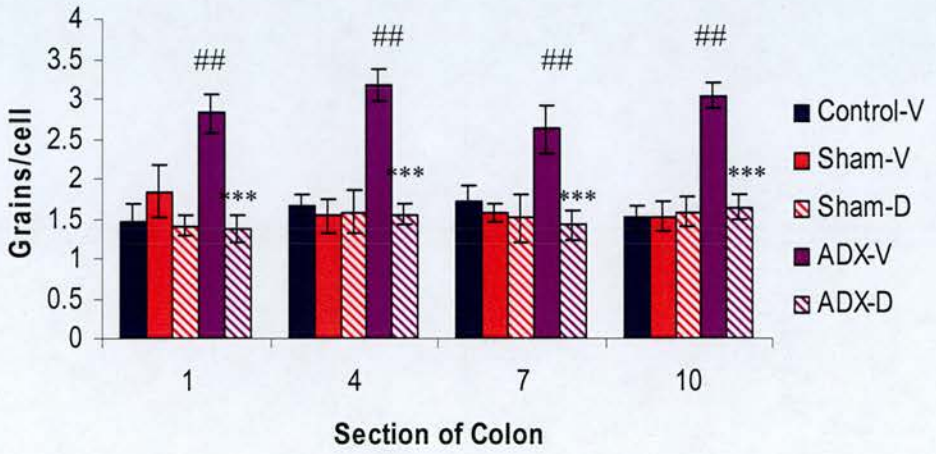


Figure 3.6: Dexamethasone treatment of Sham/ADX animals significantly reduced GR mRNA levels in epithelial cells along the entire length of the colon (sections1-10, proximal to distal) compared to Sham/ADX animals given vehicle (*p<0.05, **p<0.005, ***p<0.001). Vehicle treated ADX animals compared to control animals given vehicle had a significant increase in expression in all areas of the colon (#p<0.001). (b) MR mRNA expression was unaffected by dexamethasone, although adrenalectomy did increase expression within epithelial cells along the colon which was returned to baseline by DEX (#p<0.01, ##p<0.001).

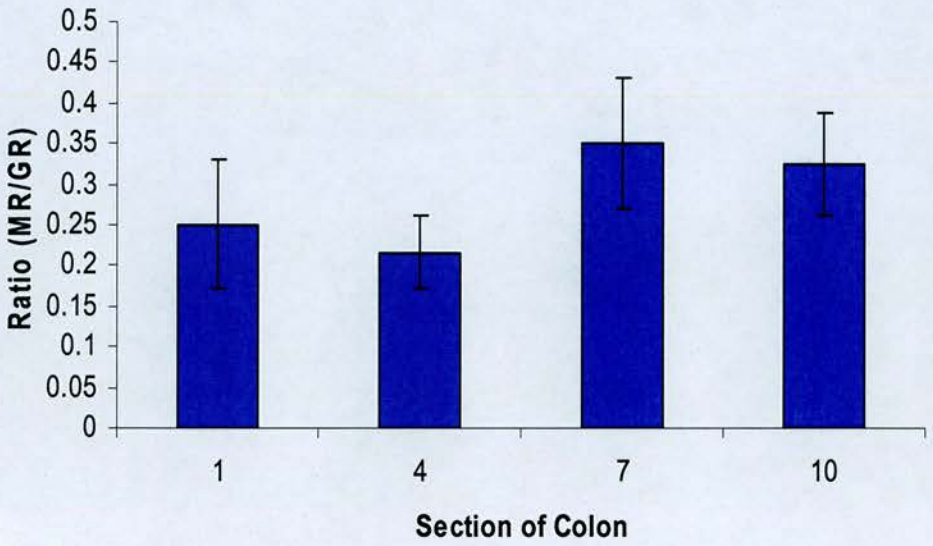
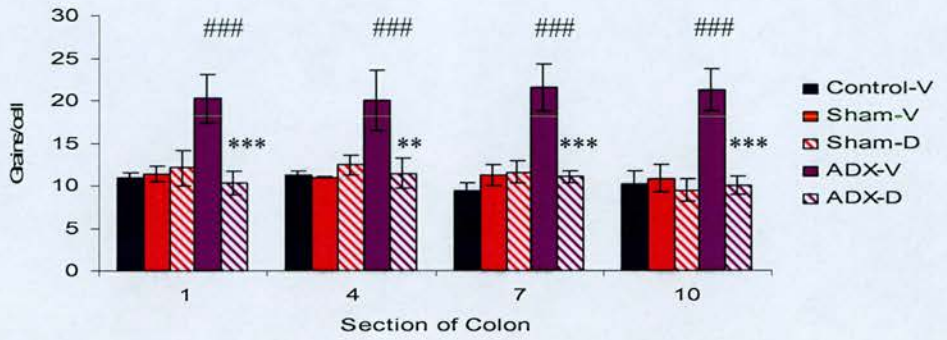
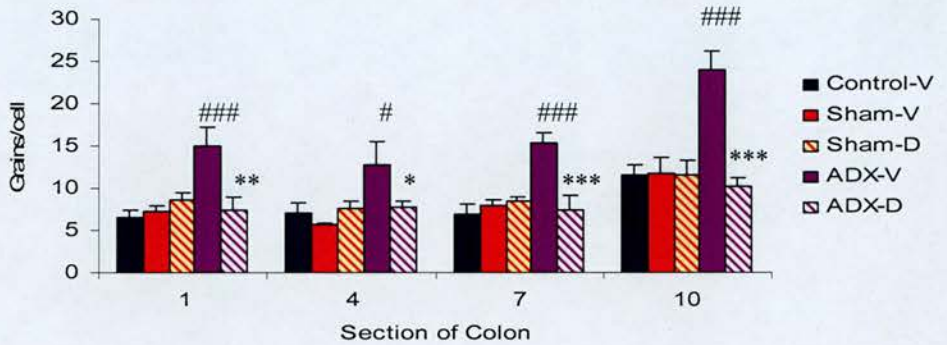


Figure 3.7: Graph illustrating the MR/GR mRNA ratio along vehicle treated-unoperated healthy rat colon.

a)



b)



c)

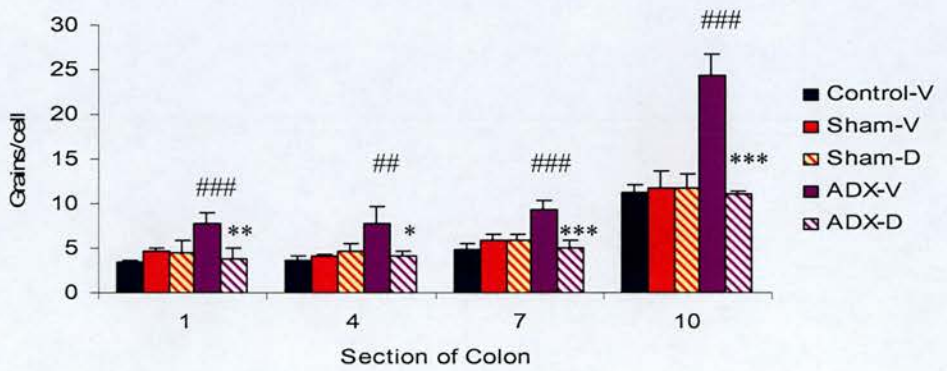
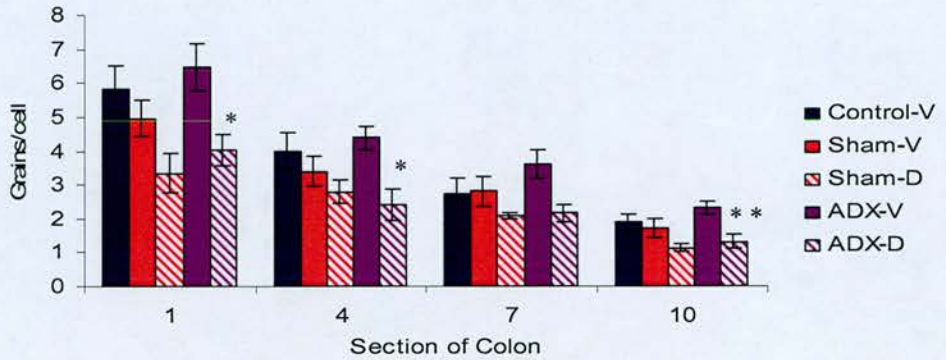
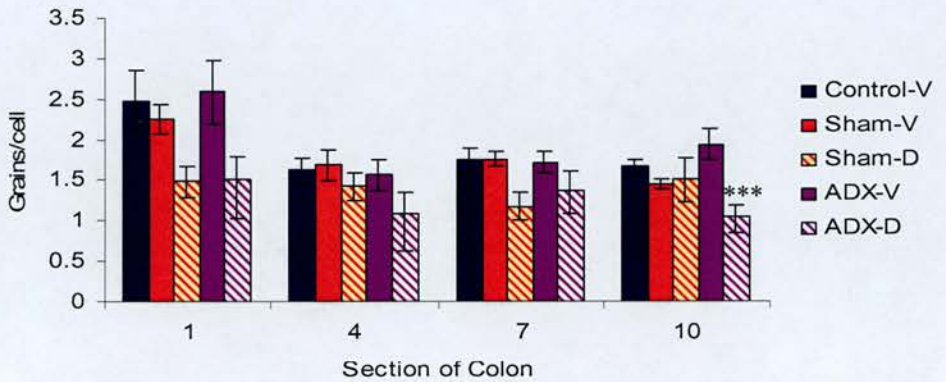


Figure 3.8: Graphs illustrating 11β-HSD2 expression in epithelial cells within the tip a), mid b) and basal c) areas of the colonic crypt. All epithelial cells within the crypt axis and along the colon (sections1-10, proximal to distal), has increased 11β-HSD2 levels in the vehicle treated ADX group compared to control animals (#$p<0.01$, ##$p<0.005$, ###$p<0.001$). This increased was return to basal levels by DEX (*$p<0.05$, **$p<0.005$, ***$p<0.001$).

a)



b)



c)

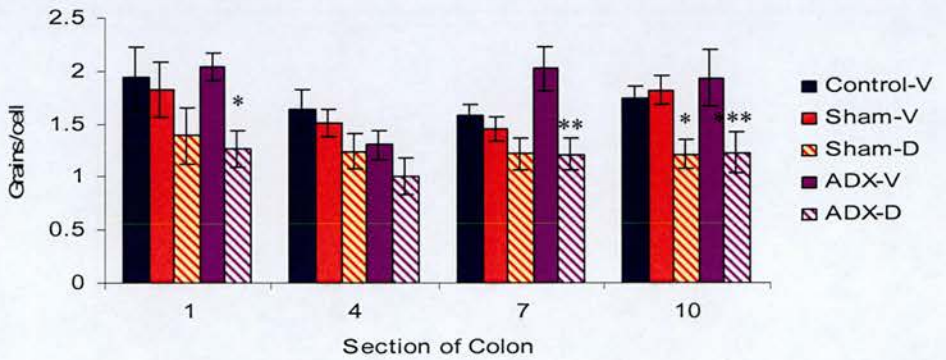


Figure 3.9: Graphs illustrating that dexamethasone-treated animals compared to vehicle-treated Sham/ADX group significantly decreased the expression of mdr1a mRNA in epithelial cells within the tip a), mid b) and basal c) areas of the crypts along the colon (section 1-10, proximal>distal), markedly in the proximal colon (section 1) where expression was highest (*$p<0.05$, **$p<0.005$, ***$p<0.001$).

3.3.3 Effects of GC manipulation on GR protein expression

A power failure was responsible for the defrosting of the freezer containing all colonic samples and therefore these samples had been freeze-thawed prior to Western analysis. Western blots performed on proximal and distal sections of the colon from 4 animals from each group (ADX, Sham and control animals treated with/out dexamethasone) produced inconsistent results (data not shown). It was hypothesised that the inconsistent results were due to protein degradation during this period. To ensure the method was viable and inconsistencies in protein levels within each group were due to degradation, fresh protein samples from hippocampus, liver on proximal colon were analysed (see section 3.1.1.2). Table 3.1 shows a significant decrease in hippocampal GR levels in dexamethasone-treated animals ($p=0.034$). There is also a trend towards a decrease in colonic GR protein (table 3.1) in response to dexamethasone, unlike in liver where GR levels seem to be unchanged (table 3.1).

In the following chapter, colonic GR protein and P-glycoprotein expression was analysed using tissue homogenates from vehicle-treated Wistar rats used in subsequent experiments, and therefore further supports the hypothesis that the inconsistent western results noted in the present chapter must have been due to protein degradation.

TISSUE HOMOGENATE	VEHICLE TREATMENT (Optical density ± SEM)	DEXAMETHASONE TREATMENT (Optical density ± SEM)
HIPPOCAMPUS	2.392 ± 0.1763	1.088 ± 0.3717
PROXIMAL COLON	2.912 ± 0.9408	2.274 ± 0.7945
LIVER	2.248 ± 0.6155	2.398 ± 1.097

Table 3.1: Optical density of GR protein expression in rat tissues.

GR protein expression in hippocampal, proximal colon and liver homogenates were analysed by Western blotting. GR expression in the hippocampus significantly decreased after 3 days dexamethasone treatment ($p<0.034$). In the proximal colon GR protein levels were seen to decrease, however this was not significant, and GR protein expression was unaltered in the liver after DEX treatment.

3.4 DISCUSSION

3.4.1 Mapping the longitudinal and axial distribution of GR, MR, 11βHSD2 and mdr1a along the healthy rat colon:

The colon represents a key target tissue for corticosteroid actions. GR stimulates electroneutral NaCl absorption and inhibits electrogenic Na absorption, whereas MR induces the opposite effects (Meyer & Schmidit 1994). Thus each receptor produces overlapping as well as distinct physiological effects. Our data show that both that GR and MR are ubiquitously expressed in the epithelial cells along the colon, implying that the colon is a major site for corticosteroid affects. The activity of steroids in the colon is likely not only is to involve maintenance of salt and water balance (GR and MR) but also involve immuno-regulation (predominantly GR) since the colon in contact with a large population of luminal immunogens. The greater MR/GR ratio distally may facilitate the predominant mineralocorticoid actions (salt retention, potassium loss) in the distal colon to mirror effects seen in the distal nephron (Escoubet *et al.* 1996). Indeed, in the hippocampus, another structure with high expression of both GR and MR, the MR/GR ratio appears key to determining the nature of target genes regulated by the two receptor subtypes and hence overall GC function (de Kloet *et al.* 1998).

Unlike the hippocampus which has no 11ß-HSD2 (Li *et al.* 1996), the high levels of the 11β-HSD2 in the colon suggests it engenders the known aldosterone selectivity of the rat colonic MR (Whorwood *et al.* 1993). Our data illustrate increased expression of the enzyme in the distal colon, concurring with the higher MR/GR ratio in this locus. This presumably ensures the documented maximum mineralocorticoid effects (absorption of fluid and salts) distally, as occurs in the distal nephron (Bocchi *et al.* 2003). Here however, like in hippocampus perhaps, it may be the ratio of MR/GR that also drives the effects of specific corticosteroids. The increased levels of 11ß-HSD2 at the tips of the crypts in the proximal colon may reflect the increasing differentiation of the apical cells (Pácha *et al.* 2002), or simply an increased exposure to electrolytes at this site.

The present investigations have also provided the first evidence for the regional distribution of mdr1a mRNA expression along the rat colon, showing higher levels in the proximal colon. The explanation behind the colonic distribution of mdr1a remains uncertain. Particularly in the proximal colon, bacterial growth is rapid and there is also an abundance of substrates for metabolism by bacteria which generate toxins. The higher levels of mdr1a expression may help protect colonic tissue from these substances (Guarner & Malagelada 2003). In contrast to the present results, a study in mice showed higher mdr1a protein expression in the distal compared to the proximal colon (Stephens *et al.* 2002). These conflicting results might be explained by differences between species, by differential effects of xenobiotics substrates on the various mdr subtypes, or simply by differences between protein and mRNA levels due to post-translational effects.

The differential distribution of mdr1a mRNA expression within the crypts, with highest expression in the tips in proximal colon, supports previous immunohistochemistry studies in healthy human colonic tissue (Meiger *et al.* 1999). These data are likely to reflect greater epithelial cell differentiation in the upper crypt compared with the basal layers, though why more differentiated epithelia require greater mdr1a expression is unclear. One possibility is the cells within the tips of the crypts simply have the greatest xenobiotic exposure. If this were the case, it would also support our11ß-HSD2 data where increased expression was noted within this area of the crypt, and help clarify previous studies implicating this enzyme in the detoxification of xenobiotics (Maser & Bannenberg 1994).

3.4.2 GC manipulations in the healthy colon:

3.4.2.1 Effects of GC manipulations on GR and MR expression

Our results suggest that colonic GR is under autoregulatory (negative) control by basal levels of endogenous GCs, effects reversed with dexamethasone 'replacement' (GR mRNA was increased after the removal of endogenous corticosteroids by adrenalectomy, but was reduced when DEX was given to ADX animals), confirming previous data (Meyer & Schmidit 1994). The changes in GR were not due to surgical stress. Presumably such autoregulatory control adjusts GR levels to modulate the

signal to the colon from widely varying circulating GC levels in response to stress, diurnal cues, etc. The implication is that chronic GC pharmacotherapy may partly de-sensitise the whole colon to GC action, presumably with the balance of GC efficacy determined by the individual product of GC levels and local GR density.

In contrast, although ADX increased colonic MR mRNA, there was no reversal with the GR agonist dexamethasone, mirroring differential control in the hippocampus over this timescale (Meyer & Schmidit 1994). Experiments in the hippocampus have illustrated that the analogous increase in MR mRNA after ADX is reduced to base levels by reintroducing GCs (GR agonists) but not aldosterone (MR agonist) (Holmes *et al.* 1995). The explanation for these observations is unclear. It is possible that the increase in colonic MR mRNA with ADX may in fact be indirectly mediated rather than being directly regulated by GCs. The present observations may thus reflect a mechanism by which colonic cells can maintain control of local salt and electrolyte homeostasis despite being exposed to varying concentrations of endogenous and exogenous GCs.

3.4.2.2 Effect of GC manipulations on 11β-HSD2 expression

A novel finding is that exogenous and endogenous GCs regulate colonic 11β-HSD2 gene transcription as ADX increased enzyme mRNA levels, a change reversed with dexamethasone. GC-mediated down-regulation of 11ß-HSD2 has not ubiquitously been noted in other tissues expressing this isozyme. GC-driven down-regulation of 11ß-HSD2 favours increased GC sensitivity by reducing inactivation of active GC substrates including dexamethasone (Li *et al.* 1996; Whorwood *et al.* 1993).

3.2.2.3 Effect of GC manipulations on mdr1a expression

There remains intense interest in the importance of P-glycoprotein in the gastrointestinal tract. Langmann and colleagues have shown MDR expression to be reduced in patients with UC and this mirrors data from animal models of colitis where a decrease in mdr1a expression was noted (Lizasa *et al.* 2003). The pregnane X receptor (PXR), a transcription factor essential for controlling xenobiotic metabolism

and known for controlling MDR transcription (Ho *et al.* 2003; Langmann *et al.* 2004), has been implicated in the regulation of MDR in patients with UC (Langmann *et al.* 2004). This study illustrated a decrease in PXR mirroring a reduction in MDR in these patients. A decrease in PXR and P-glycoprotein expression may increase xenobiotics exposure in intestinal epithelial cells, consistent with data implicating P-gp as a protective barrier in the intestine. This hypothesis is further supported by studies in mdr-/- mice, where all animals develop intestinal inflammation (Panwala *et al.* 1998).

Here we show that dexamethasone decreases expression of mdr1a mRNA in the healthy colon. The trend of increased mdr1a mRNA expression after adrenalectomy compared to Sham-operated animals, and significant reductions only noted in dexamethasone treated ADX animals opposed to dexamethasone-treated Sham animals, implies a role for endogenous GCs in the regulation of mdr1a, supporting *in vitro* studies (Li *et al.* 1999). The reduction in mdr1a with dexamethasone complements results seen in cultures of rat hepatocytes (Fardel *et al.* 1993), but contrasts with observations in hepatoma cell lines (Zhao *et al.* 1993), healthy liver (Demeule *et al.* 1999), and also work in chapter 7 using IEC-6 cells where dexamethasone increased P-glycoprotein expression. There are a number of possible explanations for these discrepancies, which may reflect differences in cell culture methods, tissue specific regulation of mdr1a and/or differential regulation in health and disease. Recent data has shown dexamethasone treatment did not affect mdr1a mRNA expression in the rat colon (Mei *et al.* 2004), however this could simply be due to treatment duration as we treated with DEX for 7 days whereas Mei and colleagues treated for 3 days. Therefore Mei and colleagues may not have treated long enough to induce changes in expression of mdr1a mRNA. Also, dexamethasone was given orally at 1 and 20mg/kg/day. The cellular concentration of dexamethasone may be different in these animals compared to those animals given dexamethasone subcutaneously, and may therefore explain the differences noted between studies.

Intriguingly, mdr1a is induced by the 'inflammatory' transcription factors AP-1 (Ikeguchi *et al.* 1991; Teeter *et al.* 1991) and NFκB (Thevenod *et al.* 2000). GCs alter the expression and function of both transcription factors in a variety of settings (Barnes 1998). Hence in the colon dexamethasone may prevent AP-1 and NFκB from

initiating transcription of the mdr1a gene. In consequence, dexamethasone could reduce drug/xenobiotic exclusion from colonic epithelia and ultimately increase its own pharmacological effect within cells. Indeed, in the healthy colon, dexamethasone appears to both potentate its pharmacological effect by decreasing both 11β-HSD2 to basal levels and also reduce the efflux of GCs via mdr1a. As noted above, our data suggest the former predominates distally and the latter proximally along the colon. Against this, reduced GR may attenuate signal. Further studies of target gene effects are needed to dissect the overall balance in specific subregions of the colon in health and disease.

The limitations of this study are principally the lack of parallel protein data due to the freeze-thawing of the samples. However the western blotting results from adrenalectomised rats treated for 3 days with dexamethasone indicate a significant decrease in GR protein levels in the hippocampus, and less evidently in the colon. This complements previous studies in hippocampus (O'Donnell *et al.* 1995), and therefore discrepancies in western blotting data from the rat samples were due to protein degradation and not blotting technique. The reduction in colonic GR protein mirrors our mRNA data, although the reduction in protein was not significant. This could be solely due to differences in treatment length between experiments, and if dexamethasone treatment had been for 1 week compared to 3 days then GR may have been decreased significantly. The lack of effect of DEX in liver samples could also be explained by the short treatment length as previous work has shown liver GR decreases after one week of dexamethasone.

In conclusion, mdr1a and 11β-HSD2 mRNA expression is highest within the colonic crypt and decreases longitudinally along the healthy rat colon. In contrast, GR and MR are uniformly distributed throughout the crypt axis. Dexamethasone regulates mdr1a, 11ß-HSD2 and GR, but not MR mRNA expression. These changes in expression may influence the action of GCs in the healthy rat colon, and may even have implications for disease states where DEX treatment is effective.

