GEOGRAPHICAL AND DISEASE INFLUENCES ON INTESTINAL ANTIBODIES IN MAN

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Declaration

I declare that all the work in this thesis, unless otherwise indicated, is entirely my own.

Dedication

To the Memory of Professor Anne Ferguson, Who Always Wanted to See Me as a Leader in the Field of Mucosal Immunity and Infection

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ABSTRACT

Gastrointestinal infections are a major cause of mortality and morbidity especially in the developing world. It is nearly a century since it was first observed that antibodies were present in the gut and it is now clear that secreted antibodies in the gut mucosa are crucial for host defence against pathogens. In addition, they may be important in the interaction of the host with commensal bacteria and oral vaccines.

The development of a new clinical technique, whole gut lavage, has facilitated the collection of large amounts of gut fluid containing antibodies for the study of immune responses in the GI tract in health and disease.

The aims and related hypotheses of this thesis were:

a) To develop an antibody test that could be used as a tool to examine the gut humoral immune response to bacteria of the gut flora. In order to develop the antibody test, various antigens were prepared from the bacterial cell wall and their biological potentials were examined with human and murine cell lines. These tests could be combined with studies of other facets of gut immunity for which methods were already available, in order to explore active immunity and tolerance in the mucosal and systemic compartments.

b) To examine and compare humoral immunity in the gut and serum of immunologically normal people from Edinburgh and Dhaka, in order to establish and test the following hypotheses:

1. In view of the probable higher antigenic load from a potentially contaminated environment, there would be evidence of gut damage and inflammation in the healthy people of Dhaka, and thus high levels of IgA, compared to people in Edinburgh.
2. In the developing country, the drive for production of high levels of humoral responses to bacteria would have the additional effect that antibodies to other gut antigens, such as foods, would be absent or of low titre.

By studying patients with chronic colitis (inflammatory bowel disease), it might be possible to investigate how chronic gut inflammation affects specific antibodies and to identify the sources of gut antibodies, ie. serum- derived or locally produced. Prompted by the case of a patient with hypereosinophilic syndrome, high gut IgA concentrations and ulcerative colitis, the stimuli for eosinophil migration into the gut and eosinophil activation were examined and possible interaction with the regulation of humoral immunity investigated.

My literature review concentrates on a monograph on 'The gut as an immune organ' and illustrates the important features of intestinal immunoglobulins and antibodies. In the first chapter a section on bacterial structure and antigens has been included and the current knowledge on the regulation of eosinophil migration has been discussed. There is a small section on an appraisal of the whole gut lavage procedure that has been used in this thesis. Chapter 2 includes characteristics of patients and healthy volunteers, laboratory methods and technical development. A pilot study to confirm technical competence and reproducibility of methods is presented in chapter 3. Chapter 4 describes various gut damage and immune parameters in people from Edinburgh and Dhaka. Despite higher antigenic load, no evidence of gut damage, but high IgA, was

found in the Dhaka groups. Results of anti-endotoxin and antibacterial antibodies, including those to various core types of *E.coli* LPS are presented in chapter 5. A section on the purity of the antigens and their biological potential by producing nitric oxide, inducing various cytokines in murine and human cell line have been presented. For the first time it has been found that IgA antibodies against various core types of LPS of *E.coli* are present in the gut, and their potential in the therapy of sepsis syndrome and for oral vaccine development has been discussed. Chapter 6 describes the of humoral immunity, role of gut bacteria, and cytokines controlling local and systemic immunity and pathogenicity in chronic inflammatory bowel disease. Evidence has been presented in chapter 7 to support the view that the source of IgG antibody detected in the gut of patients with active IBD is locally produced and not serum derived.

The drive for production of a high humoral response to bacteria may be responsible for absent or low titre antibodies to other gut antigens, such as food. The antigen-driven bystander suppression or recently described Th1/Th2 paradigm is the purported mechanism for these results. The implications of these results in therapeutics and strategies for oral vaccine against infectious disease have been discussed in chapter 8. The stimuli of eosinophil migration in to the gut and their role in the mucosal inflammation of IBD have been discussed in chapter 9. For the first time it has been shown that the eosinophil specific chemoattractant, eotaxin, is secreted into the gut lumen; this may help in our understanding of the diseases related to eosinophil accumulation.

The final chapter is an overview with some speculation on the relationship of the findings to future developments.

Table of Contents

Abbreviations

α-1-AT	Alpha-1- anti-trypsin
AIDS	Acquired immunodeficiency syndrome
Alb	Albumin
APCs	Antigen-presenting cells
BSA	Bovine serum albumin
B.fragilis	*Bacteroides fragilis*
CD	Crohn's disease
CDAI	Crohn's disease activity index
CI	Confidence interval
CRP	C-reactive protein
CT	Cholera toxin
CSF	Colony-stimulating factor
DEA	Diethanolamine
DH	Dhaka
dIgA	Dimeric IgA
E.coli	*Escherichia coli*
ECP	Eosinophil cationic protein
EDTA	Ethylene diamine tetraacetic acid
EIEC	Enteroinvasive *E.coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E.coli*
ESR	Erythrocyte sedimentation rate
ETEC	Enterotoxigenic *E.coli*

GE	Granulocyte elastase
GI	Gastrointestinal
GM	Granulocyte-macrophage
Hb	Haemoglobin
HLA	Human leukocyte antigens
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IPAA	Ileal pouch-anal anastomosis
LPS	Lipopolysaccharide
LT	(Heat) Labile toxin
M cell	Microfold cells
MNCs	Mononuclear cells
mRNA	Messenger ribonucleic acid

NK	Natural killer
OD	Optical density
Ova	Ovalbumin
P	Probability
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PIgA	Polymeric immunoglobulin A
r	Correlation coefficient
Rc mutant	Rough mutant type c
RIA	Radioimmunoassay
SC	Secretory component
SD	Standard deviation
SDS	Sodium lauryl sulphate
SIgA	Secretory IgA
ST	(Heat) stable toxin
TGF-β	Transforming growth factor-beta
Th	T helper
TNF	Tumour necrosis factor
UC	Ulcerative colitis
V.cholerae	*Vibrio cholerae*
VTEC	Vero cytotoxin producing *E.coli*
WGH	Western General Hospital (Edinburgh)
WGL	Whole gut lavage
WGLF	Whole gut lavage fluid

L	Litres
mL	Millilitres
µL	Microlitres
kg	Kilograms
mg	Milligrams
µg	Micrograms
ng	Nanograms
pg	Picograms
cm	Centimetres
mm	Millimetres
MU	Median units
µm	Micrometres
U	Unit

Presentations and publications based upon this Thesis

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3. **Hoque SS,** Ghosh S, Poxton IR, Ferguson A. Evidence of high local production of IgG in the gut lumen of patients with active inflammatory bowel disease. Am J of Gastroenterology, 94(4), 1999.

4. Poxton IR, Currie CG, McCallum K, **Hoque S,** Ferguson A. Mucosal and Systemic Immune Responses to The Lipopolysaccharide of *E.coli* 0157. Annual conference of the society of General Microbiology, Leeds, September, 1999.

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Presentations

1. **Hoque SS**, Drumond H, Ghosh S, Ferguson A. Increased Eosinophil Cationic Protein and Eosinophil Specific Chemokine'Eotaxin' in Gut Lavage Fluid from patients with Inflammatory Bowel Disease. British Society of Gastroenterology, Annual Meeting, March 20-23, 1999.

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7. **Hoque SS**, Poxton IR, Ghosh S. Differences in Intestinal Humoral Immunity Between Healthy Volunteers from UK and Bangladesh. **British Society of Gastroenterology Annual Meeting, Birmingham (21-23rd March, 2000)**

8. **Hoque SS**, Poxton IR, Ghosh S. Gut Mucosal Humoral Immunity Against Core types of LPS of *E.coli*- A Potential Novel Approach of Mucosally Presented Vaccine. **British Society of Gastroenterology Annual Meeting, Birmingham (21-23rd March, 2000)**

General Introduction

Gastrointestinal infections are a major cause of disease and death, particularly in the developing world. It has been estimated that about 5-10 million deaths annually are related to gastrointestinal infections. The discovery of aetiological agents including bacteria, viruses, and protozoa, and the appearance of new diseases such as AIDS have illustrated the enormous burden of infectious diseases throughout the world.

Historically, the field of immunology, in particular the study of humoral immunity, developed in the context of resistance to infectious disease. The humoral immune system is compartmentalised; antibodies in mucosal secretions, such as in the gastrointestinal tract, differ from those in the serum; this was recognised by Besredka in the early part of the century. The local humoral immunity concept was much clarified after the discovery of IgA in 1953 and its isolation and characterisation in 1959. It is now clear that secreted antibodies are crucial to the host/bacterial interactions and are as relevant to commensal bacteria as to pathogens and to the development of vaccines. However, despite the existence of multiple mechanisms which control microbial populations at mucosal surfaces, the mucous membranes remain the most important portal of entry for microbial infections. Therefore the ability to enhance mucosal resistance to infection has enormous clinical importance.