Chapter 4

Effect of altering bacteria flora on the expression of P-glycoprotein and the Glucocorticoid Receptor along the healthy rat colon

4.1 INTRODUCTION

The bacterial flora in the intestine plays an important role in the development and maintenance of homeostasis in the gut. The flora are involved in metabolism of non-digestible food residues as well as the regulation of mucus production, protection against invasive pathogens, epithelial cell proliferation and differentiation (Guarner & Malagelada 2003). The mucus layer covering the intestinal epithelium prevents the influx of bacterial products and toxins, and it is thought that a defect in this barrier may facilitate the penetration of pathogenic bacterial substances which activate the mucosal immune system (Garcia-Lafuente *et al.* 2001).

The importance of bacteria in the initiation of inflammation has been illustrated in genetically-engineered models of intestinal inflammation, as well as in other induced models of colitis. Animals in germ-free conditions do not develop inflammation, whereas in specific-pathogen free condition inflammation develops (Sartor 2004a). Noteworthy, exceptions to this paradigm include the IL-2 knockout mouse model in which animals still show signs of mild inflammation in germ-free conditions; and also the dextran sodium sulphate (DSS)-induced colitis model, in which animals develop aggressive inflammation in a sterile environment (Sartor 2004a; Sartor 2004b). Antibiotics have been shown to prevent and treat inflammation in both rat and mouse models of intestinal inflammation (Sartor 2004b). Broad spectrum antibiotics (vancomycin and imipenium) can prevent disease in animals when administered prior to bacterial colonisation (Sartor 2004a) and also reverse established intestinal inflammation in both HLA-B27 transgenic rats and IL-10 knockout mouse animal models. Importantly, studies in IL-10 knockout mice have also shown selective-spectrum antibiotics used to treat aerobic and anaerobic bacteria (ciprofloxacin and metronidazole respectively) differentially reduced inflammation in the caecum and colon respectively (Hoentjen *et al.* 2003). This study emphasises that different subsets of bacteria may be involved in initiating inflammation in particular regions of the intestine.

The body has a complex mucosal defence system which protects the intestinal epithelium from potentially harmful substances in the luminal contents. The

epithelium functions both as a barrier and as a detoxifying organ. To do so, the intestinal epithelium secretes substances which reduce the access of potentially toxic substances to cells; has a number of effective rapid repair mechanisms after injury; and of great importance, is capable of transporting substances back into the lumen. Foreign substances (xenobiotics) which penetrate the cells undergo a process of biotransformation to less-toxic substances prior to excretion (Meddings *et al.* 2003). A number of molecular mechanisms are involved in this process, most notably the Pregnene X Receptors (PXR-which recognises foreign material); metabolising enzymes (such as cytochrome P450, glutathione-S-transferases which breakdown xenobiotics into less harmful substances); and transporter pumps (which excrete xenobiotics and their metabolites of out the cells) (Dietrich *et al.* 2003). Xenobiotics and steroidal drugs such as dexamethasone, have been shown to increase intestinal and hepatic expression of the metabolising enzyme - cytochrome P450 in rats and humans as a result of PXR activation (Geick *et al.* 2001; Hartley *et al.* 2004; Kliewer *et al.* 2002; Langmann *et al.* 2004; Pascussi *et al.* 2000; Watkins 1997). Recent data have also shown intestinal MDR1 to be regulated by PXR activation (Langmann *et al.* 2004). Therefore a mechanism is present to protect against and eliminate xenobiotics and pharmacological drugs from the body.

Previous animal studies have shown that colonisation with specific bacteria differentially regulate expression of mdr1a mRNA and the metabolising enzyme glutathione-S-transferase (GST) mRNA in the mouse intestine (Hooper *et al.* 2001). Germ-free (GF) mice colonised with *E. coli* and *B. infantis* were characterised by an increased mdr1a and GST mRNA expression; however no increase was noted after GF mice were conventionally raised or recolonised with microflora (Hooper *et al.* 2001). Data in the previous chapter suggest an increase in mdr1a mRNA in proximal compared to distal rat colon mirrors bacterial density. This provides indirect evidence that bacterial flora may regulate mdr1a expression. Intestinal xenobiotic metabolism, and subsequent expression of mdr1a, may be altered due to differences in the composition of bacterial flora.

As discussed previously, GR has been shown to be involved in the production of anti-inflammatory cytokines as well as inhibiting the pro-inflammatory transcription factor NF-κB (Barnes 1998). A defect in GR, whereby the receptor has reduced

affinity for dexamethasone, has been implicated in steroid insensitivity and therefore reflects the importance of this receptor in the anti-inflammatory process (Farrell & Kelleher 2003). As bacteria initiate inflammation, it would be hypothesised that colonisation may increase expression of GR as a counter-regulatory mechanism. However, previous studies have shown endotoxin down-regulates GR in the liver, lung and heart (Webster & Sternberg 2004). These data imply bacterial colonisation maybe reduce GR expression in the colon.

The experiments described in the present chapter have investigated whether a reduction in bacterial load following treatment with a combination of the antibiotics ciprofloxacin and metronidazole may alter colonic expression of GR and mdr1a/P-glycoprotein. It was hypothesised that reduced bacterial flora and therefore xenobiotic production would be accompanied by a reduction in PXR activation and a decrease in mdr1a/P-glycoprotein activation and subsequent expression. As bacterial colonisation seems to down-regulate GR expression, reducing bacterial load would increase colonic GR expression.

4.2 METHODS

4.2.1 Animals

12 male Wistar rats (300-350g, of 12 weeks of age) were housed in 4 cages, each containing 3 animals. Six rats received vehicle, and 6 received a suspension of 2 oral antibiotics and 1 antifungal in Ribena for 1 week. The antibiotic suspension was administered in Ribena to mask the taste of the drugs, encourage drinking and prevent dehydration. The suspension consisted of 125mg/l ciprofloxacin, 500mg/l metronidazole and 2.5g/l amphotericin. These antibiotic concentrations had previously been shown to modulate bacterial load in mice, and amphotericin was used to prevent colonic recolonisation by fungi (Panwala *et al.* 1998; Velder *et al.* 2003). All rats were given fresh Ribena +/- antibiotic suspension every 2 days, and bottles were weighed to ensure ingestion of the drugs. On day 8, all were sacrificed by CO_2 asphyxiation and subsequent decapitation. Colons were removed, sectioned into 10 equal length sections (1-10, proximal to distal colon) and snap-frozen.

4.2.2 Bacterial Analysis

4.2.2.1 Sample preparation

Fresh faecal samples were taken before and after antibiotic treatment and immediately placed in an air-tight container. Samples were homogenised in pre-reduced nutrient broth (2g/ml) as soon as possible after collection.

4.2.2.2 Plating and identification of bacteria

To identify both aerobic and anaerobic bacteria, samples were incubated in both conditions on different agar types to ensure optimal growth of all bacteria present in the faecal homogenate. See table 4.1. Blood agar comprised of 1% Columbia agar base (Oxoid) with 5% defibrinated horseblood (Oxoid Limited, Hampshire, UK)

added, and MacConkey agar plates comprised of 1.2% MacConkey agar base (Oxoid Limited, Hampshire, UK). Agar was sterilised using an autoclave, and allowed to cool to 50°C prior to the addition of the supplements. Agar was poured into plates and left to set. Once set the surface was dried and anaerobic plates were placed and an anaerobic chamber overnight.

The following day samples were collected, homogenised as in section 2.10.1 and a standard loop-full plated onto the agar plates using a standard plating technique. Aerobic and anaerobic plates were incubated at 37°C for 24/48hr respectively and colonies analysed by Mr Robert Brown, Department of Medical Microbiology, University of Edinburgh.

4.2.3 *In situ* hybridisation

Colonic sections 1, 4, 7 and 10 were cut and mounted onto silane-coated slides and *in situ* hybridisation performed to analyse mRNA levels using transcribed antisense ^{35}S-UTP riboprobes for GR and mdr1a as described in section 2.6. Expression was analysed by measuring autoradiograph films, where the optical density (units were given arbitrarily) was measured for whole tissue (GR) or epithelial areas (mdr1a) using the MCID-M4 Image Analysis V.3.0 Rev 1.5 program.

Agar Type	Growth Condition	Bacteria Selected
Blood agar	Aerobic	Total aerobic and facultative bacteria
MacConkey agar	Aerobic	Coliforms lactose and non lactose fermenting eg *E.coli*
Blood agar	Anaerobic	Anaerobic and facultative bacteria
Blood agar plus gentimicin	Anaerobic	Anaerobic bacteria

Table 4.1 illustrating bacterial selection using different growth conditions.

4.2.4 Western Blotting

Protein levels were measured using Western blotting. Colonic sections 1, 4, 7 and 10 were homogenised and analysed as previously described in section 2.7. GR and P-glycoprotein levels were analysed using the sample antibody preparations as shown in table 2.3. Loading discrepancies were controlled by measuring tubulin in each sample, using the same antibodies and dilutions as described in section 2.3. All bands were analysed by densitometry using the MCID-M4 Image Analysis programs and results normalised against tubulin expression.

When analysing the gradient of P-glycoprotein and GR along the colon of vehicle-treated animals, protein homogenates from colonic sections 1, 4, 7 and 10 from 2 different animals were run together on one gel. This was repeated so the colonic gradient from 4 animals were analysed.

When investigating the effect of altering bacterial load on P-glycoprotein and GR expression, protein homogenates from 3 vehicle- and 3 antibiotic-treated animals from the same colonic section were run together on one gel. All 4 colonic sections were simultaneously run and analysed together. This was repeated so all 6 animals were analysed.

Please note that although the same samples (animals given vehicle) were used to analyse the gradient of GR and P-gp along the colon as well as investigating the effect of reduced bacterial load in Wistar rats, as the samples were run on separate gels and developed on different films, direct comparisons between the results cannot be made. This is due to differences in the transferring stage and film developing stages of Western blotting technique; the gels were not simultaneously transferred, and therefore differences in the amount of protein transferred may have occurred. Also, in the film development stage, each film would have been developed for differing lengths of time causing differences in film densities. Therefore as the conditions could not be similar for each experiment, direct comparisons cannot be made.

4.2.5 Statistics

All results are expressed as the mean ± S.E.M. Differences between values were compared using one-way ANOVA with post-hoc Tukey's pair-wise comparisons when analysing three or more groups. Unpaired Students t-tests were performed when analysing differences between two groups. Correlation values were measured using linear regression. Values of $p<0.05$ were taken as significant.

4.3 RESULTS

4.3.1 Average Fluid Intake

Figure 4.1 illustrates the average fluid intake per rat per day for each treatment. It was assumed that all rats per cage had equal fluid intakes. For the vehicle-treated group, it was estimated each rat consumed 66mls/day. For animals drinking the antibiotic/fungal solution, rats consumed an average of 33mls/day.

4.3.2 Bacterial Flora Alteration by Oral Antibiotics

Faecal samples analysed before drug treatment was commenced showed no differences in bacterial content between all animals. Mr Robert Brown from the Medical Microbiology department, University of Edinburgh gram-stained bacteria and concluded that after drug treatment, ciprofloxacin cleared gram negative facultative bacteria including *Escherichia coli* and *Proteus* from the gut, although gram positive cocci such as *Lactobacilli* and *Bifidobacteria* were still present. Metronidazole cleared the gut of anaerobes, with the notable exception of a *Bacteroides* species.

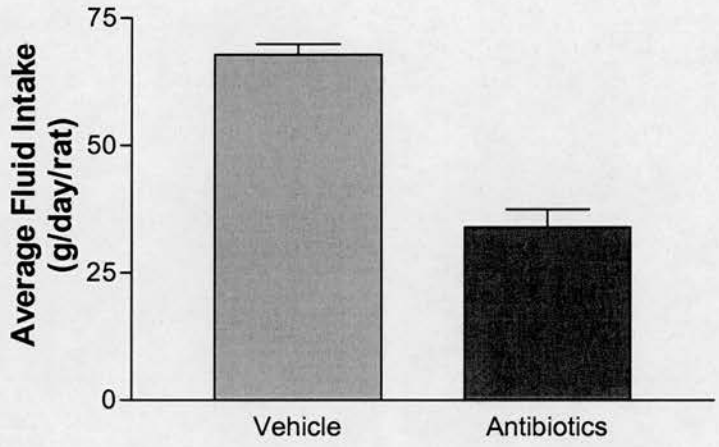


Figure 4.1: Average daily fluid intake for vehicle- and antibiotic-treated animals. As there were 3 animals per cage, and it was assumed all had equal fluid intakes, vehicle-treated animals drank approximately 66mls/day, and 33mls/day for the antibiotic/antifungal-treated animals.

4.3.3 Colonic gradients of mdr1a/P-glycoprotein and GR expression in healthy rats

In the previous chapter mdr1a and GR mRNA expression along the colon in the Wistar rat was investigated as well as MR and 11β-HSD2 mRNA expression. However, protein data could not be studied as protein degradation had occurred due to a power failure affecting the laboratory freezer, and all tissue samples had been freeze-thawed. As a similar strain of rat was used in the antibiotic experiment described in the present chapter, colons from vehicle-treated animals were taken to study P-glycoprotein and GR expression along the healthy colon and investigate whether mRNA and protein expression correlate. MR and 11β-HSD2 protein expression was not studied as these genes are not known to be involved in the inflammatory/disease process.

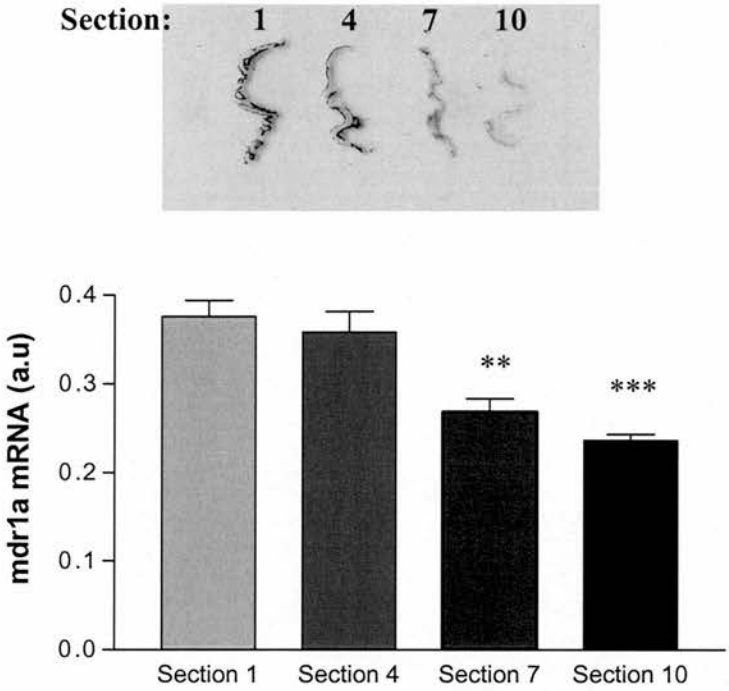
4.3.4 mdr1a and P-glycoprotein gradient along the colon

Figure 4.2 shows mdr1a mRNA and P-glycoprotein expression along the rat colon in vehicle treated animals. A gradient for both mRNA and protein expression along the colon was present; decreasing from proximal to distal colon, as noted previously in chapter 3. mdr1a mRNA was significantly decreased in section 7 and 10 compared to section 1 of the colon ($p<0.01$ and $p<0.001$ respectively). P-glycoprotein expression was significantly decreased in section 4 ($p<0.05$), 7 ($p<0.01$), and 10 ($P<0.01$) compared to proximal colonic section 1. The correlation between mdr1a and P-gp expression was measured using linear regression, and found to have an R^2 value of 0.8157, with $p=0.0968$.

4.3.5 Glucocorticoid Receptor mRNA and protein gradient along the colon

There was no difference in GR mRNA expression along the rat colon in the present experiments, consistent with data presented in chapter 3. However, figure 4.3 shows GR protein expression increased significantly towards distal colon, with significance reaching $p<0.01$ in section 7, and $p<0.001$ in section 10 when compared to proximal section 1. Therefore unlike mdr1a and P-glycoprotein, GR mRNA and protein expression did not correlate ($p=0.33$).

(a)



(b)

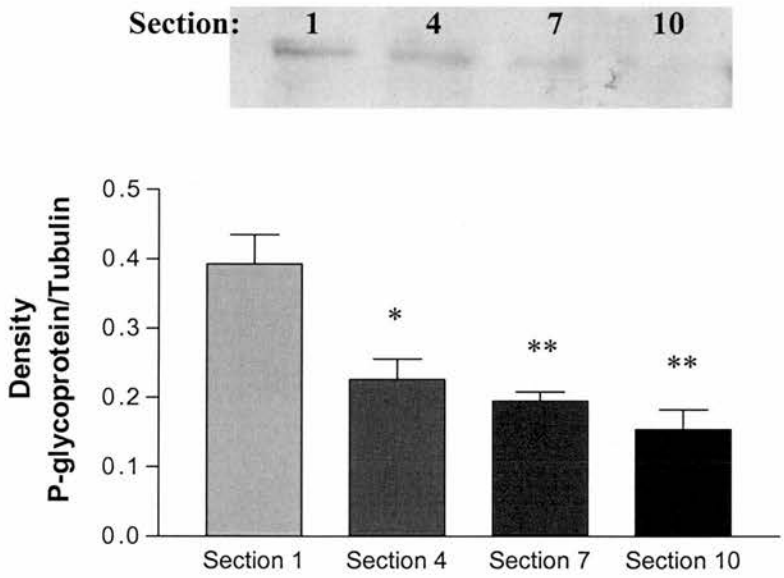


Figure 4.2: (a) A representative autoradiograph and corresponding graph showing mdr1a expression decreased along the rat colon (n=6/group) (b) Western blot and associated graph showing P-glycoprotein along the rat colon (n=3). Both mdr1a and P-glycoprotein expression significantly decreased towards distal colon, mirroring mRNA results in chapter 3. (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to section 1). Comparison of mdr1a and P-gp expression was performed using linear regression and shown to have an R^2 value of 0.8157, with $p=0.0968$.

(a)

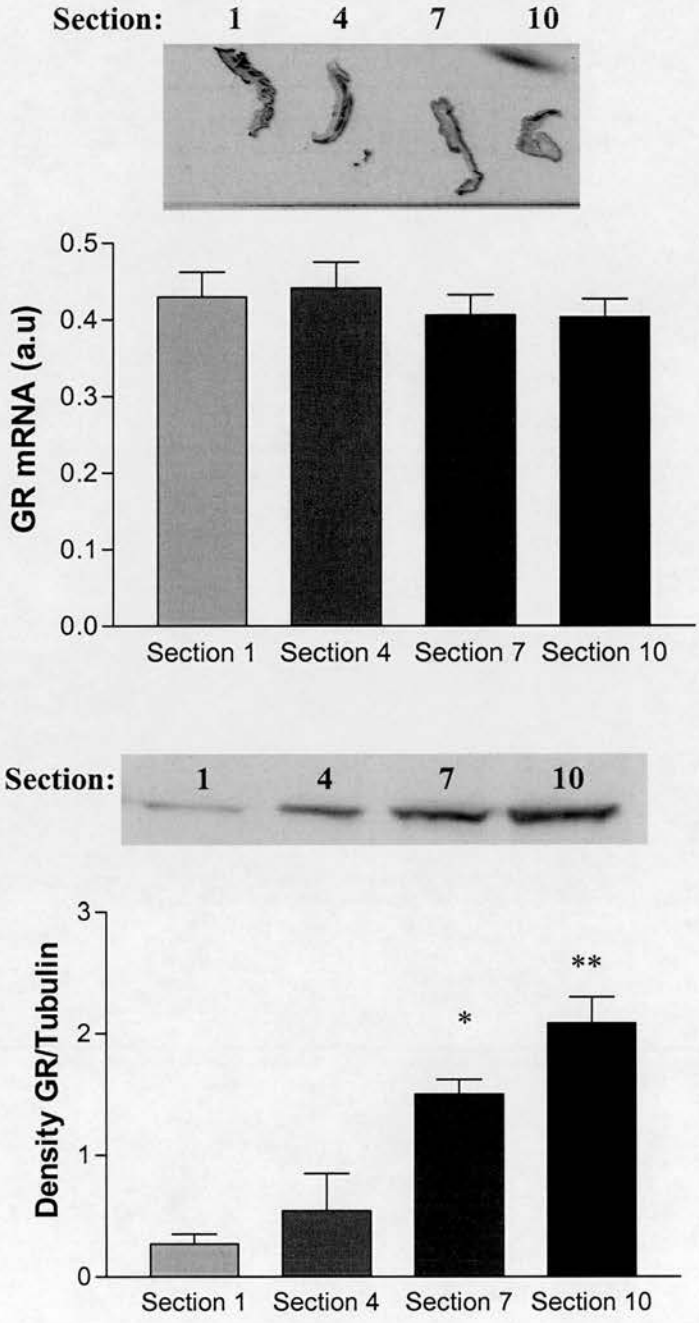


Figure 4.3: GR mRNA (n=6) (a) and protein expression (n=3) (b) with a representative autoradiograph and western film respectively, along the rat colon. There was no difference in mRNA expression along the colon however GR protein levels increased distally (*$p<0.01$, **$p<0.001$ compared to section 1).

4.3.6 mdr1a and P-glycoprotein expression after antibiotic treatment

There was no statistically significant different in mdr1a or P-glycoprotein expression in any region of the colon in animals which had received antibiotic treatment when compared to vehicle-treated animals (figure 4.4). However there was a trend towards decreased P-glycoprotein expression in the distal colonic sections after antibiotic treatment.

4.3.7 Glucocorticoid receptor mRNA and protein expression after antibiotic treatment

There was no significant difference in either GR mRNA or protein expression when animals treated with antibiotics were compared to vehicle-treated animals. However there was a trend towards an increase in GR protein expression in the antibiotic-treated group compared to vehicle animals in distal colonic section 10, although this just failed to be statistically significant ($p=0.0565$) (figure 4.5).

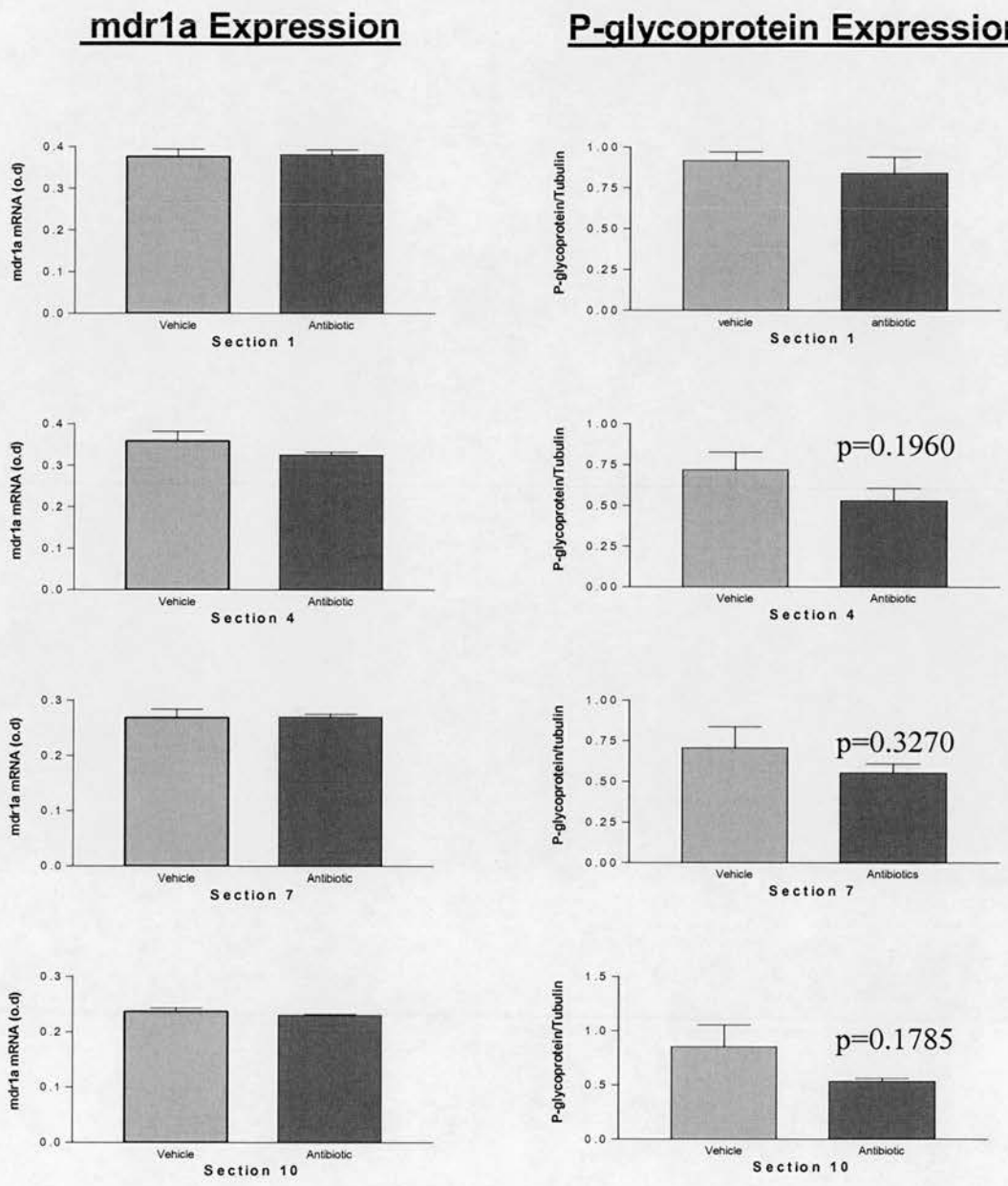


Figure 4.4: Effect of antibiotics on mdr1a (left) and P-glycoprotein (right) expression in sections 1, 4, 7 and 10 (top to bottom of the page), of the rat colon. There was no significant difference between treatment groups in any colonic section (n=6/group), although a trend towards a reduction in P-gp expression was noted in colonic sections 4, 7 and 10 (towards distal colon, which just failed statistical significance) when analysed using unpaired Students t-tests.

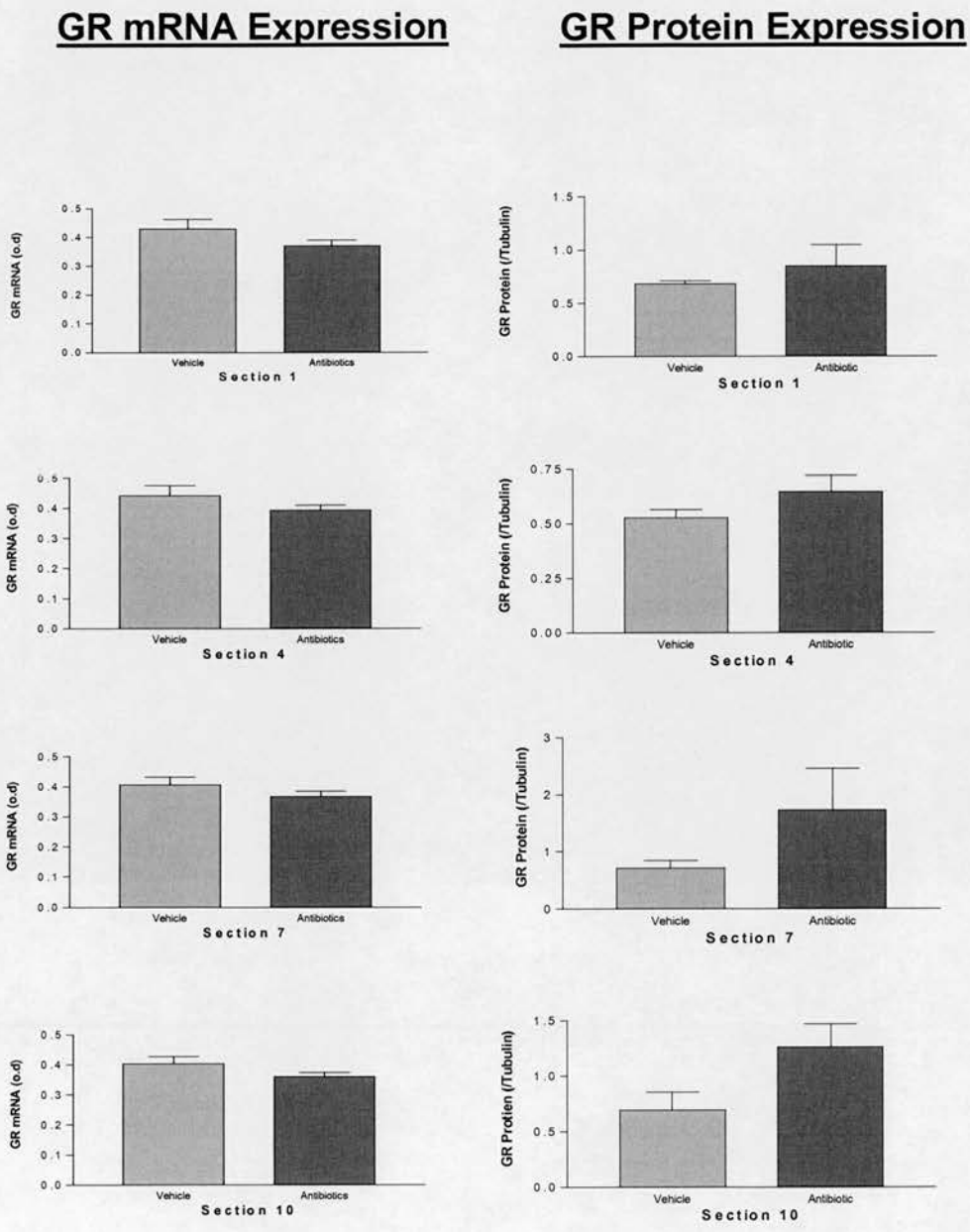


Figure 4.5: GR mRNA (left) and protein (right) expression along the rat colon (proximal>distal, top to bottom). There was a trend towards increased GR protein levels only in colonic section 10 after antibiotic treatment which failed to reach significance when analysed by Student t-tests (unpaired) (p=0.0565) (n=6/group).

4.4 DISCUSSION

The intestine is more densely populated with micro-organisms than any other organ, and therefore the epithelium plays a pivotal role in maintaining gut homeostasis in response to pathogenic and commensal organisms (Hooper *et al.* 2001). As discussed previously, there are a series of innate defence mechanisms which protect the intestine against potentially toxic substances/organisms ingested, or produced by metabolising bacteria. The work described in this chapter primarily investigated the effects of altering bacterial load on the expression of P-glycoprotein, a pump implicated in transporting xenobiotics out of cells (Ambudkar *et al.* 1999), and also the glucocorticoid receptor which is involved in the anti-inflammatory process . However to support data presented in the previous chapter where mdr1a and GR mRNA expression along the colon was studied but not protein expression (due to a freezer failure), P-glycoprotein and GR protein expression in the healthy rat colon was also investigated.

4.4.1 Colonic Expression of mdr1a/P-glycoprotein and Glucocorticoid Receptor

4.4.1.1 mdr1a/P-glycoprotein

Data presented in this chapter describing P-glycoprotein expression along the rodent colon complement the mRNA mdr1a data noted in this and the previous chapter. The increased expression in proximal compared to distal colon may be due to the high bacterial concentrations noted in this colonic region (Guarner & Malagelada 2003). We hypothesised an increase in bacterial concentrations in proximal colon may lead to increased xenobiotic production, and as a compensatory mechanism to protect the intestine from these substances, transcription of mdr1a and subsequent translation of P-glycoprotein would be induced. To investigate this, the intestinal bacterial load was reduced by treating with antibiotics, and expression of mdr1a and P-gp studied (see section 4.4.2).

4.4.1.2 Glucocorticoid Receptor

The increased expression of GR protein in distal compared to proximal colon was inconsistent with mRNA data noted in this and the previous chapter, as no difference in mRNA expression was noted along the colon. The discordance between mRNA and protein data may reflect post-translational modifications, RNA stability or differential rates of mRNA/protein turnover.

Our data showed increased GR protein expression in the distal compared to proximal colon. The explanation behind this novel finding remains to be established. A recent study has shown endotoxin decreases GR expression in the heart, liver and lung (Webster & Sternberg 2004), and therefore suggests bacteria down-regulate GR expression. This supports our findings where decreased GR expression was noted in proximal compared to distal colon, inversely correlating with bacterial density. A possible explanation for bacteria down-regulating GR could be due to the 'autoregulatory' mechanism associated with the regulation of the glucocorticoid receptor (Meyer & Schmidit 1994). GR is activated in proximal colon in response to pro-inflammatory mediators produced by bacteria, and thus GR is down-regulated via a 'negative feedback loop'. However in distal colon, where less bacteria and therefore less pro-inflammatory mediators produced, GR is not activated to the same extent as in proximal colon, and therefore GR expression is increased via a 'positive-feedback loop'.

4.4.2 Effect of altering bacterial flora on colonic mdr1a and P-glycoprotein expression

Data presented in this chapter showed metronidazole (selective against anaerobic bacteria including gram-negative such as *Bacteroides* species) and ciprofloxacin (antibiotic against aerobic bacteria with an extend spectrum against anaerobic gram-positive bacteria) (Rath *et al.* 2001) decreased colonic bacterial content. However, a *Bacteroides* species and other gram positive cocci were still present in the gut. Previous studies in healthy mice (Velder *et al.* 2003) showed the doses and time of treatment of metronidazole and ciprofloxacin used in this study cleared anaerobic and

aerobic bacteria respectively from the intestine, as measured by real-time PCR and bacterial isolation.

In the present study, the decrease in bacterial load was not correlated with a significant decrease in mdr1a or P-glycoprotein expression. However in distal colon, there was a trend towards decreased P-gp expression in animals treated with antibiotics, further supporting the hypothesis that intestinal bacteria regulate P-glycoprotein expression.

Previous studies in healthy mice have shown *Escherichia coli* increase whereas *Bacteroides thetaiotaomicron* decrease ileal mdr1a expression compared to mice in germ-free conditions (Hooper *et al.* 2001). Hooper and colleagues also showed mdr1a expression was increased in mice conventionally raised, albeit to a lesser extent than noted during *E. coli* mono-association studies. Studies in IL-10 knockout mice have also shown *E. coli* to induce a rapid-onset caecal inflammation, whereas *E. faecalis* caused a slow-onset distal colitis (Sartor 2004b). Taken together these data suggest that, in mice, intestinal mdr1a expression may be regulated by bacterial flora and also emphasise the importance of different subsets of bacteria in regulating mdr1a expression. These data complement the results of mono-association studies in HLA-B27 transgenic rats which have shown *B. vulgatus and B. thetaiotaomicron,* but not *B. distasonis* induced colitis (Sartor 2004a; Sartor 2004b). *E. coli* in these rats did not induce colitis (Rath *et al.* 2001; Sartor 2004a). However monoassociation studies using IL-10 knockout mice have shown *E. coli* but not *B. vulgatus* induced colitis (Sartor 2004a). These studies highlight the complexity and species specific role for bacteria in inducing colitis in animal models.

In the present study we may not be seeing a significant decrease in colonic mdr1a/P-gp expression because bacterial clearance was incomplete and critical strains maintained. Moreover, a trend towards decreased protein expression was observed only in distal colon, a possible explanation could be due to different subsets of bacteria colonising specific areas of the colon (Sartor 2004a). It seems likely that *Bacteroides* species and other bacteria still present in the intestine may predominantly

be present in proximal colon, and this may provide an explanation why no decrease in P-glycoprotein expression was noted in this colonic area.

4.4.3 Effect of altering bacterial flora on colonic GR mRNA and protein expression

In the distal colon, there was a trend towards increased GR protein expression after antibiotic treatment. It was hypothesised an increase in bacteria, and therefore pro-inflammatory mediators, would increase GR expression due to the glucocorticoid receptor being involved in the resolution of inflammation. Data present in this chapter are in direct opposition to this hypothesis, as a *decrease* in bacterial load increased GR protein expression in distal colon. As discussed previously, recent data has shown endotoxin reduces heart, lung and liver GR expression (Webster & Sternberg 2004), and these studies would be consistent with data presented in this chapter. The mechanism behind this phenomenon is unclear, although a possible explanation for increased GR expression in response to reduced bacterial concentrations in distal colon could be due the 'feedback loop' regulating GR expression, as discussed earlier in section 4.4.1.2. Another possible reason for the increase in GR protein expression in the distal colon could simply be due to the antibiotics directly up-regulating GR, although no studies analysing the effects of ciprofloxacin and metronidazole on GR expression have been undertaken.

In summary, data in this chapter suggests a possible role for bacteria in the regulation of P-glycoprotein and GR in Wistar rats. To further clarify the role of bacteria in the regulation of these genes, subsequent germ-free and mono-association studies (a condition unavailable during this study) are required. This would help establish whether these genes are regulated by bacteria, and also if specific bacteria are involved in the regulation of mdr1a/P-gp and GR in these rats. If bacteria were involved in the regulation of these genes, it would provide possible explanations on how specific subsets of bacteria alter the barrier function of the intestinal epithelium and initiate inflammation.

Chapter 5

Expression and regulation of P-glycoprotein and Glucocorticoid Receptor in the colon of HLA-B27 transgenic rats

5.1 INTRODUCTION

As discussed in chapter 1, animal models of inflammation can be categorised into four groups based on the method of induction- drug- or chemically-induced, spontaneous, genetically engineered or T-cell transfer. The genetically engineered models can be further subdivided into the type of immune response mediating inflammation-Th1 (primarily involved in cell-mediated immunity and involving the cytokines IL-2 and IFN-γ) and Th2 (responsible for antibody responses and involving the cytokines IL-4, IL-5 and IL-10).

There are a limited number of rat models of colitis, and these include the drug induced models of colitis, such as the indomethacin and trinitrobenzene sulfonic acid (TBNS)/alcohol models, as well as the HLA-B27 transgenic rat model (Sartor 2004b). Animal models of intestinal inflammation have provided vital information on the pathogenesis of inflammatory bowel disease, notably providing compelling evidence for the role of bacteria in the initiation of disease, since inflammation is reduced after antibiotic treatment, prevented/attenuated when treated with probiotics/prebiotics, or is absent when housed in germ-free conditions (Sartor 2004b).

Expression of the HLA-B27/β_2 microglobulin transgene in rats (referred to as the HLA-B27 transgenic rats) is associated with a Th1 pattern of intestinal inflammation as well as extra-intestinal magnifications such as axial arthritis by 12 weeks of age when housed in a specific pathogen-free (SPF) environment (Sartor 2000). When these animals are housed in germ-free (GF) conditions, intestinal inflammation and arthritis are absent, although skin and nail lesions are still present. There have also been a variety of gnotobiotic studies carried out in this model, with specific strains of bacteria (*Bacteroides vulgatus* and *Bacteroides thetaioaomicron*) shown to preferentially induce colitis, with the caecum being the site of the most intense inflammation (Rath *et al.* 1999). Studies using selective and broad spectrum antibiotics have also shown inflammation to be attenuated and even reversed in HLA-B27 transgenic rats with established inflammation (Sartor 2004a).

The mdr1a -/- mouse model is also associated with a Th1 pattern of inflammation (Sartor 2004b). mdr1a is known to play a role in the transport of drugs and other potentially toxic substances including bacterial products, out of cells, and thereby may be involved in the detoxification of xenobiotic compounds (Langmann *et al.* 2004). Moreover, the MDR1 gene is now strongly implicated in human IBD (Farrell *et al.* 2000; Langmann *et al.* 2004). Expression genetics have also shown a correlation between polymorphisms in the MDR1 gene determining disease extent as well as susceptibility to IBD (Ho *et al.* 2003; Ho *et al.* 2005). As with the HLA-B27 transgenic rats, studies have shown these mice develop colitis by 20 weeks of age when housed in SPF conditions (Panwala *et al.* 1998). Treatment with antibiotics attenuates colitis, and when housed in a GF environment, no colitis is present in this animal model (Sartor 2004a). These data implicate a protective role for mdr1a in the intestinal epithelium against potentially harmful/toxic substances (Panwala *et al.* 1998). Recent studies in mice, where intestinal inflammation was induced using dextran sodium sulphate (DSS), have shown a decrease in mdr1a in diseased animals (Lizasa *et al.* 2003), and this implies that during disease, the intestinal epithelial barrier is disrupted.

Interestingly, even though the glucocorticoid receptor plays a series of important roles in the anti-inflammatory process in disease, including both inhibiting pro-inflammatory transcription factors and inducing the transcription of anti-inflammatory cytokines, the effects of disease on expression of GR within the inflamed intestinal tissue of these transgenic animal models have not been studied (Barnes 1998). There are conflicting results surrounding GR expression in patients with IBD. Patients with CD have been shown to have reduced GR mRNA expression in peripheral blood lymphocytes (Hori *et al.* 2002), whereas GR mRNA was increased in peripheral blood lymphocytes in UC patients in remission compared to healthy controls (Flood *et al.* 2001). Expression of GR in the mucosa of IBD patients were seen to be reduced (Rogler *et al.* 1999). The effect of inflammation on intestinal GR expression has not been previously studied in animal models of disease. It is worthy of note that GR expression in hepatic T-cell lymphocytes has been shown to be reduced in rats with experimental cholangitis (Tjandra *et al.* 2003). Primary

sclerosing cholangitis is a Th-1 driven response disease, similar to the type of inflammation produced in HLA-B27 transgenic rats (Tjandra *et al.* 2003).

To complement studies documented in previous chapters where intestinal expression of mdr1a/P-glycoprotein and GR was investigated in healthy adult Wistar rats, the expression of these genes in a rat model of colitis was investigated. In the investigation described in the present chapter, the HLA-B27 transgenic rat model was chosen in view of the well-characterised bacterial studies undertaken in this model. We hypothesised that the regional variation of expression of mdr1a/P-glycoprotein and GR in the rat colon discussed previous chapters was a consequence of the density of bacterial colonisation. Expression of mdr1a/P-glycoprotein and GR from caecum to rectum was studied in the Fischer 344 HLA-B27 transgenic and non-transgenic rats to determine whether a) a gradient in expression of these genes was present in the colons of these rats; b) expression was associated with the density of bacterial colonisation; and finally c) to investigate the effect of active colitis on expression of P-glycoprotein and GR.

5.2 METHODS

5.2.1 Transgenic and Non-transgenic HLA-B27 Rats

Tissue from these animals was a gift from Professor Sartor, North Carolina, USA. Transgenic (TG) and non-transgenic (NT) Fischer 344 rats in germ-free (GF) and specific pathogen free (SPF) conditions were sacrificed at 4-5 months of age. Sections from the caecum, proximal colon and rectum dissected and frozen in OCT medium for RNA analysis and adjacent tissue sections were also taken and frozen on dry ice for protein analysis. All samples were sent on dry-ice.

5.2.2 *In situ* hybridisation

Caecal, proximal colonic and rectal sections from HLA-B27 rats were cut and mounted onto saline-coated slides. *In situ* hybridisation was performed to analyse mRNA levels using transcribed antisense ^{35}S-UTP riboprobes for GR and mdr1a as described in section 2.6. Expression was analysed by measuring the autoradiograph films, in which the optical density (units were given arbitrarily) was measured for whole tissue (GR) or epithelial areas (mdr1a).

5.2.3 Western Blotting

Protein levels were measured using Western blotting. Tissues from caecum, proximal colon and rectum from HLA-B27 rats were homogenised and analysed as previously described in section 2.7. GR and P-glycoprotein expression were analysed using the sample antibody preparations as shown in table 2.3. Discrepancies in loading were assessed by measuring tubulin expression in each sample using the antibody dilution as described in table 2.3. All bands were analysed by densitometry using the MCID-M4 Image Analysis programs and results normalised against tubulin.

For analysing the gradient along the colon, caecal, proximal colon and rectal homogenates from 1 animal from each of the 4 groups (TGSPF, NTSPF, TGGF, NTGF) were run on a single gel, and 3 gels were analysed (n=3).

When analysing differences between TG/NT in GF/SPF conditions in specific areas of the colon, specific tissue homogenates (caecal, proximal colon or rectum) from 3 animals from each group (TGSPF, NTSPF, TGGF, NTGF) were run together on a single gel. All 3 gels, containing either caecal, proximal colon and rectal sections, were run and analysed together. This experiment was repeated so all 6 animals/group were analysed (n=6).

5.2.4 Statistics

All results are expressed as the mean ± S.E.M. Differences between values were compared using ANOVA with post-hoc Tukey's pair-wise comparison. P values of less than 0.05 were considered significant.

5.3 RESULTS

5.3.1 Histology

Only transgenic (TG) animals in specific-pathogen free (SPF) conditions developed inflammation in all regions of the colon, most notably the caecum. Tissue sections taken from these animals showed elongated crypts with an infiltration of mononuclear cells compared to non-transgenic animals in the same condition or those in a germ-free (GF) environment, which are typical characteristics of intestinal inflammation. Figure 5.1 represents histology pictures taken from the caecum of TG and NT rats in SPF and GF conditions.

5.3.2 P-gp and mdr1a gradient along the colon

P-glycoprotein and mdr1a expression decreased from caecum to rectum in all animal groups except diseased animals (TGSPF), in which all sections had low mdr1a and P-glycoprotein expression, as shown in figure 5.2. In NT animals housed in either SPF or GF conditions, the rectal sections had significantly lower mdr1a ($p<0.001$, $p<0.01$ respectively) and P-glycoprotein expression ($p<0.05$ in both areas) compared with the caecum. TG animals in GF conditions (non-diseased) had reduced mdr1a ($p<0.001$) and P-glycoprotein ($p=0.051$) expression in the rectum compared to the caecum.

5.3.3 GR mRNA and protein gradient along the colon

Figure 5.3 shows GR protein expression along the colon. In NT animals in both GF and SPF conditions, there was a trend towards reduced GR mRNA in rectum compared to caecum, which reached significance ($p<0.05$). In contrast, non-diseased animals with bacterial flora (NTSPF) had increased GR expression from caecum to rectum ($p<0.01$). In animals with disease (TGSPF), no gradient was noted.

Both TG and NT animals in bacterial free conditions (GF) (TGGF, NTGF; all non-diseased) showed no difference in GR protein expression along the colon.

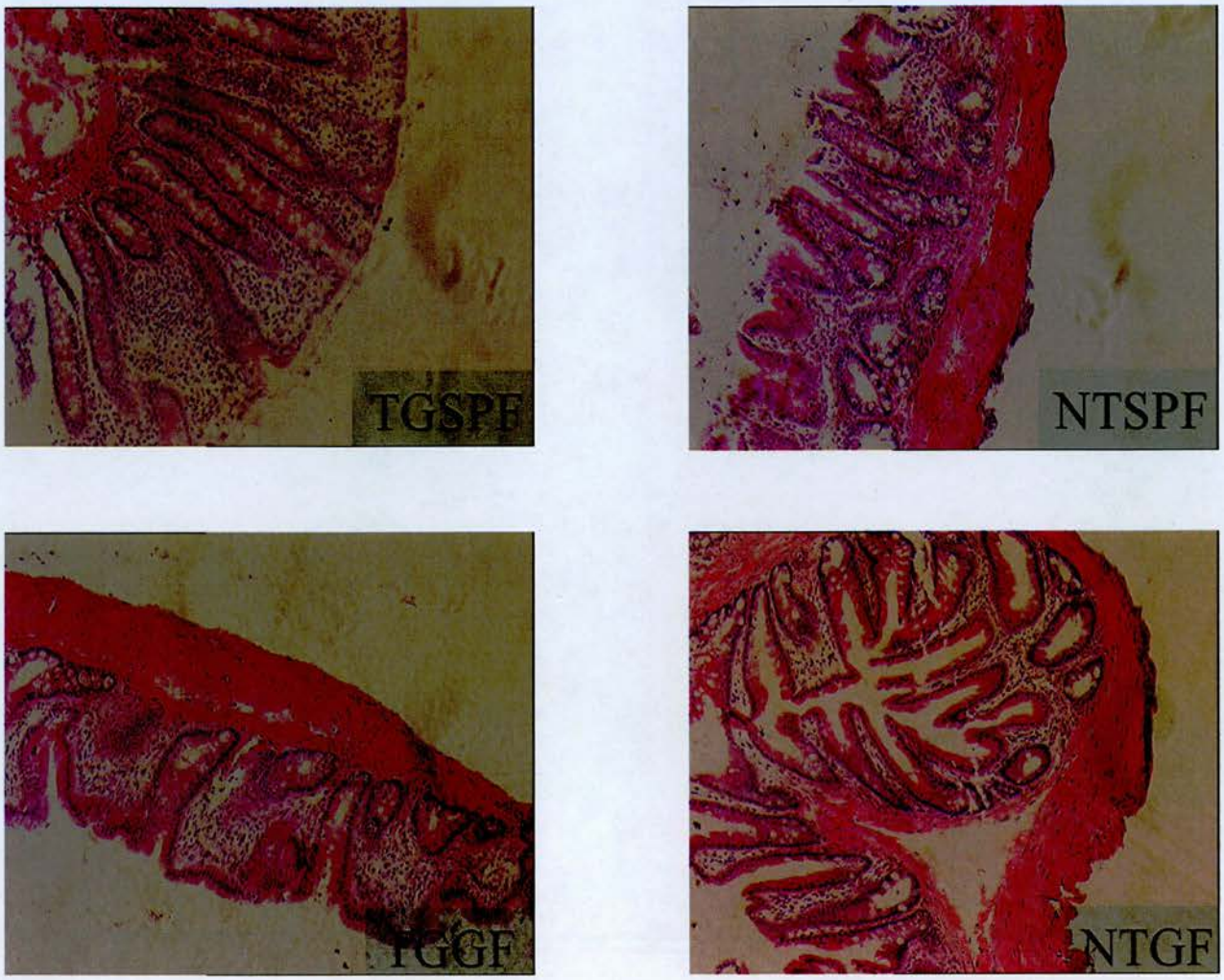


Figure 5.1: Haematoxylin and eosin staining of caecum from Transgenic (TG) and non-transgenic (NT) animals in specific-pathogen free (SPF) and germ-free (GF) conditions taken at x10 magnification. In sections taken from animals with disease, TGSPF, crypts were seen to be elongated compared to NT animals in SPF conditions (NTSPF), and there was increased nuclear staining of cells in the TGSPF rat mucosa compared to NTSPF, suggesting an infiltration of mononuclear cells in response to inflammation.

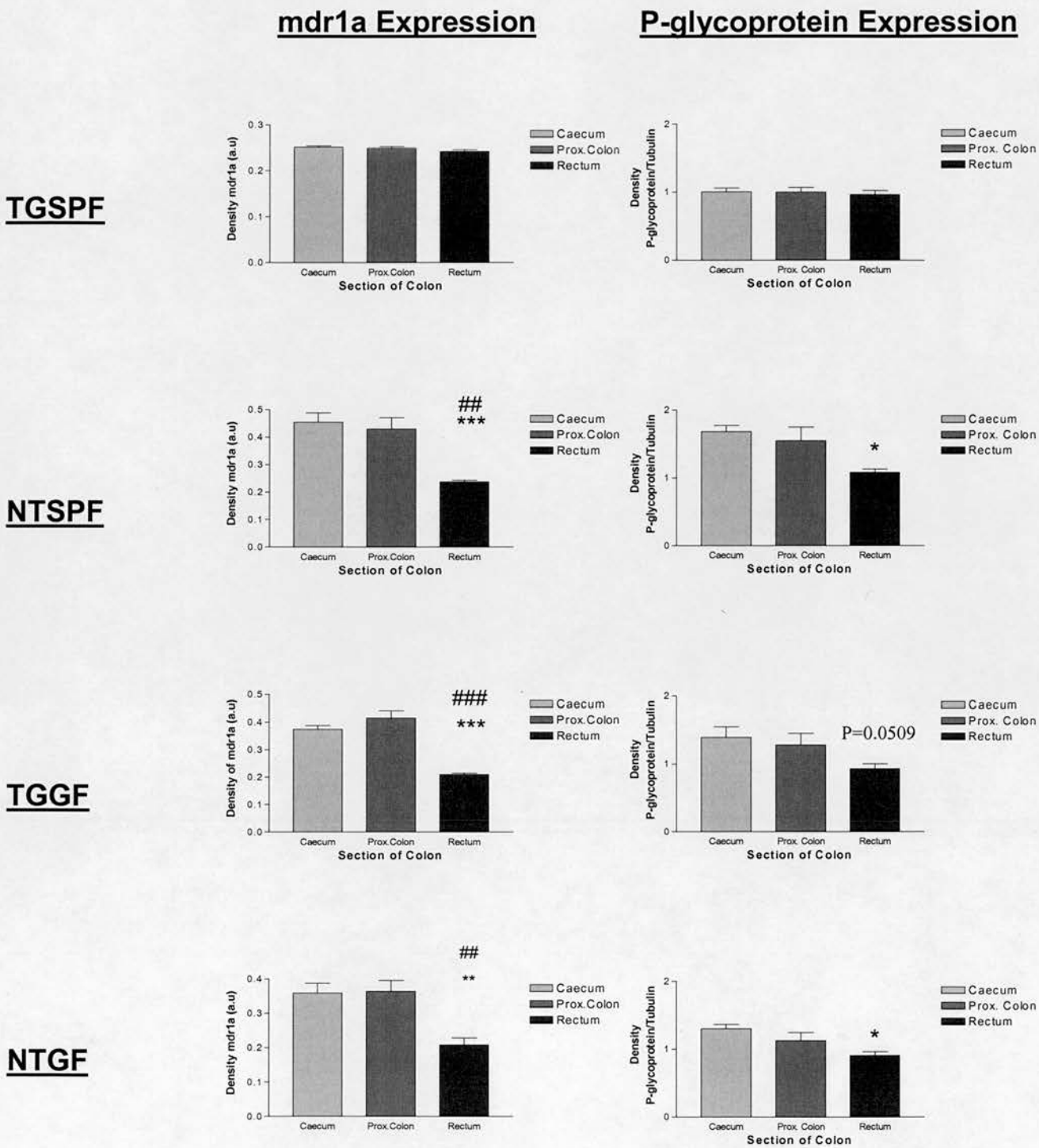


Figure 5.2: Colonic *mdr1a* mRNA (n=6/group) (left) and P-glycoprotein expression (n=3/group) in TG and NT rats in SPF and GF conditions. Diseased animals (TGSPF) had low mdr1a and P-glycoprotein expression in all colonic areas. NT animals in SPF conditions (non-diseased) had significantly reduced mdr1a and P-gp levels in the rectum compared to caecum ($p<0.001$, $p<0.05$ respectively) and proximal colon ($p<0.01$). TG and NT animals in germ-free conditions also had reduced mdr1a expression in rectal compared to caecal and proximal colon ($p<0.001$, $p<0.01$ respectively). P-gp expression was reduced in NT animals in GF conditions ($p<0.05$). Compared to caecum* and proximal colon #.

(*/# $p<0.05$, **/## $p<0.01$, ***/### $p<0.001$)

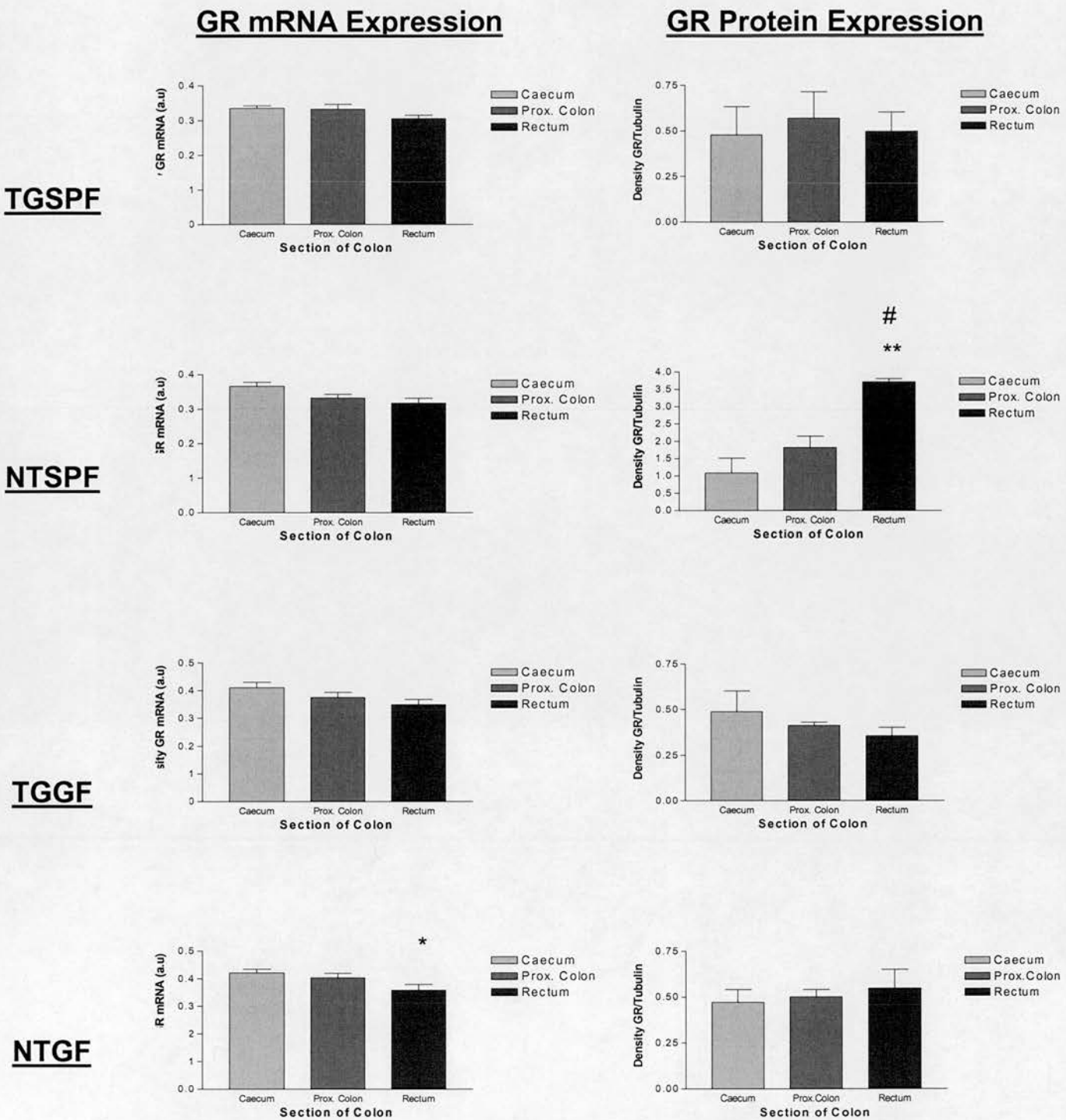


Figure 5.3: GR mRNA (n=6/group) (left) and protein expression (n=3/group) in TG and NT HLA-B27 rats housed in either SPF or GF conditions. There was a trend towards decreased GR mRNA in rectal sections from animals in GF environments and non-diseased animals in SPF conditions (NTSPF). A significant decrease in GR mRNA expression was noted in NT animals GF conditions ($p<0.05$). This contrasted to GR protein data, where NT animals in SPF conditions (non-diseased) had increased GR expression in rectal compared to caecal and proximal colonic sections ($p<0.01$, $p<0.05$ respectively). However in diseased animals (TGSPF) and animals in GF conditions, no GR protein gradient from caecum to rectum was noted. *compared to caecum #compared to proximal colon.

*/# $p<0.05$, **$p<0.01$

5.3.4 mdr1a and P-glycoprotein expression in the caecum, proximal colon and rectum

5.3.4.1 Caecum

TG rats in SPF conditions (rats with disease) had significantly lower mdr1a and P-glycoprotein ($p<0.001$) expression compared to NT rats in the same condition (NTSPF). There was no difference in mRNA or protein expression between TG and NT rats kept in germ-free conditions (TGGF compared to NTGF). Figure 5.4 shows P-glycoprotein was significantly decreased in NT rats in GF compared to SPF conditions ($p<0.05$).

5.3.4.2 Proximal Colon

As with the caecum, mdr1a and P-glycoprotein levels were significantly decreased in TG compared to NT rats in SPF conditions ($p<0.01$). Figure 5.4 also shows no difference between TG and NT rats in germ-free conditions (TGGF compared to NTGF). mdr1a expression was increased significantly ($p<0.05$) in TG rats without disease when compared to TG rats with disease (TGGF compared to TGSPF).

5.3.4.3 Rectum

Figure 5.4 shows mdr1a levels were similar in TG and NT rats in GF and SPF conditions. P-glycoprotein was significantly decreased in diseased animals (TG) compared to non-diseased (NT) rats in SPF conditions. There was no significant difference between P-glycoprotein expression rats in germ-free conditions.

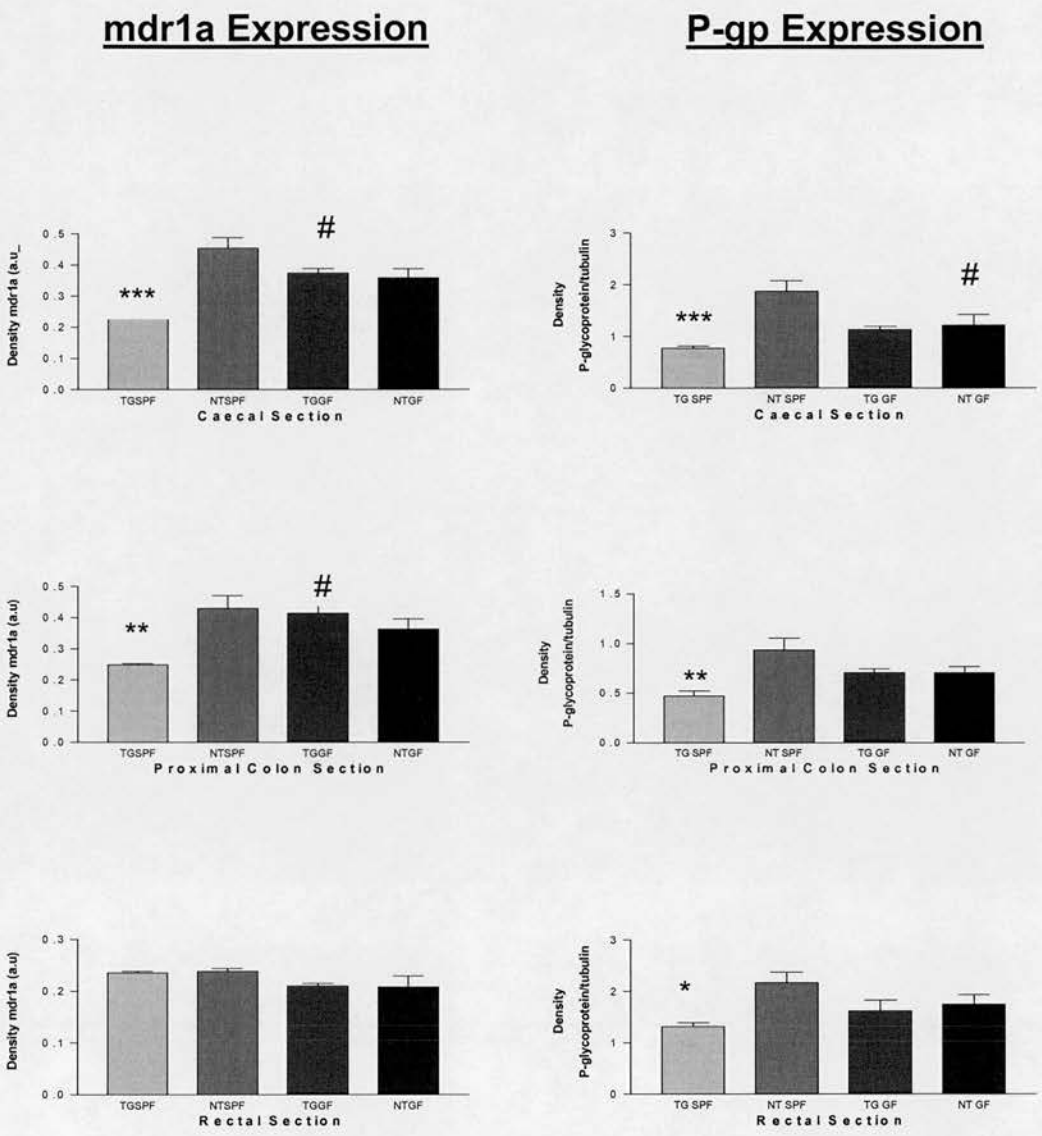


Figure 5.4: Graphs showing mdr1a (left) and P-glycoprotein expression (right) in caecum, proximal colon and rectal sections (top to bottom of the page). TG rats in SPF conditions had significantly lower mdr1a and P-glycoprotein expression in caecal, proximal colon and rectum compared to NT rats in the same environment (***$p<0.001$, **$p<0.01$, *$p<0.05$ respectively). P-glycoprotein expression was lower in NT rats in GF compared to SPF conditions and this reduction was significant in caecum (#$p<0.05$).

* comparison between TGSPF versus NTSPF or NTGF versus TGGF

comparison between TGSPF versus TGGF or NTSPF versus NTGF

5.3.5 GR mRNA and protein expression in the caecum, proximal colon and rectum

5.3.5.1 Caecum

Figure 5.5 shows GR mRNA expression increased significantly when TG and NT rats were housed in GF compared to SPF conditions ($p<0.01$, $p<0.05$ respectively). Protein expression was also increased when rats were housed in GF conditions, and this increase was significant in the TG rat groups.

5.3.5.2 Proximal Colon

GR mRNA expression increased in rats housed in GF compared to SPF conditions, and this increase was significant in the NT rat group ($p<0.05$) (figure 5.5). This contrasts to GR protein expression, as rats in GF environments had significantly lower GR protein expression than NT rats in SPF conditions ($p<0.001$). Diseased rats (TGSPF) had significantly lower GR expression than NT rats (non-diseased) in the same environment (NTSPF; $p<0.001$). There was no difference between the groups in GF conditions.

5.3.5.3 Rectum

Figure 5.5 shows no difference in GR mRNA expression between NT and TG rats with/out bacteria. GR protein expression was similar to expression in the proximal colon, where NT rats in SPF conditions (non-diseased) had significantly increased GR expression compared to GF conditions and also TG rats in the SPF environment (diseased rats) ($p<0.001$). There was no difference between NT and TG rats in GF conditions.

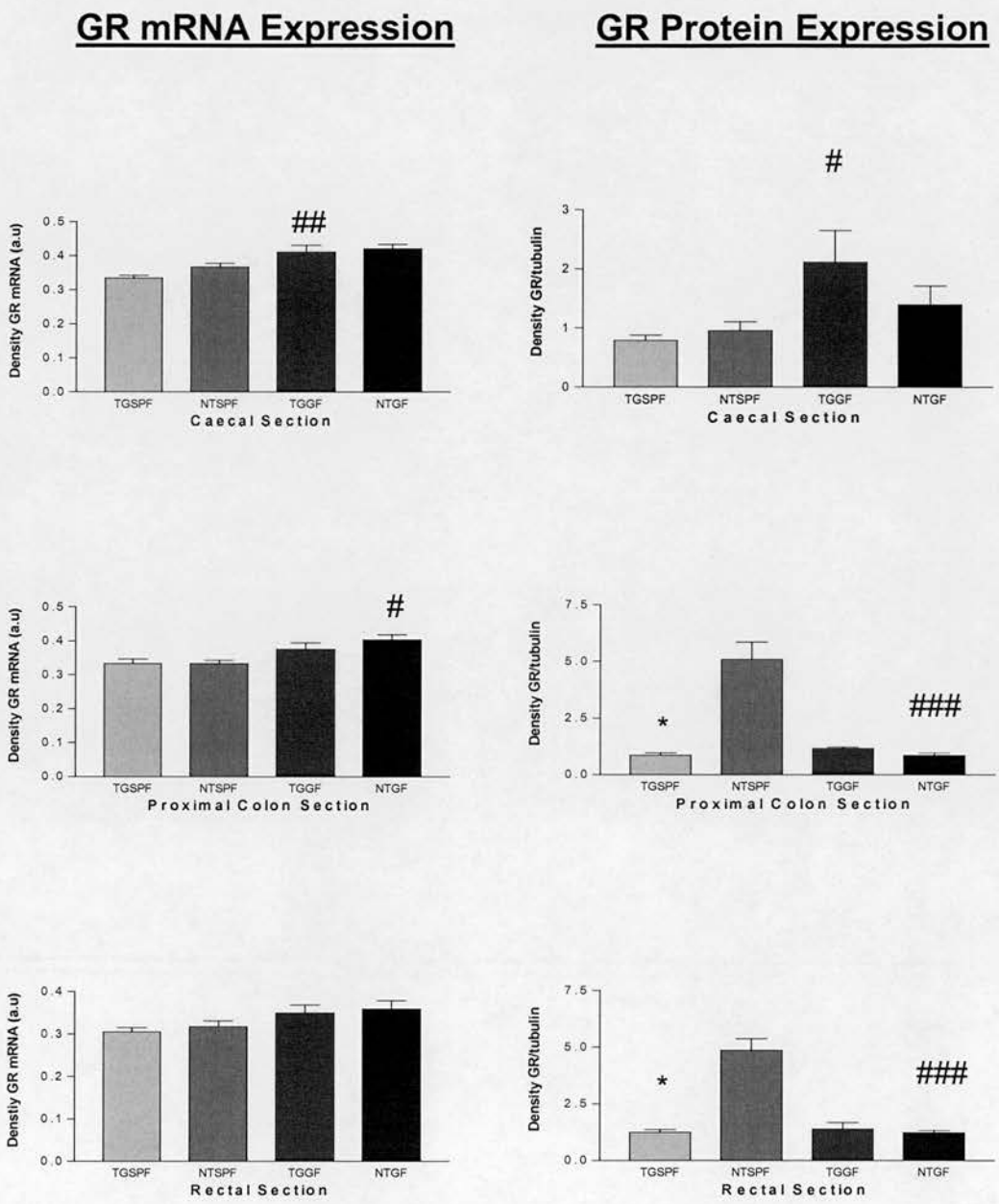


Figure 5.5: GR mRNA (left) and protein (right) expression in caecum, proximal colon and rectum (top to bottom of the page). GR mRNA levels were significantly increased in GF compared to SPF conditions in TG caecal, NT proximal colonic sections ($p<0.05$, $p<0.01$ respectively). Caecal GR protein levels were increased ($p<0.05$) in TG animals housed. However in the proximal colon and rectum, animals housed in GF conditions had decreased GR protein expression compared to non-diseased animals in SPF conditions (NTSPF). Diseased rats also had reduced GR protein levels compared to non-diseased rats (NTSPF). No differences were found between groups in GF environments in any colonic section.

* Comparison between TGSPF versus NTSPF or NTGF versus TGGF

\# Comparison between TGSPF versus TGGF or NTSPF versus NTGF

(*$p<0.001$, #$p<0.05$, ##$p<0.01$, ###$p<0.001$)

5.4 DISCUSSION

The emergence of animal models of intestinal inflammation has provided vital information on the role of bacteria involved in initiating disease. It is increasingly accepted that P-glycoprotein may be involved in the protection of the epithelium from potentially toxic substances, and absence of this pump in mice induces disease (Panwala *et al.* 1998). It is also well documented that the glucocorticoid receptor is involved in the anti-inflammatory process (Barnes 1998), and a non-functional glucocorticoid receptor (GR-β) has been implicated in steroid resistant disease (Honda *et al.* 2000). The expression of these genes, however, has not previously been studied in HLA-B27 transgenic rat models of colitis, and in this chapter the effect of inflammation and disease on expression of mdr1a/P-glycoprotein and GR was investigated.

5.4.1 Colonic expression of mdr1a/P-gp and GR in transgenic and non-transgenic animals

5.4.1.1 mdr1a and P-glycoprotein

Studies described in the previous chapter involving healthy Wistar rats concluded that mdr1a and P-glycoprotein expression was increased in proximal compared to distal colon, and this conclusion is mirrored by data presented in this chapter. Colons from healthy Fischer rats, regardless of the presence/absence of bacteria, were also characterised by a gradient of expression, with increased expression seen in caecum and proximal colon compared to rectum. In the previous chapter it was hypothesised that the gradient of mdr1a/P-gp expression may mirror the density of bacterial colonisation, allowing the transport of xenobiotic substances and the protection of the intestinal epithelium. However, data presented in this chapter suggest that this gradient may be specific phenomenon noted in rat, as previous studies in healthy FVB mice have shown increased P-gp expression in distal compared to proximal colon (Stephens *et al.* 2002): no data are yet available in man.

In diseased rats (TG in SPF conditions), no expression gradient was noted, and it seems likely that disease is associated with reduced mdr1a and P-gp expression. To

further investigate the effect of disease and bacteria on the expression of mdr1a/P-gp, expression of mdr1a/P-gp in caecum, proximal colon and rectum was studied and compared between TG and NT animals housed in GF and SPF conditions.

5.4.1.2 Glucocorticoid Receptor

Consistent with findings described in the previous chapter, healthy Fischer rats (NT) housed in an environment with bacteria present had increased GR protein expression in distal compared to proximal colon, although no differences were noted in mRNA expression along the colon. The differences between mRNA and protein expression may be a result of post-translational modifications, RNA stability or the difference in the rates of turnover of mRNA/protein.

When NT rats were housed in the absence of bacteria, the gradient was absent suggesting bacterial flora may regulate GR expression in the colon. As with the findings of mdr1a/P-gp expression, diseased rats (TG rats in SPF conditions) did not have a GR protein gradient and suggests the effect of disease down-regulated GR expression. To further clarify the effect of bacteria and disease, the expression of GR in NT and TG rats housed in SPF or GF conditions were compared individually for each colonic region (caecum, proximal colon and rectum).

5.4.2 Effect of bacteria and disease on colonic mdr1a/P-gp and GR expression

5.4.2.1 mdr1a/P-glycoprotein expression

Data presented in this chapter have shown for the first time that mdr1a and P-glycoprotein expression was reduced in the inflamed colon from HLA-B27 transgenic rats. Previous studies in mice, where colitis had been induced by DSS and turpentine, have also shown inflammation to reduce P-glycoprotein expression in the large intestine and liver respectively (Sukhai *et al.* 2000). Moreover, recent data in man have noted that patients with UC have reduced colonic MDR1 mRNA expression than healthy controls (Langmann *et al.* 2004). Collectively, these observations imply

inflammation may down-regulate mdr1a/P-glycoprotein expression in both humans and animals.

The mechanism underlying the down-regulation of mdr1a/P-glyocoprotein has not been fully established. Experiments *in vitro* have shown IL-6 and IL-1β decreased P-glycoprotein expression in rat hepatocytes, and inflammation induced by turpentine in mice also resulted in reduced hepatic mdr1a expression (Hartmann *et al.* 2001; Sukhai & Piqutte-Miller 2000). Endotoxin-treated rats have also been shown to have reduced levels of mdr1a in the small intestine (Kalitsky-Szirtes *et al.* 2004). Therefore the decrease in P-glycoprotein noted in HLA-B27 transgenic rats housed in SPF conditions (where inflammation is present) may be due to the release of pro-inflammatory cytokines from the intestinal mucosa, which down-regulates P-glycoprotein expression.

Inflammation in mice, induced by endotoxin and IL-6, has been shown to decrease the expression of the hepatic Pregnane X Receptor (PXR). In this model, the decrease was involved in the down-regulation PXR-mediated genes including the drug metabolising enzyme cytochrome P450 (Teng & Piqutte-Miller 2004). This receptor and enzyme is involved in the mucosal defence system. PXR recognises foreign substances (xenobiotics) and activates metabolising enzymes, including cytochrome P450, which breakdown xenobiotics into less harmful substances (Dietrich *et al.* 2003). P-glycoprotein is involved in the transport of these substances into the lumen to be excreted by the body. Recent studies have implied PXR may be involved in regulating P-glycoprotein expression (Langmann *et al.* 2004; Teng & Piqutte-Miller 2004). Therefore inflammatory cytokines produced during disease in these transgenic rats may be down-regulating PXR expression which subsequently reduces mdr1a/P-gp levels.

Another possible reason for this down-regulation could be due to the release of corticosterone in a stressed/diseased state (de Kloet *et al.* 1998). *In vitro* and *in vivo* studies have implied mdr1a may also be regulated by corticosterone (Murakami *et al.* 2002), and a trend towards increased mdr1a mRNA expression after adrenalectomy compared to Sham-operated animals in chapter 3 implies a role for endogenous GCs

in the regulation of mdr1a. Therefore an increase in endogenous corticosterone from the adrenal glands may be involved in the down-regulation of mdr1a and P-glycoprotein in these diseased animals.

We hypothesised a priori that bacteria may influence mdr1a/P-glycoprotein expression as a means to protect the intestinal epithelium from potentially toxic xenobiotics produced by bacteria. Work presented previously in this chapter still shows an mdr1a/Pgp gradient from proximal to distal colon in germ-free conditions, and finding apparently at odds with our prior hypothesis. Nonetheless, when sections from the caecum, proximal colon and rectum from all the different bacterial conditions were analysed separately, a significant decrease was noted in germ-free compared to healthy non-transgenic animals housed in specific-pathogen free conditions (figure 5.4). This implies that in caecum (where bacterial density is highest) but not proximal colon or rectum, bacteria increase P-glycoprotein levels and support our initial hypothesis which implicated bacteria in the regulation of mdr1a/P-glycoprotein.

However the reason as to why this significant decrease was only noted in the caecum remains to be eluded. A possible explanation could be due to specific subsets of bacteria regulating mdr1a/P-gp expression where these bacteria are localised in the caecum. A recent study has shown the therapeutic activities of different antibiotics in treating rodent intestinal inflammation are site-specific; with caecal and colonic inflammation resolved using ciprofloxacin and metronidazole respectively (Hoentjen *et al.* 2003). It is interesting to note that transgenic animals housed in SPF conditions develop intestinal inflammation, with the most aggressive site noted in the caecum. This may be due to increased bacterial flora in this region, or as discussed, the presence of specific bacteria which are involved in the development of disease (Sartor 2004a). These data highlight the differences in the composition of bacterial flora within the large intestine, which may not only play a role in regulating mdr1a/P-gp, but also in the initiation of disease.

5.4.2.2 Glucocorticoid Receptor

In contrast to mdr1a and P-glycoprotein expression, GR mRNA and protein expression did not correlate. There are a number of possible explanations underlying differences between mRNA and protein expression, including the effect of post-translational modification or even the stability/degradation of both mRNA and protein. Notwithstanding these differences, we are the first to show that in HLA-B27 transgenic rats with disease, GR expression was significantly reduced in the inflamed intestine. GR expression has been shown previously to be decreased in response to inflammation, however this was in hepatic T-lymphocytes and also in mononuclear cells of patients with Crohn's disease (Hori *et al.* 2002; Tjandra *et al.* 2003). In this chapter, the inflammation-induced reduction in GR was only present in proximal colon and rectum, but not the caecum of the colon. The reason for the site-specific reduction is unknown. As discussed previously, GR expression levels in the caecum of healthy NT rats housed in an environment containing bacteria (SPF) are lower than elsewhere in the colon. Therefore GR expression levels in both healthy and diseased rats are similar because of the low expression in the caecum. The lack of GR protein expression in caecum could be due the presence/lack of specific subsets of bacteria which drive GR expression (Hoentjen *et al.* 2003; Rath *et al.* 2001; Sartor 2004b). This would account for the differences noted in specific areas of the colon between diseased and non-diseased animals in SPF conditions.

The glucocorticoid receptor is under 'auto-regulation' by the HPA axis, and corticosterone is known to down-regulate GR expression (Meyer & Schmidit 1994). As discussed earlier, inflammation in diseased animal models increases corticosterone levels (Murakami *et al.* 2002). As corticosterone activates GR, the negative feedback control of this gene would down-regulate GR in order to prevent over-activation, and therefore this could be a reason for the decreased expression noted in diseased animals.

Data presented in this chapter imply bacteria regulate GR expression within the colon. This was shown by the decrease in GR expression in germ-free NT animals compared to healthy NT animals in SPF conditions. As described above, this

decrease was localised to proximal and distal colon and the reason for this may be due to the presence of specific bacteria which drive GR expression being localised to different areas of the colon. These observations contradict work in the previous chapter where antibiotic treatment increased GR expression in distal colon. These discrepancies could be due to inherent genetically determined differences in GR regulation in rodent models; the composition of bacterial flora in Fischer compared to Wistar rats; or even an effect of antibiotics on directly regulating GR.

The explanation for the decreased GR expression in GF animals remains uncertain. However a possible explanation may be that animals in GF environments have a relatively underdeveloped immune system; a delayed hypersensitivity to T-cell responses as well as under developed Peyer's patches, mesenteric lymph nodes (MLN) and mononuclear cell population have been shown to be present in GF animals (Sartor 2004b). Bacteria may be required to 'prime' the immune system and activate GR expression in the mucosa. Therefore, as the mucosa of these animals have never been in the presence of bacteria, GR is lowly expressed. To clarify this theory, the colon of healthy rats in a conventional environment should be cleared of micro-organisms in order to study whether mucosal GR expression is changed when the immune system has been previously activated in these animals.

Interestingly, T-cells have been shown to express GR where it functions as an inducer of apoptosis in these cells; increased expression sends these cells into apoptosis (Geley *et al.* 1996). If the signal is the same in epithelial cells, this may provide a reason as to why in the mucosa of diseased animals have elongated crypts, as GR expression in these animals are reduced and therefore cells do not have an apoptotic signal, so cells continue to grow and crypts become elongated.

In summary, data in this chapter show mdr1a/P-glycoprotein and GR protein expression varies in a gradient from proximal to distal colon in healthy Fischer 344 rats, as previously shown in Wistar rats. Disease and the presence of bacterial flora regulate both mdr1a/P-gp and GR protein expression, with disease down-regulating both mdr1a/P-gp and GR protein expression, whereas the flora influence expression in a site-specific manner. To further clarify the role of bacteria in regulating

expression of these genes, gnotobiotic studies should be undertaken to investigate the effect of different types of bacteria on P-gp and GR expression in different regions of the colon. This may provide information as to which bacteria are involved in both initiating disease and altering P-gp and GR levels. Changes in expression of these genes may be involved in inflammation.

Chapter 6

Regulation of Intestinal P-glycoprotein and Glucocorticoid Receptor Expression by Dexamethasone in the HLA-B27 Transgenic Rat Model of Colitis

6.1 INTRODUCTION

Animal models of intestinal inflammation have provided vital insights into the pathogenesis of intestinal disease, from investigating the involvement of specific bacteria in the initiation of inflammation through to the investigation of immune-regulatory pathways and efficacy of possible therapeutic agents (Sartor 2004a). As discussed in chapter 5, HLA-B27 transgenic rats develop colitis under specific pathogen-free (SPF), but not germ-free conditions. Inflammation is fully developed by 12 weeks of age in an SPF environment, however if a transgenic adult (8-12 weeks) rat is re-housed from a GF to an SPF environment, an aggressive caecal colitis develops within 2-4 weeks after bacterial colonisation (Rath 2003; Sartor 2004a). This suggests the onset of disease is age-dependent, and could possibly be due to the maturation of the intestinal epithelium.

There have been a substantial number of studies undertaken in these animals regarding the role of bacteria in disease, including the effect of antibiotic treatment on intestinal inflammation (Sartor 2004a; Sartor 2004b). However the effect of glucocorticoids, such as dexamethasone, has not been studied in this model. This is interesting as corticosteroids remain a mainstay of treatment for patients with IBD (Arnott *et al.* 2003), and the inflammation seen in this animal model resembles that noted in patients with active IBD.

P-glycoprotein, a transporter pump present in the intestinal epithelium, is thought to be involved in the detoxification process and is known to transport various drugs, including dexamethasone, and other potentially toxic molecules out of cells and into the intestinal lumen (Ho *et al.* 2003). Moreover, inflammation has also been shown to be associated with a reduction in MDR1 mRNA and mdr1a/P-glycoprotein expression in patients with IBD and animal models respectively (Langmann *et al.* 2004; Lizasa *et al.* 2003). This reduction in expression and consequent increase in the intracellular concentration of dexamethasone, may affect the severity of inflammation and therefore alter P-gp expression in these rats.

Data presented in the previous chapter have shown, in these animals, inflammation is associated with a reduction in GR expression. Dexamethasone initiates its effects

after binding to GR in the cytoplasm where it translocates to the nucleus and increases the transcription of anti-inflammatory molecules, as well as directly interacting with pro-inflammatory transcription factors (Barnes 1998). Therefore a decrease in intestinal GR expression may reduce the sensitivity of the intestinal epithelium to steroids, and render these animals steroid insensitive.

The work described in this chapter has investigated a) the effect of dexamethasone treatment on the expression of mdr1a/P-glycoprotein and GR expression in healthy HLA-B27 null rats b) the effect of steroid treatment on the expression of these genes in diseased HLA-B27 transgenic rats.

6.2 METHODS

6.2.1 Animals

As in chapter 5, TG and NT Fischer animal tissues were a gift from Professor R.B. Sartor, Chapel Hill University, North Carolina, USA. Transgenic and non-transgenic rats were subcutaneously injected with dexamethasone dissolved in ethanol/saline (et/OH) (125μg/kg/day) or vehicle (et/OH) for 1 week. On day 8, rats were sacrificed and caecal, proximal colon and rectal sections frozen in OCT medium for use in situ hybridisation, and adjacent sections snap frozen on dry ice for use in Western blotting. All sections were shipped on dry-ice and stored at minus 80°C.

6.2.2 *In situ* Hybridisation

Caecal, proximal colon and rectal sections were mounted on saline-coated slides. Transcribed antisense ^{35}S-UTP riboprobes for GR and mdr1a were used to determine mRNA expression as described in section 2.6. Expression was quantified by analysing the autoradiograph films where the optical density (units were given arbitrarily) was measured for whole tissue (GR) or epithelial areas (mdr1a).

6.2.3 Western Blotting

GR protein and P-glycoprotein levels were measured by Western blotting. All sections were homogenised and loaded onto polyacrylamide gels as described in section 2.6. Homogenates from 3 animals from each of the 4 groups (DT-DEX treated transgenic, ST-saline treated transgenic, DN-DEX treated non-transgenic, SN-saline treated non-transgenic animals) from the same intestinal section were ran on one gel. All 3 gels containing either caecal, proximal colon or rectal sections were blotted and analysed together and this was repeated so all 6 animals per group were analysed.

GR and P-glycoprotein levels were analysed using the same antibody preparations as shown in table 4.1. Loading discrepancies were assessed by measuring tubulin expression for each sample, using the same antibodies and dilutions as described in section 4.2.3. All bands were analysed by densitometry using the MCID-M4 Image Analysis programs and results normalised against tubulin.

6.2.4 Statistics

All values are shown as the mean ± S.E.M. Differences between values were compared using ANOVA with Tukeys post-hoc comparisons. P values of less than 0.05 were considered significant.

6.3 RESULTS

6.3.1 The effect of dexamethasone on inflammation in diseased animals

3 of the 6 diseased animals (transgenic) treated with dexamethasone had reduced inflammation associated with arthritis. Inflammation was seen to reduce and mobility increased in these animals. Figure 6.1 shows histological pictures taken from dexamethasone- and saline treated transgenic rats.

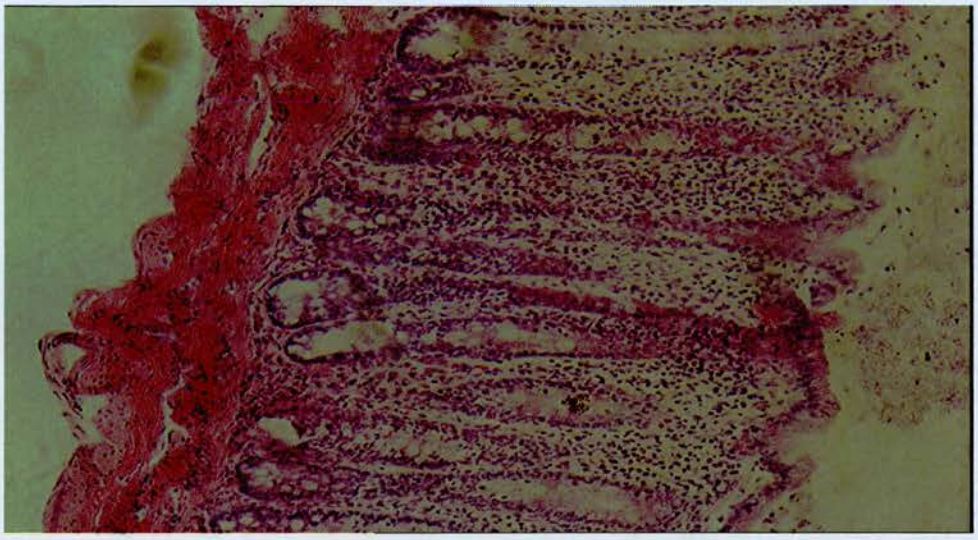
6.3.2 The effect of dexamethasone on mdr1a and P-glycoprotein expression

Rats with intestinal inflammation (transgenic rats) had significantly reduced mdr1a and P-glycoprotein compared to non-transgenic rats in all parts of the colon (figure 6.2). Dexamethasone did not significantly alter mdr1a or P-glycoprotein expression in the caecum, proximal colon or rectum.

6.3.3 The effect of dexamethasone on Glucocorticoid Receptor mRNA and protein expression

Dexamethasone treatment did not affect GR mRNA expression in the caecum, proximal colon or rectum of either transgenic or HLA-B27 null mice (figure 6.3). Dexamethasone increased GR protein levels in the caecum of healthy NT rats ($p<0.001$) compared to saline-treated NT rats. In the proximal colon of NT rats, DEX treatment slightly reduced GR protein levels compared to saline-treated NT rats, whereas in the rectum dexamethasone did not alter GR protein expression. In the transgenic diseased rats, DEX treatment did not alter GR protein expression in caecum, although in the proximal colon and rectum, TG rats treated with DEX had increased GR protein levels compared to saline-treated TG rats, and this increase was significant in the rectum ($p<0.05$).

a)



b)

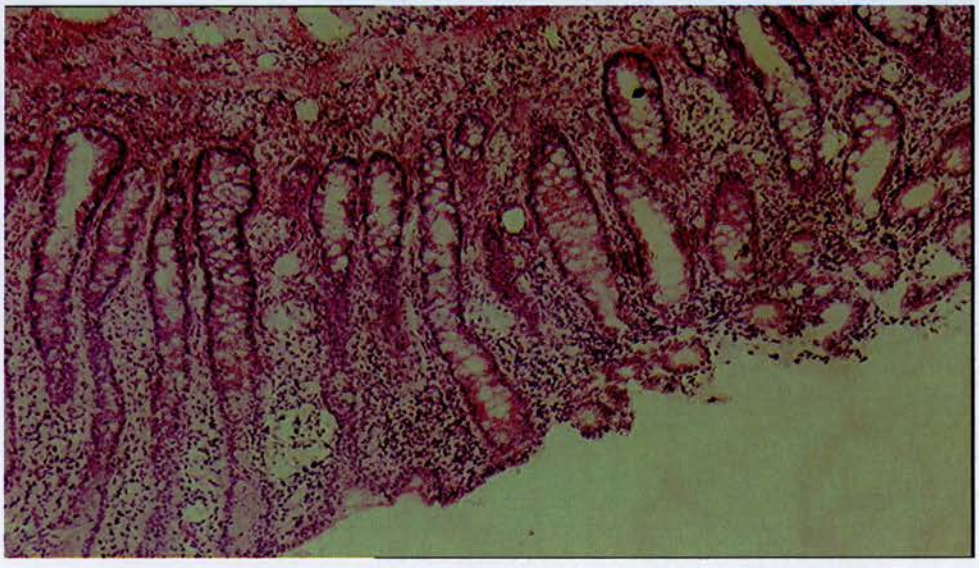


Figure 6.1: Haematoxylin and eosin staining of the caecum from transgenic rats with intestinal inflammation treated with saline (a) and dexamethasone for 1 week (b). Crypts are elongated and have infiltration of mononuclear cells (stained dark purple) in the mucosal layer compared to non-transgenic animals in SPF conditions (see page 130). Dexamethasone treatment does not seem to have reduced infiltration and crypts remain elongated.

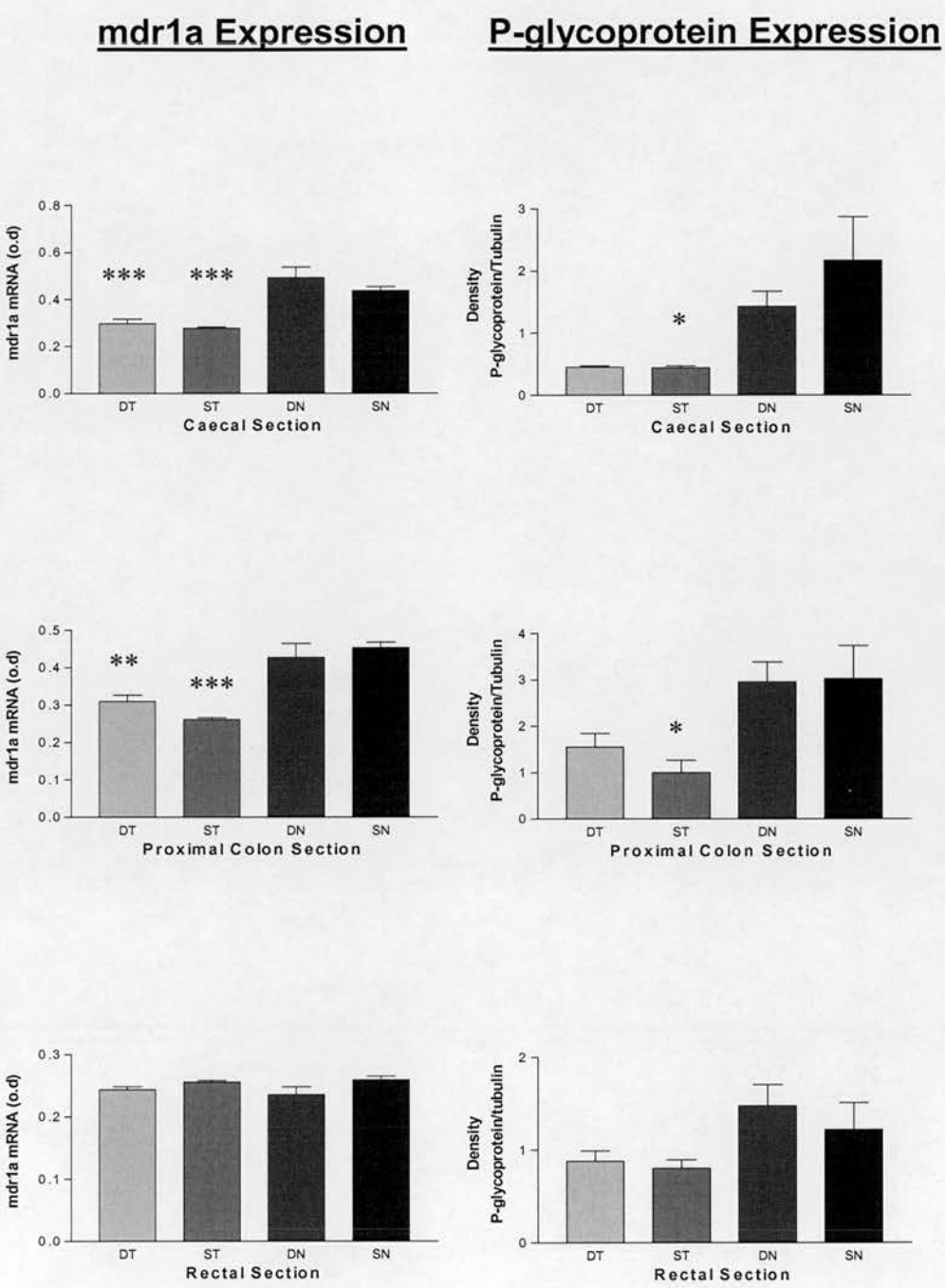


Figure 6.2: mdr1a (left) and P-glycoprotein (right) expression in caecum, proximal colon and rectum. mdr1a levels were significantly increased in healthy non-transgenic rats treated with/out dexamethasone (DN, SN) compared to transgenic diseased rats treated with/out dexamethasone (DT, ST, $p<0.01$, $p<0.001$) in caecum and proximal colon sections. There was no difference in mdr1a expression between the groups in the rectal sections. P-glycoprotein levels in all colonic sections were reduced in the transgenic diseased rats and this reduction was significant in the caecum and proximal colon ($p<0.05$). Dexamethasone treatment did not affect P-glycoprotein expression in either the TG or NT rats in any section.

* comparison between ST versus SN or DT versus DN

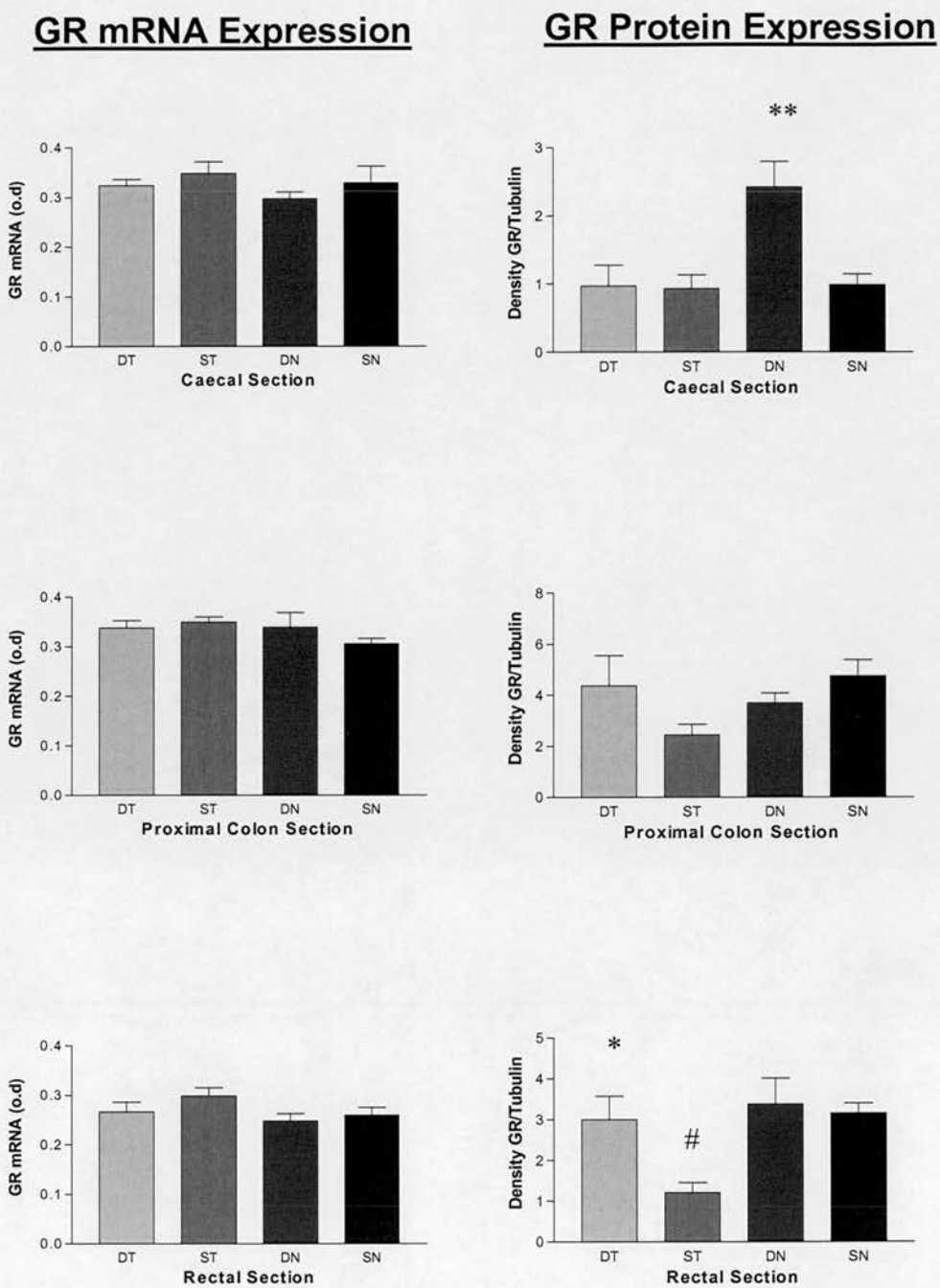


Figure 6.3: GR mRNA (left) and protein (right) expression in caecum, proximal colon and rectum. GR mRNA expression was unaltered by dexamethasone treatment in the TG or NT groups (DT, DN respectively). In the caecum, DEX treatment significantly increased GR protein levels in healthy NT rats (DN) (**$p<0.01$) when compared to saline-treated NT animals (SN). No difference was noted between DEX-treated and saline-treated diseased TG rats (DT, ST respectively). In proximal colon GR protein levels slightly decreased after DEX treatment in NT rats however in the rectum of the same rats, DEX treatment did not change GR protein expression. TG diseased rats treated with dexamethasone had an increase in GR protein levels in the proximal colon and rectum, which was significant in the rectal sections (*$p<0.05$).

* comparison between SN versus DN or ST versus DT
# comparison between SN versus ST

6.4 DISCUSSION

Glucocorticoid therapy is effective at reducing inflammation in both IBD and other inflammatory disorders including asthma (Faubion *et al.* 2001; Wikström 2003). Steroid-resistance and steroid-dependence are common problems in clinical practice. Alterations in the expression of P-glycoprotein and the glucocorticoid receptor have been implicated in explaining inter-individual responsiveness in efficacy (Farrell *et al.* 2000; Langmann *et al.* 2004; Rogler *et al.* 1999). In the previous chapter inflammation was shown to reduce both P-gp and GR expression, and these changes maybe involved in the initiation or perpetuation of disease. In this chapter we have investigated the effect of dexamethasone-treatment on expression of these genes during inflammation.

6.4.1 Effect of dexamethasone on intestinal inflammation

In these experiments dexamethasone was seen to decrease the symptoms of arthritis however inflammation noted in the colon, particularly the histology of elongated crypts with an infiltration of mononuclear cells, was seen to be unaltered after therapy. Previous studies have shown that the dose of dexamethasone administered decreased inflammation in joints and intestinal mucosa in Lewis rats where inflammation had been previously induced by dextran sodium sulphate (Herfarth *et al.* 1998). The differences may be explained by the induction method of inflammation or even differences in steroid sensitivity between Lewis and Fischer rats.

6.4.2 Effect of dexamethasone on mdr1a/P-glycoprotein expression in inflamed and non-inflamed colon

As noted previously, inflammation significantly decreased mdr1a and P-gp expression in all areas of the rat colon. However dexamethasone treatment had no effect on expression of these genes in either the healthy or diseased colon. This is of note as dexamethasone treatment was seen to decrease mdr1a mRNA expression in the healthy Wistar rat colon, whereas a recent *in vivo* study has shown DEX treatment did not alter mdr1a expression in the rat colon (Mei *et al.* 2004). Mei and colleagues

showed oral dexamethasone treatment of 1mg/kg/day and 20mg/kg/day for 3 days, increased mdr1a and P-gp expression in the small intestine but not colon, suggesting tissue-specific mdr1a/P-glycoprotein regulation.

Discrepancies between the effect of dexamethasone on mdr1a/P-gp expression in the healthy rat colon in these studies could be due to differences in steroid regulation and sensitivity in specific stains of rats, or even due to the differences in treatment administration-oral versus subcutaneous injection. In chapter 3, Wistar rats were treated with 200μg/kg/day whereas in the present chapter rats were treated with 125μg/kg/day, therefore a possible explanation behind the lack of effect of dexamethasone on mdr1a/P-gp expression may be due to the dose of dexamethasone, where the dose was effective at reducing the symptoms of inflammation, but not at an effective dose to alter mdr1a/P-gp expression.

The lack of effect of dexamethasone on mdr1a/P-gp expression in the healthy Fischer rat colon may alternatively be due to differences in steroid regulation of these genes between the strains or even steroid sensitivity. Fischer 344 rats are known to be more sensitive to the endogenous steroid oestrogen (Putz *et al.* 2001), however the sensitivity to exogenous glucocorticoids have not been studied. If we presume these rats have a heightened sensitivity for all steroids, the reduced dose of dexamethasone given to Fischer 344 rats may have been of the same efficacy as the increased dose given to Wistar rats, where dexamethasone decreased mdr1a expression.

In the diseased colon, dexamethasone treatment also did not alter mdr1a/P-gp expression in any section of the colon. Again the lack of effect could be due to the reasons cited above; because the dose given is at a therapeutic level to decrease symptoms associated with inflammation (joint inflammation) but not alter gene expression; or because the inflammation present was altering the efficacy of steroids by down-regulating GR expression (see next section).

6.4.3 Effect of dexamethasone on GR expression in the inflamed and non-inflamed rat colon

In chapter 3, dexamethasone was seen to reduce GR mRNA expression in proximal and distal colon, however in these healthy Fischer 344 rats, steroid treatment did not alter colonic GR mRNA expression but differentially regulated GR protein expression in a site-specific manner. Differences in mRNA expression between the rat strains may be due to differences in steroid regulation of GR in these animals, whereas discrepancies between mRNA and protein expression may be explained by post-translational modifications or even the stability of the mRNA/protein.

Interestingly, dexamethasone was seen to differentially regulate colonic GR protein expression in healthy Fischer 344 rats: GR increased in the caecum, slightly decreased in proximal colon and was unaltered in the rectum. This novel finding indicates the complexity of steroid regulation in the rat colon. Previous work has shown dexamethasone treatment did not alter GR mRNA in distal colon (Escoubet *et al.* 1996) in Sprague-Dawley rats and further supports the diversity of steroid regulation noted in specific rat stains and also work presented in this chapter. Site-specific changes in GR expression in response to steroids may alter the sensitivity of these areas of the colon to steroid treatment, and this may be important when treating colonic inflammation.

In the inflamed rat colon, GR levels were seen to increase to 'healthy' levels in the rectum by dexamethasone, but not in any other area studied. The lack of effect of DEX in caecum and proximal colon may be due to the severity of inflammation noted in the caecum and proximal colon. It is known that the most severe inflammation is noted in caecum compared to rectum, and GR levels are reduced in the inflamed colon (see chapter 5) and therefore the efficacy of dexamethasone in the diseased colon may be reduced. As inflammation in rectum compared to the other colonic areas is less severe, the efficacy of dexamethasone in this area may be sufficient to resolve inflammation and therefore returning GR protein expression to 'healthy' levels. This observation may be important when using dexamethasone as a treatment for colonic inflammation, as steroids may not be as effective in treating caecal or proximal colonic disease.

Work presented in this chapter shows differences in the regulation of colonic genes determining steroid sensitivity in health and disease, and the diversity of steroidal-gene regulation between rodent strains. The site-specific differences in GR regulation by dexamethasone in inflamed and non-inflamed colon may be important in determining the effectiveness of steroid treatment in colitis, and possible reasons behind steroid-insensitivity in inflammatory disease.

Chapter 7

Intestinal Epithelial Cells: The Regulation of P-glycoprotein and Glucocorticoid Receptor by Dexamethasone

7.1 INTRODUCTION

In order to investigate further the mechanisms underlying our previous work, in which we observed that dexamethasone decreased both GR and mdr1a mRNA in rat colonic epithelial cells *in vivo* (chapter 3), we have studied the effects of dexamethasone *in vitro* using the cell line-IEC-6. This is a rat jejunum non-transformed cell line. This cell line was used as previous studies have shown IEC-6 express both functional GR and P-glycoprotein (Göke *et al.* 2002; Li *et al.* 1999), and also because there is not a non-transformed rat colonic cell line.

As discussed in chapter 1, dexamethasone elicits its anti-inflammatory response by increasing transcription of anti-inflammatory cytokines and decreasing transcription of pro-inflammatory cytokines as a result of a direst interaction with pro-inflammatory transcription factors AP-1 and NF-κB. It also increases the transcription of IκB, which inhibits NF-κB by sequestering it in the cytoplasm (Jobin & Sartor 2000; Neurath *et al.* 1998).

There have been conflicting studies reported regarding the involvement of these two transcription factors in the regulation of the mdr1a gene. In mouse hepatocellular carcinoma AP-1 was seen to function as a negative regulator of mdr1a; however in the hamster, AP-1 functions as a positive regulator (Ikeguchi *et al.* 1991; Teeter *et al.* 1991). AP-1 has been shown to be up-regulated in human breast cancer cells that over-express P-gp (Ratnasinghe *et al.* 2001). NF-κB has also been shown to increase P-gp and protect kidney tubules from apoptosis (Thevenod *et al.* 2000), as well as increasing P-glycoprotein in colon cancer cells (Bentires *et al.* 2003). However the involvement of these transcription factors in the regulation of rodent intestinal P-gp has not been documented.

Moreover, the effect of dexamethasone on GR and P-glycoprotein expression within IEC-6 cells has not been studied. Previous work has shown dexamethasone regulates P-gp in a tissue- and disease-specific manner. Dexamethasone treatment of rat hepatocytes decreased P-gp levels (Fardel *et al.* 1993), whereas in mouse non-hepatoma cells mdr1a was unchanged by DEX . However in mouse hepatoma cells, dexamethasone increased mdr1a levels (Zhao *et al.* 1993). *In vivo* studies has shown

DEX increases P-gp in liver, lung and intestine, but decreases expression in the kidney (Demeule *et al.* 1999; Murakami *et al.* 2002). There are also limited data on GR levels in response to dexamethasone in IEC-6 cells, although DEX has been shown to induce morphological and proliferation changes in these cells (Göke *et al.* 2002; Quaroni *et al.* 1999).

Therefore this study aimed to determine (a) whether the dexamethasone-mediated effects on P-glycoprotein and GR expression *in vitro* correlate with results seen *in vivo*, (b) if these effects are mirrored by changes in NF-κB/AP-1 levels and (c) whether alterations in transcription factor protein levels correlate with nuclear activation.

7.2 METHODS

7.2.1 Cell Lines

IEC-6 cells were cultured and plated as described in section 2.3.2. Cells were grown to 90% confluency prior to treatment. Total cell lysates were collected as described in section 2.7.1.

7.2.2 Treatments

For all experiments dexamethasone and RU486 (see below) were dissolved in 100% ethanol, filter sterilised, and diluted 2 x100 in stripped serum.

To investigate the effect of dexamethasone on P-gp and GR expression over a 24hr period, dexamethasone (1μM) was added 24hr, 8hr, 4hr and 2hr prior to harvesting. Total cellular NF-κB and AP-1 expression in these samples were also measured. A maximum time point of 24hr was used to prevent cell overgrowth, as this may have interfered with cellular response to dexamethasone.

To study whether dexamethasone was acting through the glucocorticoid receptor rather than other steroid receptors, for example MR, RU486 was used. This is a competitive GR antagonist, and works by preferentially binding to GR when present at a higher concentration than dexamethasone, and therefore prevents dexamethasone binding to GR and initiating its effects. RU486 was added at 100μM (a concentration x100 of dexamethasone) 4hr prior to addition of dexamethasone/vehicle to encourage binding to GR before adding dexamethasone. Thereafter, fresh stripped serum containing RU486 (100μM) with/out dexamethasone (1μM) was added 24hr before harvesting.

To investigate whether changes in NF-κB expression noted after 24hr dexamethasone-treatment occurred in the nucleus or cytosol, cells were treated with/out DEX (1μM) for 24hr, and nuclear and cytosolic fractions were collected as described in section 2.7.1. To study whether NF-κB was sequestered in the

cytoplasm through binding to its inhibitor protein IκB-α, levels of this protein in these samples were also measured.

7.2.3 Western Blotting

Western Blotting was performed on the protein samples as described in section 2.7.2. Protein samples from each experiment were run together on individual gels, and each experiment performed in triplicate. All 3 gels were analysed together to prevent differences between the density of films.

To analyse the effect of dexamethasone and RU486 at various time points, antibodies against P-glycoprotein (ID labs, Canada), GR (Santa Cruz, CA), AP-1(Santa Cruz, CA) and NF-κB (Santa Cruz, CA) were used in combination with a horseradish-peroxidase linked-secondary antibody (see table 2.3) as described in section 2.7.3. For each blot discrepancies in loading was controlled by analysing tubulin expression. Blots were stripped and a primary anti-mouse monoclonal tubulin antibody (1:5000 dilution), followed by an HRP-linked anti-mouse secondary antibody (1:1000 dilution) added. Protein was detected by ECL as previously described in section 2.7.4, and the density of bands analysed and loading corrected against the density of tubulin for each sample. When analysing cytosolic and nuclear fractions, loading was controlled by staining the membrane with 0.4% pyronin, and measuring the density of a non-specific protein band.

7.3.4 Statistics

All data are expressed ± S.E.M. All experiments were performed in triplicate. One-way ANOVA with post-hoc Turkey comparisons was used to compare differences between groups. P values of less than 0.05 were considered significant.

Experiment	Primary Antibody & Dilution	Secondary Antibody & Dilution
Time Course	Monoclonal-C219 P-glycoprotein 1:200 Polyclonal - Glucocorticoid Receptor 1:400 Monoclonal -AP-1 1:300 Monoclonal-NF-κB (p65) 1:300	Anti-mouse 1:1000 Anti-rabbit 1:1000 Anti-mouse 1:1000 Anti-mouse 1:1000
RU486	Monoclonal-C219 P-glycoprotein 1:200 Polyclonal- Glucocorticoid Receptor 1:400	Anti-mouse 1:1000 Anti-rabbit 1:1000
Cytosolic and Nuclear Extracts	Polyclonal-NF-κB (p65) 1:200 Monoclonal- IκB-α 1:200	Anti-rabbit 1:1000 Anti-mouse 1:1000

Table 7.1: Primary and secondary antibody combinations and dilutions for each experiment.

7.3 RESULTS

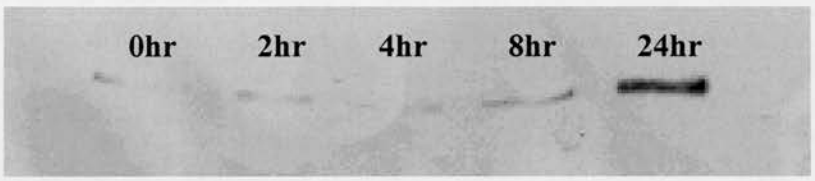
7.3.1 Effects of dexamethasone on P-glycoprotein and GR protein expression over 24 hours

Dexamethasone treatment significantly increased P-glycoprotein expression after 24hr ($p<0.001$). Trends towards increased P-gp expression after 2, 4 or 8hr treatment (figure 7.1) were also seen, but not statistically significant. In contrast, GR protein expression was significantly decreased after 2hr ($p<0.05$), 4hr ($p<0.01$) and 8hr DEX treatment ($p<0.01$), with lowest expression noted after 24hr dexamethasone-treatment ($p<0.01$) (figure 7.2).

7.3.2 GR antagonist (RU486) inhibits the dexamethasone mediated effects on P-glycoprotein and GR protein expression

Figure 7.3 shows dexamethasone significantly increased P-glycoprotein expression ($p<0.01$) after 24hr treatment, and this increase was inhibited by RU486. The significant reduction ($p<0.01$) of GR protein by DEX after 24hr was also inhibited by RU486 (figure 7.4). RU486 alone did not have an effect on expression of either P-glycoprotein or GR.

(a)



(b)

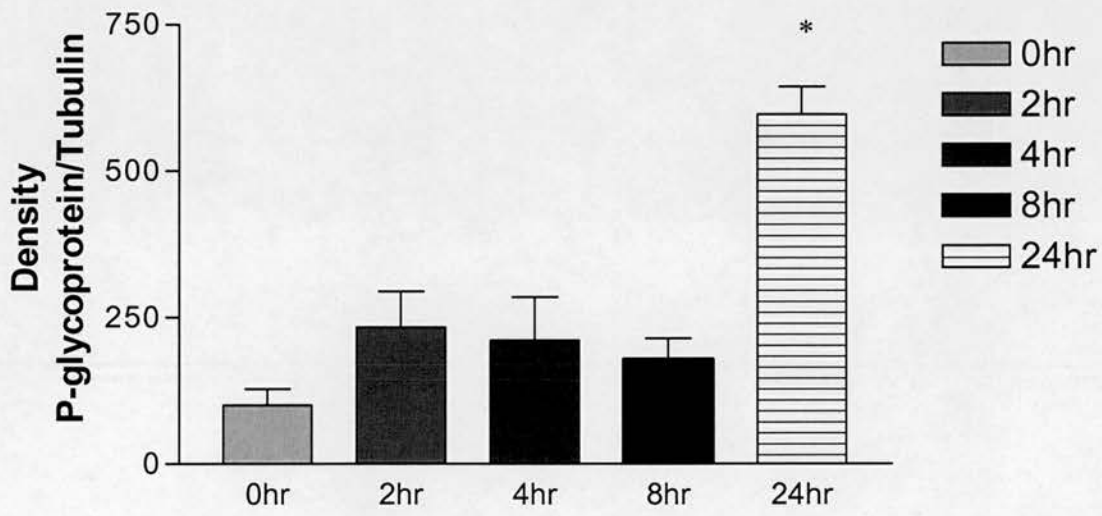
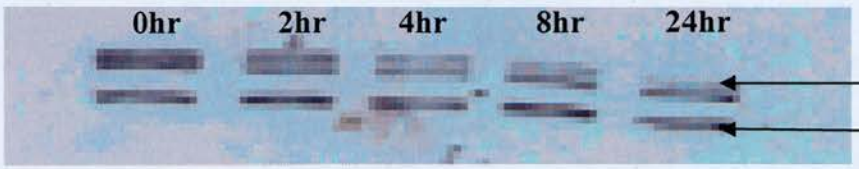


Figure 7.1: (a) Western blot showing expression of P-glycoprotein in IEC-6 cells whole-cell lysates after dexamethasone treatment (1μM) over 24hr. (b) Graph illustrating dexamethasone increased P-glycoprotein expression significantly after 24hr ($p<0.001$).

(a)



(b)

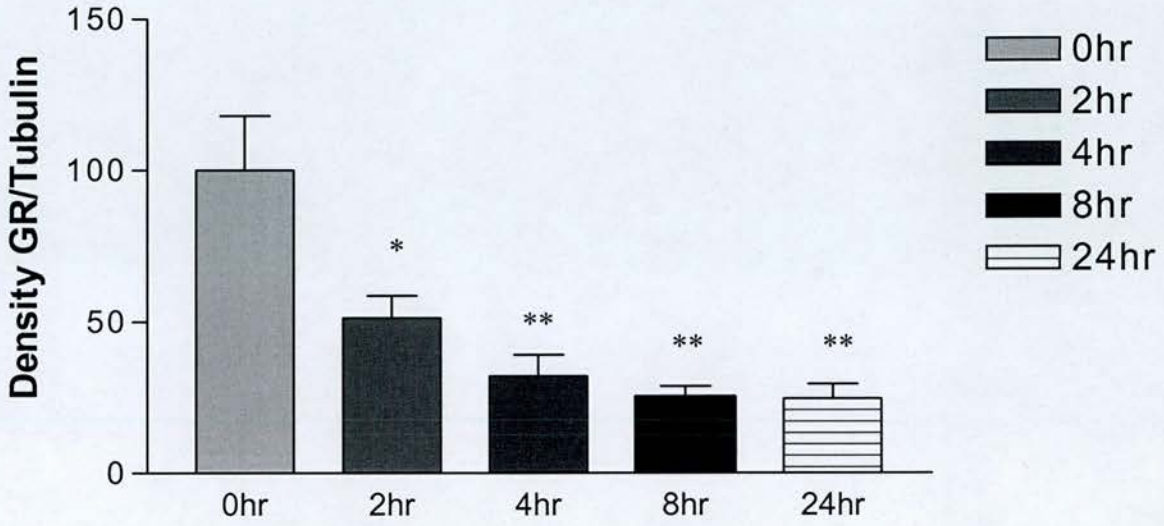


Figure 7.2: (a) A typical Western blot showing GR expression in whole cell lysates from IEC-6 cells after 0, 2, 4, 8 and 24hr dexamethasone treatment (1μM). (b) Graph illustrating dexamethasone decreased GR levels in time-dependent manner (*$p<0.05$, **$p<0.01$).

(a)

(b)

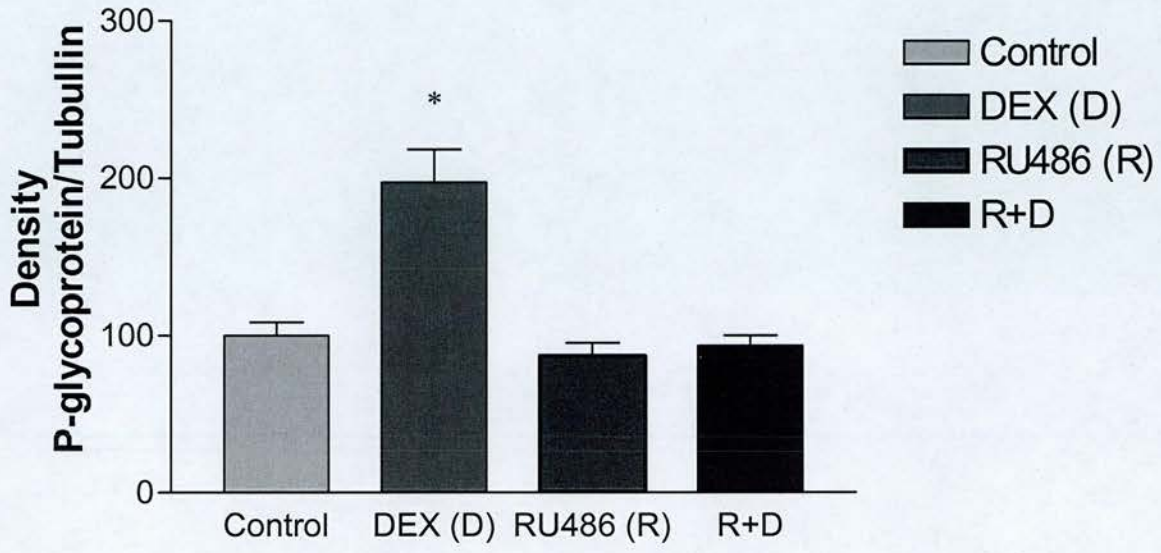


Figure 7.3: (a) A typical Western blot representing whole cell lysate P-glycoprotein expression in IEC-6 cells after dexamethasone (1μM) treatment. The dexamethasone-mediate effects are inhibited by the GR antagonist RU486 (100μM). (b) Graph showing 24hr dexamethasone treatment increased P-glycoprotein levels (*$p<0.01$), and this increase was inhibited when GR was blocked using RU486. RU486 did not have an effect on P-glycoprotein expression.

(a)

(b)

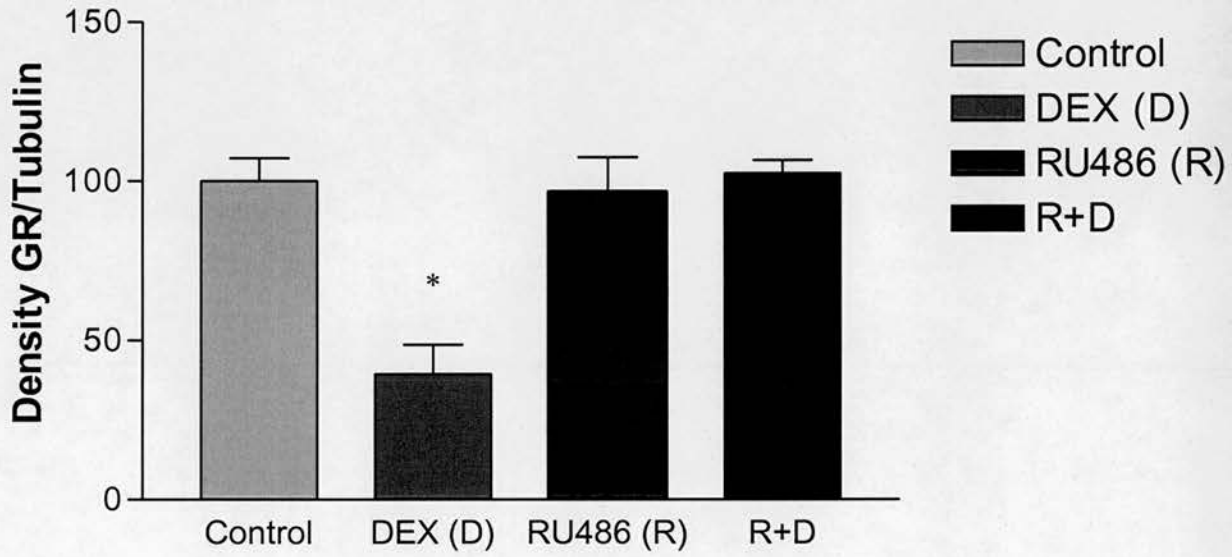


Figure 7.4: (a) Western blot showing whole cell lysate GR protein expression in IEC-6 cells. GR protein levels were decreased in IEC-6 cells by 60% (*$p<0.01$) after 24hr dexamethasone treatment (1μM). This decrease was inhibited by the GR antagonist RU486 (100μM) (b).

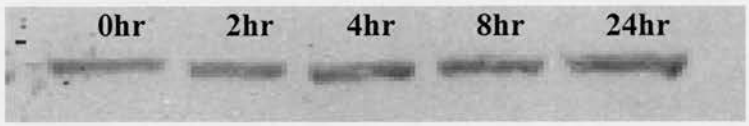
7.3.3 AP-1 and NF-κB protein expression after dexamethasone treatment

Whole cell lysate AP-1 protein levels were unaffected by 2hr, 4hr, 8hr and 24hr dexamethasone treatment (figure 7.5). However figure 7.6 shows DEX significantly increased total NF-κB expression by 50% and 45% after 8hr and 24hr respectively ($p<0.05$).

7.3.4 Nuclear and Cytosolic NF-κB & IκB-α protein expression after dexamethasone treatment

Dexamethasone treatment significantly altered both P-gp and NF-κB expression only after 24 hour treatment. To investigate the compartmentalisation and activity of increased NF-κB after dexamethasone treatment (NF-κB is sequestered in the cytosol by binding to IκB) and suggest a role for NF-κB in the regulation of P-gp in intestinal epithelial cells, NF-κB expression in cytosolic and nuclear fractions were studied, as well as levels of IκB-α . In untreated IEC-6 cells, NF-κB expression was similar in cytosolic and nuclear fractions. NF-κB expression was also similar in cytosolic extracts regardless of treatment. After cells were stimulated with dexamethasone for 24hr, figure 7.7 shows nuclear NF-κB increased by approximately 75% compared to the cytosolic fractions. In the same samples IκB-α levels were also analysed. IκB-α was found in abundance in the cytosolic extracts, and dexamethasone treatment did not alter levels. Nuclear fractions had approximately 85% less IκB-α compared to cytosolic preparations (figure 7.8).

a)



b)

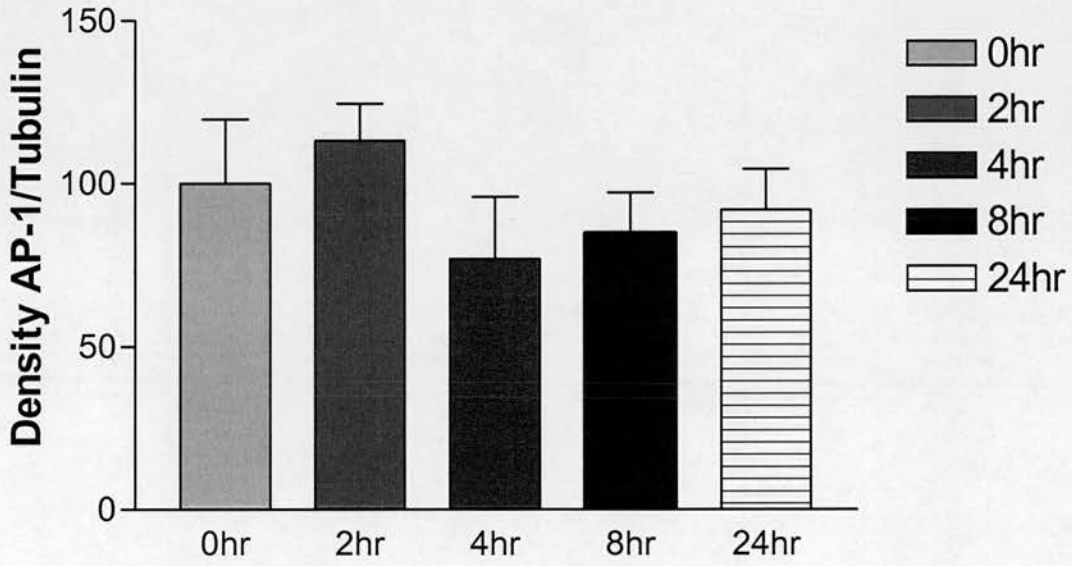
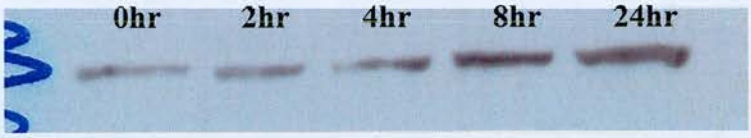


Figure 7.5: a) A typical Western blot showing whole-cell lysate AP-1 protein expression in IEC-6 cells. b) AP-1 expression was unaltered after 2hr, 4hr, 8hr and 24hr dexamethasone treatment ($P>0.05$).

(a)



(b)

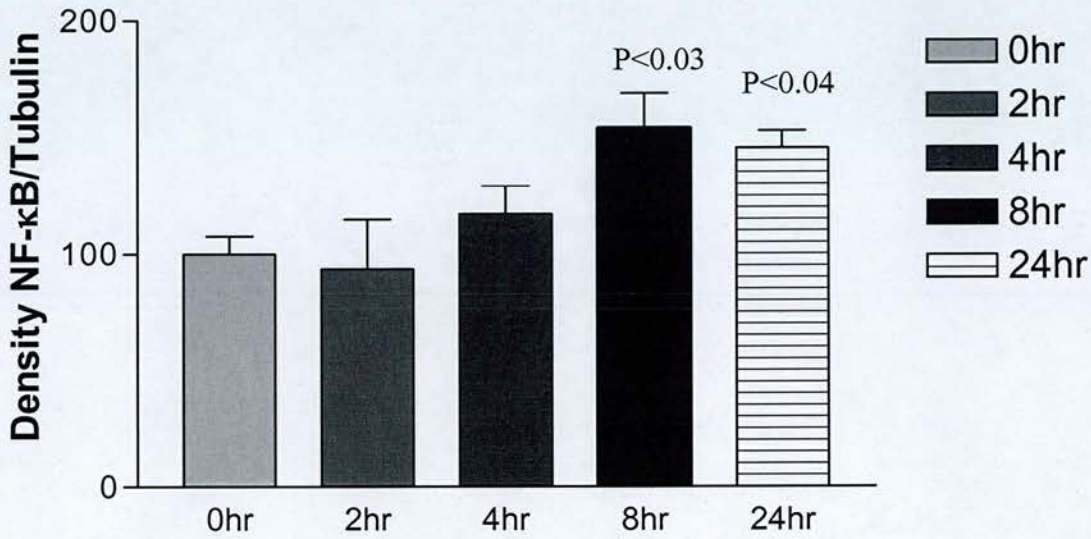
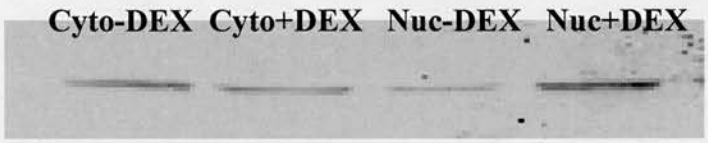


Figure 7.6: (a) Western film showing NF-κB protein expression in IEC-6 cells after 0, 2, 4, 8 and 24hr dexamethasone treatment. (b) NF-κB expression in whole cell lysates significantly increased after 8 and 24hr dexamethasone treatment ($p<0.03$, $p<0.04$ respectively).

(a)



(b)

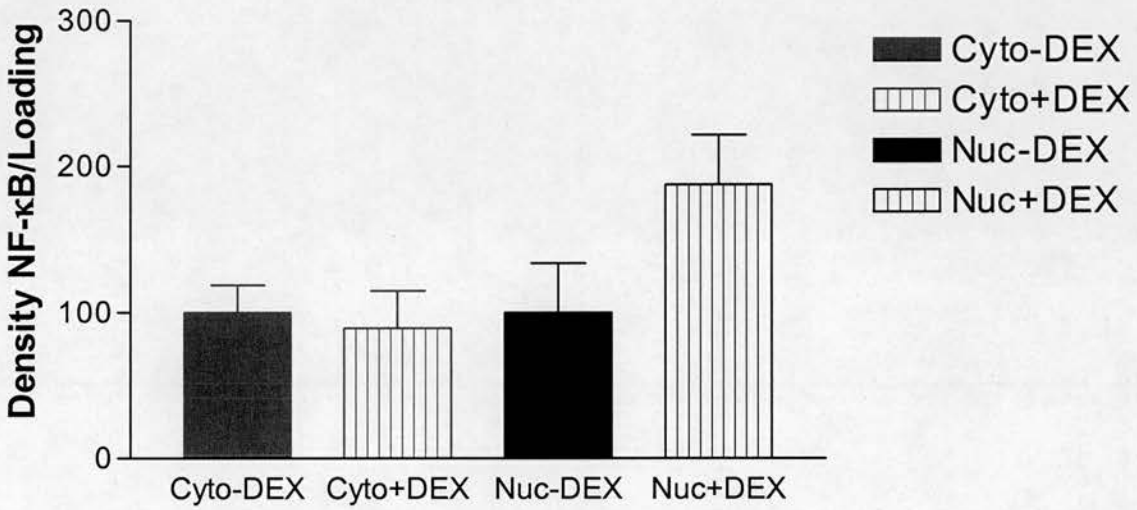


Figure 7.7: (a) A representative Western blot showing NF-κB protein expression in cytosolic (Cyto) and nuclear (Nuc) IEC-6 cell fractions treated +/- dexamethasone (1μM) for 24hr. (b) Similar protein expression was noted in cytosolic fractions treated with/out dexamethasone (Cyto-DEX, Cyto+DEX). There was no difference in NF-κB expression noted in nuclear and cytosolic extracts without DEX (Nuc-DEX and Cyto-DEX, respectively). However after dexamethasone treatment, there was a trend towards increased NF-κB protein expression in nuclear extracts (Nuc+DEX) compared to DEX-treated cytosolic fractions (Cyto+DEX) (p=0.1).

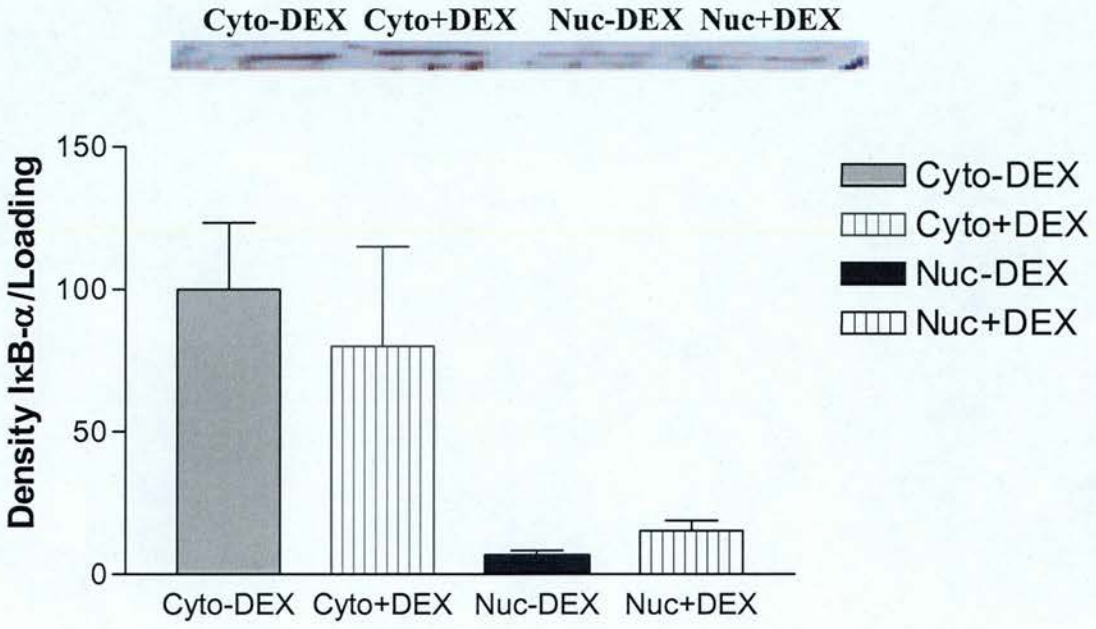


Figure 7.8: A representative Western film showing cytosolic and nuclear IκB-α protein expression. IκB-α expression was predominantly in the cytosol (Cyto), with nuclear (Nuc) extracts having approximately 85% less than cytosolic fractions. 24hr dexamethasone treatment (1μM) (+DEX) did not alter expression in either fraction.

7.4 DISCUSSION

Chapter 3 provided data on the effects of dexamethasone on rodent colonic epithelial cell expression of mdr1a and GR *in vivo*. However, before investigating the effects of altered epithelial cell expression of P-glycoprotein and GR on steroid sensitivity in health and disease, we have addressed the mechanisms by which steroids regulate these genes. This chapter presents novel data on the regulation of P-gp and GR in the rat intestinal epithelium, and also contains data concerning the possible transcriptional mechanisms underlying glucocorticoid action in epithelial cells.

7.4.1 Effects of dexamethasone on P-glycoprotein and GR expression *in vitro*

These data presented in this chapter demonstrate that P-glycoprotein expression was increased in IEC-6 cells after 24hr dexamethasone treatment, an effect inhibited by the glucocorticoid receptor antagonist. The effect of dexamethasone on IEC-6 P-gp expression, is consistent with previous *in vitro* studies using both rat and mouse hepatoma cells in which dexamethasone increased P-glycoprotein expression in the hepatoma cells (Fardel *et al.* 2001; Zhao *et al.* 1993), but contradicts work in non-hepatoma cells where dexamethasone treatment did not alter P-gp expression (Zhao *et al.* 1993). These discrepancies may possibly be due to differences in the mechanism of action of dexamethasone in specific tissues or disease states.

Recent *in vivo* work has shown oral dexamethasone (1 and 20 mg/kg/day), given for 3 days, increased mdr1a and P-gp in the rat small intestine (Mei *et al.* 2004). These data may be contrasted with work presented in chapter 3 where a decrease in colonic epithelial cell mdr1a mRNA after dexamethasone treatment was noted. Therefore a possible explanation for differences between mdr1a/P-gp expression *in vitro* and our *in vivo* data may be due to the differential regulation of mdr1a/P-gp by dexamethasone in the rat intestine (small intestine versus colon). Another reason for these discrepancies could involve cell-cell interactions. *In vivo,* intestinal epithelial cells are in close contact with cells of the lamina propria, and these cells could produce mediators which signal to the epithelial cell lining. This in turn may alter the regulation of these genes in response to dexamethasone.

The GR antagonist-RU486 is a competitive antagonist which will bind GR and prevent GR agonists, such as dexamethasone, from binding to the receptor and initiating its effects. Data present in this chapter illustrate that dexamethasone elicits its effects on P-glycoprotein expression by binding to GR, as the addition of the inhibitor RU486 prevented the increase in P-gp expression in these cells. Previous work supports these findings, where studies in rat hepatoma cells showed RU486 blocked dexamethasone activation of the mdr1b transcript (Schuetz *et al.* 1995). A possible explanation for the lack of effect on P-gp expression by dexamethasone prior to 24hr may be the result of *de novo* protein synthesis. GR bound dexamethasone may initiate the transcription and subsequent translation of a protein, and this 'second messenger' may then bind mdr1a. If GR was binding directly to mdr1a, changes in P-gp expression would be expected to have occurred prior to 24hr as noted with GR, where 2hr dexamethasone-treatment decreased GR expression (see below).

The regulation of GR by dexamethasone has been studied in a number of rodent tissues, including the hippocampus, liver and colon as noted in chapter 3 (Ghosh *et al.* 2000; Meyer & Schmidit 1994). These data suggest that GR may be under 'auto-regulatory' control; removing GCs by ADX increases GR levels, and reintroducing dexamethasone induces a subsequent decrease in the expression of GR (Holmes *et al.* 1995; Meyer & Schmidit 1994). The effect of dexamethasone on GR expression in IEC-6 cells has not previously been documented, although previous studies in IEC-6 cells have shown the K_D of GR (the concentration of ligand required to occupy half of the receptors in the cell) to be 1nM (Quaroni *et al.* 1999). Thus we can conclude that during these experiments our dose of DEX (1μM) saturated GR and therefore produced maximal effects.

In this chapter our data suggest that the same 'auto-regulatory' mechanism for control of GR in IEC-6 cells as previously postulated for rat hippocampal and colonic tissue-dexamethasone significantly decreased GR protein levels in a time dependent manner. This is consistent with data derived from studies with human ovarian carcinoma cells line (Pedersen & Vedeckis 2003) and also with rat NHI 3T3 cells (Hoeck *et al.* 1989), in which dexamethasone treatment reduced GR protein levels to around 20% of untreated cells. Furthermore, our work *in vitro* mirrors our previous

work *in vivo* (see chapter 3), where dexamethasone significantly decreased GR mRNA and reduced protein levels after 7 and 3 days DEX treatment respectively.

The data presented in this chapter showing an inhibitory effect of RU486 on GR expression further support our conclusions that GR is under 'auto-regulatory' control. As discussed in section 1, dexamethasone binds GR in the cytoplasm and causes the complex to translocate to the nucleus, where it binds GREs. This binding activates a negative-feedback loop which down-regulates GR. RU486 will compete with DEX to bind the glucocorticoid receptor. When RU486 binds GR, it prevents the translocation from cytoplasm to nucleus, binding to the GRE on the GR gene, and therefore prevents the negative feedback loop. Thus prevents a reduction in GR levels. This regulatory system controls cellular sensitivity to GC hormones and also steroidal drugs.

7.4.2 Effect of dexamethasone on transcription factors-AP-1 and NF-κB

There have been detailed investigations into the transcriptional regulation of human MDR and mouse mdr1a and mdr1b genes (Labialle *et al.* 2002; Sukhai & Piqutte-Miller 2000) but to date, such detailed studies have not been carried out in detail for the rodent mdr1a and 1b genes. Binding sites in the promoter region for NF-κB and AP-1 have been found in human MDR1 and murine mdr1a and 1b genes (Sukhai & Piqutte-Miller 2000) hence we decided to investigate whether changes in P-glycoprotein expression by dexamethasone were correlated with an alteration in the expression and activation of these transcription factors. In the resting cell, NF-κB is sequestered in the cytoplasm by its inhibitor IκB (Neurath *et al.* 1998). After the cell is stimulated by an activator of NF-κB, IκB is degraded and allows NF-κB to translocate to the nucleus and induce transcription (Schmid & Adler 2000).

Data presented in this chapter shows a significant increase in total NF-κB after 24hr dexamethasone treatment mirroring an increase in P-glycoprotein expression. As the increase in NF-κB levels was in the nuclear opposed to cytosolic fraction, it implies NF-κB is in its active state and therefore could potentially induce mdr1a gene transcription. The increase in nuclear NF-κB by dexamethasone was perhaps

unexpected as it a potent anti-inflammatory drug, shown to reduce inflammation by inducing the transcription of the inhibitor protein IκB-α, and directly binding to NF-κB in the cytoplasm and thus preventing the transcription of pro-inflammatory genes (de Bosscher *et al.* 2003). Dexamethasone does not increase the transcription of NF-κB directly as there no GRE sequences are present in the promoter region, which suggests dexamethasone must induce the transcription and subsequent translation of a protein which increases the transcription of NF-κB.

Consideration of the time course involved is pertinent in understanding the relevance between NF-κB activation and IκB-α degradation. Previous work in Caco-2 cells have shown IL-1β stimulation caused IκB-α to be degraded after 10min with a maximum peak in nuclear NF-κB DNA binding activity 15min after stimulation (Russo *et al.* 2004) therefore showing direct regulation of NF-κB by IL-1β. Another study involving human myeloid U937 cells showed a decrease in IκB-α, and an increase in nuclear NF-κB protein, 60min after activation by pervanadate (Mukhopadhyay *et al.* 2000). In our study, if dexamethasone had been regulating NF-κB directly, an increase in NF-κB protein expression would have been expected prior to 24hr. The synthesis of this 'second message' would account for the time delay between adding dexamethasone and the change in expression.

Another indicator of NF-κB activity is the level of IκB. IκB proteins included α, β, γ or ε (see chapter 1). IκB-α is known to bind the p65 subunit of NF-κB and sequester NF-κB in the cytoplasm (Neurath *et al.* 1998) . Upon activation, IκB-α is degraded and a reduction in protein levels is an indicator of NF-κB translocating to the nucleus. As expected, data in this chapter showed low levels of expression in the nucleus: IκB-α is a cytosolic protein, and this observation supports the conclusion that nuclear NF-κB is in its active state. As an increase in nuclear NF-κB expression was noted, one may have expected a decrease in cytosolic IκB-α to account for translocation from cytosol to nucleus, if this finding is of functional relevance. Once again the time course is critical, as previous studies have shown that although IκB-α is degraded within 10min after NF-κB stimulation, there is a subsequent increase in IκB-α expression to 'basal' levels within 90-180min. This is probably due to *de novo* protein synthesis generated by the 'negative feedback loop' from activated NF-κB

(Mukhopadhyay *et al.* 2000; Russo *et al.* 2004). We found similar levels of IκB-α in cytosolic fractions regardless of treatment, and we suggest that the most plausible explanation for this is the time point measured. It is known that dexamethasone and NF-κB directly increase the transcription of IκB-α. Therefore if IκB-α had been degraded, the dexamethasone-mediated *de novo* protein synthesis of IκB-α and the 'negative feedback loop' generated by an increase in NF-κB expression, would have returned IκB-α to resting levels 90-180min after stimulation. A decrease in expression at 24hr is of uncertain significance. Using a shorter time point, or a protein synthesis inhibitor (cycloheximide), may have revealed a decrease in IκB-α expression correlating with an increase in nuclear NF-κB activation.

To further investigate the role of these transcription factors in the regulation of these genes in IEC-6 cells, there is a need for more in depth experiments. The use of a protein synthesis inhibitor such as cycloheximide would clarify whether the increase in P-gp was indeed due to *de novo* protein synthesis, and would elucidate whether GR is directly or indirectly regulating P-glycoprotein expression. Also the direct inhibition of NF-κB activation using an adenoviral vector, where the protein inhibits a part in the activation pathway of NF-κB such as phosphorylation of IκB-α, would show whether preventing NF-κB nuclear translocation prevents increased P-glycoprotein expression. Although no difference in total NF-κB expression was seen after 2hr dexamethasone treatment, alterations in the cellular localisation (nuclear versus cytosolic) may be noted and warrants further studies.

In conclusion, dexamethasone decreased GR protein levels in the rat jejenal IEC-6 cells in a time dependent manner and complements previous work *in vivo*. P-glycoprotein expression was increased by dexamethasone treatment contrasting previous *in vivo* work in the colon. The inhibition of the dexamethasone-mediated effects by the GR antagonist RU486 shows DEX is mediating its effects through binding GR. Intriguingly, there was a significant increase in total and nuclear NF-κB, but not AP-1, mirroring an increase in P-glycoprotein expression at 24hr but not at an earlier time-point. The NF-κB inhibitor IκB-α was lowly expressed in the nucleus. This data implies *de novo* protein synthesis by dexamethasone increased and subsequently activated NF-κB. It is possible that elevated levels of in NF-κB induced

P-glycoprotein expression in these cells however, but future studies are required to address the critical question: whether transcription of mdr1a is regulated by NF-κB.

Chapter 8

Implications

The main aim of this thesis was to study the molecular mechanisms involved in the regulation of key genes determining corticosteroid access to cells and actions in the rat colon, particularly P-glycoprotein and GR. The effect of steroids *in vivo* and *in vitro*, as well as the role of bacteria, on the regulation of these genes in the healthy and diseased rat colon was investigated. Alterations in expression of intestinal GR and P-glycoprotein may have implications for initiating disease and steroid resistance in human IBD.

8.1 The regional distribution of genes determining steroid sensitivity and effect of steroid treatment on gene expression in the healthy rat colon

In the healthy adult Wistar rat, P-glycoprotein and GR were shown to be differentially expressed along the length of the colon, with increased P-gp expression in proximal compared to distal colon. A reverse gradient was noted for GR protein expression. Systemic dexamethasone treatment in these healthy Wistar rats decreased intestinal expression of both mdr1a and GR mRNA compared to untreated animals. Corticosterone was shown to regulate these genes, and thereby these data support a role for endogenous steroids in regulating tissue sensitivity to steroids.

Increased expression of P-glycoprotein in proximal compared to distal colon may protect the proximal intestinal sections from potentially toxic ingested or bacterially produced xenobiotics, as P-gp is thought to be involved in the active transport of foreign substances out of cells. If a similar P-gp expression gradient is present in human colon it may explain the fact that ulcerative colitis almost always involved the distal colon, as an increase in xenobiotic concentrations may be involved in the initiation of inflammation.

Also worthy of note is the increased expression of GR protein, which is known to be involved in the inhibition of pro-inflammatory cytokines as well as the production of anti-inflammatory proteins (Barnes 1998), in distal colon. The decreased P-gp and increased GR expression may provide a balance between pro-inflammatory and anti-inflammatory mediators, where increased GR prevents an inflammatory reaction in

response to increased xenobiotic concentration in the distal colon, and therefore explain the lack of inflammation in the colon of healthy rats and humans.

Moreover, if this differential distribution of GR and P-gp is mirrored in humans, steroid sensitivity will vary along the colon. Increased P-gp (which excludes steroids including dexamethasone) and decreased GR expression (known to initiate the anti-inflammatory effects of steroids) in proximal compared to distal colon, would reduce the sensitivity of proximal colon to steroids. This may have implications for steroid treatment, as certain areas of the colon may be insensitive to steroids and would therefore be an ineffective at treating inflammation. Furthermore we noted decreased expression of mdr1a and GR mRNA in the healthy rat colon in response to dexamethasone treatment. If this were to occur in response to steroid treatment in humans, the decrease in GR expression may reduce the efficacy of steroids while a reduction in mdr1a expression may amplify the inflammatory reaction in response to pro-inflammatory molecules, both factors thereby contributing to 'steroid-insensitivity'.

To clarify the relevance of our data in man, the expression of these genes along the human colon needs to be studied. Furthermore, if expression were also studied in the small bowel, these data may provide vital information concerning small bowel steroid sensitivity. These investigations may have implications for the possible therapeutic steroid treatment regimes in intestinal disease, depending on the expression of these genes and therefore sensitivity of specific intestinal areas.

Other genes involved in tissue steroid sensitivity were also studied. 11-Beta Hydroxysteroid Dehydrogenase type-2 mRNA (11β-HSD2) was also shown to be differentially expressed along the colon, whereas MR was ubiquitously expressed. Removal of endogenous glucocorticoids increase expression of both these genes, however glucocorticoid replacement returned levels to base-line. The proteins encoded by these genes are involved in the water and electrolyte homeostasis in the intestinal epithelium, where 11β-HSD2 inactivates active glucocorticoids and therefore ensures aldosterone (which circulates at a much lower concentration than glucocorticoids) can bind MR and initiates its effects (Whorwood *et al.* 1994). If a similar distribution of expression is noted in human colon, the increased expression of

11β-HSD2 in distal colon may ensure aldosterone-selective control of absorption of sodium (and water) before excretion from the body and the lack of effect of steroid treatment on expression of these genes would prevent a disruption in water and electrolyte homeostasis. It would be interesting to investigate whether a gradient was noted in the small bowel, in both humans and rodents, and study whether other parts of the bowel are critical in this process.

8.2 The effect of bacteria and disease on expression of P-glycoprotein and GR in healthy and diseased colon

The presence of micro-flora initiates disease in animal models of inflammation. The effect of bacteria on expression of P-gp and GR was investigated in both healthy Wistar rats and in the HLA-B27 non-transgenic and transgenic rat model of inflammation. Reducing intestinal bacterial load in healthy adult Wistar rats using the antibiotics ciprofloxacin and metronidazole, suggested a role for bacteria in the regulation and expression of these genes, as a trend towards increased GR but decreased P-glycoprotein expression in distal colon was noted. However the intestine was not completely clear from bacteria and therefore no absolute conclusions could be drawn from this experiment. To further clarify the role of bacteria in these animals, Wistar rats in germ-free conditions (an environment unavailable at the time of investigation) should be studied, and this would show the direct effect of the presence of bacteria on GR and P-gp expression in these animals.

To further clarify the role of bacteria and disease on expression of P-gp and GR, HLA-B27 transgenic and non-transgenic rats were studied. The presence of colonic inflammation in HLA-B27 transgenic rats was associated with decreased P-glycoprotein and mdr1a expression in all areas of the colon, consistent with recent data from other animal models and UC (Langmann *et al.* 2004; Lizasa *et al.* 2003). GR expression was decreased in the proximal colon and the rectum but not caecum. Germ-free conditions were associated with a significant reduction of P-gp expression in the caecum, but not proximal colon or rectum, whereas GR levels were reduced in proximal colon and rectum, but not caecum. These data emphasise the complex gene-bacterial interactions within the colon in health and disease.

The reduction in P-gp and GR protein expression in the presence of inflammation in both humans and rodents shows disease alters the expression of both P-glycoprotein and GR protein in the intestine. The key question is to determine whether the reduction in P-gp and GR protein expression is a primary or secondary consequence of inflammation. This may be investigated by treating transgenic animals with a transcriptional inducer of mdr1a and GR expression before and after inflammation is established. The decrease in P-gp expression may be involved in the perpetuation of inflammation due to an increase concentration of pro-inflammatory cytokines within the epithelium. Moreover if the reduction in GR expression in the proximal and distal colon, as well as the low levels of GR in caecum, was also seen the human disease, this would further reduce the efficacy of steroids and therefore may have implications in the type to treatment required to resolve established inflammation. Further investigations needs to be carried out to determine intestinal expression of these genes in human disease. Inflammation may be associated with decreased expression in specific areas of the intestine and thus steroid efficacy may be differentially altered.

Worthy of note are the differences in steroid sensitivity between healthy rodent species-Wistar and Fischer rats. This has interesting parallels with man, where alterations in the genetic phenotype of MDR1 may determine extent of disease (Ho *et al.* 2005). This shows the complexity of genetic variation in rodents and more importantly in man, which may determine inter-individual responsiveness to steroids.

These studies also revealed the bacterial flora regulated colonic expression of these genes. Specific bacteria maybe involved in regulating expression, however the mechanisms behind this remain unknown. The increased expression of GR in proximal and distal colon and P-gp in caecum when bacteria are present in the gut suggests a site- and bacteria-specific regulation of these genes. Studying the effect of bacteria on expression of these genes in humans as opposed to rodents is restrictive; however the composition of bacterial flora may be important in the prevention or resolution of intestinal inflammation, as has been elegantly shown by Sartor and colleagues (Sartor 2004b). In IBD the composition of the flora has been shown to be altered as some species, especially anaerobic bacteria, have been known to be increased/decreased in these patients, and treatment with antibiotics and probiotics have been shown to aid in remission of disease in some patients (Sartor 2004b;

Shanahan 2004). Studying the composition of bacterial flora in healthy and IBD patients may aid in the development of treatments which would return the flora to that noted in healthy/non-diseased patients. Bacteria known to increase the expression of P-gp and GR may be given to IBD patients, or even specific antibiotic regimes could target bacteria known to decrease expression of these genes. These treatments would aid in returning expression levels of P-gp and GR to those noted in healthy patients, and this may aid in the resolution of inflammation.

8.3 Effect of steroid treatment on expression of P-gp and GR in healthy and diseased rat colon

Dexamethasone administered to HLA-B27 transgenic and non-transgenic animals (rats with/out intestinal inflammation respectively) differentially regulated GR expression in a site-specific manner. In the healthy colon, dexamethasone treatment increased GR expression in caecum; decreased levels in proximal colon; and was ineffective in rectum. In the inflamed colon dexamethasone treatment increased GR expression in rectum only. Colonic P-glycoprotein expression was also seen to be regulated differently by dexamethasone in these animals opposed to Wistar rats, and steroid treatment did not alter P-gp expression in the inflamed colons from transgenic animals. This may be due to a sub-optimal dose given, or this may just be another example of differences in steroid sensitivity between species, as also noted between humans.

The lack of effect of dexamethasone on the histology shown between non-inflamed and inflamed colon is worthy of discussion. Previous studies in the PG-APS Lewis animal model did show the dosing regimen that we used was a therapeutic dose at reducing histology scores in the caecum of rats 16 days after the single injection of PG-APS (Herfarth *et al.* 1998). This suggests differential steroid sensitivity between rodent models, and again supports that complex genetic variations may determine individual responsiveness to steroids. It would be interesting to explore expression of GR and P-gp once a therapeutic dose of dexamethasone is reached, and study whether resolving inflammation returns expression levels to those seen in 'healthy' animals. In the rectum of diseased but not healthy animals, dexamethasone did increase GR levels to 'healthy' levels. Alterations in expression of GR may be involved in

initiating and resolving disease. There is a need to investigate whether the present animal data may be extrapolated to man. Knowing the expression levels of GR in the inflamed human colon may be an indicator of steroid responsiveness and would enable physicians to treat patients with other therapies. The glucocorticoid-beta isoform may also determine responsiveness to steroids. Honda *et al.* suggested this isoform was increased in lymphocytes from CD patients who were insensitive to steroids, however no further studies have supported this theory, and as rodents do not express this gene, the molecular mechanisms behind its regulation in response to steroids has not been undertaken *in vivo*.

Interestingly, healthy Fischer rats showed differential regulation of GR in response to dexamethasone and these data suggests steroid sensitivity may be altered along the colon in response to dexamethasone treatment. Investigating the expression of GR in the colonic mucosa in humans in response to dexamethasone may provide further information on the regulation of this gene along the colon and mucosal steroid sensitivity, which may in turn determine the efficacy of steroids in the treatment of colonic inflammation. Prescribing steroids for other inflammatory disorders may decrease the expression of GR in the colon and as a reduction in GR may be involved in disease initiation, it may make this area susceptible to inflammation in some patients. Therefore a preventative treatment may also be given to stop the onset of colonic inflammation.

Collectively these data show the complexity of the intestinal regulation of these genes in the inflamed and non-inflamed colon, corticosteroid treatment may have selective efficacy in different regions of the colon. The data provide possible explanations governing the efficacy of steroids during inflammation in rodents and warrants further studies in man. As mentioned previously, preventing/reversing the decrease in P-gp and GR protein expression by treating both animals and humans with inducing agents of both mdr1a and GR transcription may aid in the resolution of inflammation by increasing expression of these genes to 'healthy' levels as well as increasing intestinal steroid sensitivity. Combined treatments may therefore be used to treat inflammation.

8.4 *In vitro* regulation of GR and P-gp in IEC-6 cells

To investigate the mechanisms involved in the regulation of GR and P-gp in vitro studies using the rat small intestinal cell line-IEC-6 was undertaken. In the light of previous data which have implicated the pro-inflammatory transcription factors NF-κB (Nuclear Factor-Kappa B) and AP-1 (Activating Protein-1) as regulators of transcription of the multidrug-resistance gene (Bentires *et al.* 2003; Ikeguchi *et al.* 1991; Teeter *et al.* 1991; Thevenod *et al.* 2000), the effect of dexamethasone on expression of NF-κB, AP-1, P-gp and GR levels was investigated. Dexamethasone was seen to increase P-gp and decrease GR expression, an effect initiated via the glucocorticoid receptor, as well as increase the levels of cytoplasmic NF-κB. However, the increase in P-gp expression in response to dexamethasone opposed to the *in vivo* effects as noted in Wistar rats. This lack of correlation needs further investigation, but may be another example of differential regulation of this gene between species or specific areas of the intestine.

Nonetheless, these data certainly raise the possibility that NF-κB may be involved in the regulation of P-glycoprotein, although causality has not been shown. Further in depth investigations must be undertaken to study the direct effects of altering expression/activity of NF-κB on P-gp expression. This may be achieved by using adenoviral vectors which inhibit the activation pathway of this transcription factor and study expression levels of P-gp. Moreover, primary cultures of intestinal epithelial cells from both human and rats could be used to investigate expression of both NF-κB and P-gp in healthy and diseased colon, and study whether addition of pro-inflammatory cytokines alter expression of these gene products. Studying expression in both human and rodent cultures would clarify if data can be extrapolated between man and rodent investigations. If a link between NF-κB and P-glycoprotein was noted it may provide another pathway of treatment for patients with IBD: direct inhibition of NF-κB may not only inhibit the inflammatory response but may alter P-gp expression and aid in the resolution of inflammation.

A number of the observations made in this thesis are entirely novel, and collectively these carry potentially important implications in the study of the pathogenesis and steroid efficacy in inflammatory bowel disease. Novel data include the regional

distribution and effect of dexamethasone treatment on genes determining steroid sensitivity in the healthy rodent intestinal epithelium as well as the finding that intestinal inflammation altered expression of P-gp and GR in an animal model of inflammation. Intestinal flora and corticosterone treatment was shown to modulate expression of these genes in the HLA-B27 transgenic rat models of inflammation in a site-specific manner. In addition, the complex interactions between P-gp and GR expression in response to bacteria have implications for potential mechanisms, by which inflammation develops in the colon of both rodents and man.

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