The development of a new clinical technique- whole gut lavage (WGL)-has made it possible to collect large amounts of gut-secreted antibodies, and to study the mucosal humoral responses of normal and diseased individuals.

Since infectious disease continues to be a major source of mortality and morbidity throughout the world and especially in economically less privileged areas, a better understanding of mechanisms of immunity continues to be one of the most urgent needs in medicine. There is no doubt that a better understanding of mucosal immunobiology will not only help in the effective treatment and management of infections that have a mucosal phase, but it will also help us to understand other diseases such as inflammatory bowel disease, food allergy and mucosal hypersensitivity.

Aim of the Thesis

My long- term research objectives are to investigate the gut immune system in the context of health and disease of people in the Developing World, especially in the field of malnutrition and diarrhoeal diseases. This Ph.D. project has formed a foundation for my future work.

My aims, and related hypotheses, were as follows :

1. To develop antibody tests that could be used as tools to examine the gut humoral immune responses to bacteria of the gut flora. These methods were to be combined with studies of other facets of gut immunity for which study methods were already available, to explore active immunity and tolerance in the mucosal and systemic compartments.

2. To examine humoral immunity in the gut and serum of immunologically normal people from Edinburgh and Dhaka, and test the hypotheses:

a) that there would be evidence of gut damage and inflammation in the healthy people of Dhaka, in view of a high antigenic load from the contaminated environment and thus very high levels of IgA; and

b) that in the developing country, the drive for production of a high humoral response to bacteria would have the additional effect that antibodies to other gut antigens, such as foods, would be absent or of low titre.

3. By studying patients with Inflammatory bowel disease, to investigate how chronic gut inflammation may affect specific antibody responses.

4. To use the data obtained from the above studies and establish whether the source of gut antibodies are serum-derived or locally produced.

5. A patient with hypereosinophilic syndrome and ulcerative colitis with a very high level of gut IgA, prompted me to examine the stimuli for eosinophil migration into the gut, and possible interaction with regulation of mucosal humoral immunity.

The literature review for this thesis has concentrated on ' The gut as an immune organ' which has been written as a short monograph, and has illustrated the important features of intestinal immunoglobulins and antibodies. A section has been included on bacterial structure and antigens, and previously-known information on serum antibodies against gut bacterial antigens. Current knowledge on the regulation of eosinophil migration is also discussed, and there is an appraisal of the WGL procedure which has been a critical tool in many of my studies.

Chapter 1

Review of Literature

Section 1

The Gut as an Immune Organ

Introduction

The mucosal surfaces of the body, such as the gastrointestinal, respiratory and genitourinary tracts, provide an effective barrier to the entry of potentially harmful substances and infectious agents (McGhee *et al*,1992; Staats *et al,* 1994). In Man the mucosal surface area is over 400 square metres, which is more than 200-fold greater than the surface area of the skin (McGhee *et al*, 1989; Holmgren *et al*, 1992). It should be noted that more than 80% of this large mucosal surface is occupied by the gastrointestinal tissues (i.e., gut associated lymphoid tissue [GALT]) . Considerable experimental evidence supports the existence of an elegantly integrated mucosal immune system that is largely distinct from the systemic immune system (McGhee *et al*, 1992; Kiyono and McGhee, 1994; Kraehenbuhl *et al*, 1992; Ferguson *et al*, 1994). The mucosal immune system has often been ignored on the assumption that it is a branch of the systemic immune systems. Moreover, the difficulties involved in the isolation and characterization of mucosa-associated lymphoid tissue (MALT) and measurement of secretory antibodies from mucosal secretions added further to confuse and limit our understanding of the intricacies of the mucosal immune system.

Much research on GALT has been conducted in mice. It is likely that the general principles derived from the results of such experimental work are also applicable in humans, but this has been proven for only a few aspect of immunity. Good studies of the physiology and regulation of the human GALT are urgently required.

Organization of the GI Immune System

Recent advances in the field of immunology and cell and molecular biology provide convincing evidence for a unique and independently operated immune system (Takahashi & Kiyono, 1999; Table 1).

Table 1: Unique features of the mucosal immune system

A. Common mucosal immune system (CMIS)

1. Inductive tissues: GALT, NALT (nasal-associated lymphoreticular tissue)

2. Effector tissues: lamina propria and epithelial layer of the intestinal, respiratory, and genitourinary tract glandular tissues

3. New T-cell nests for thymus-independent cells: cryptopatches

B. Secretory IgA (S-IgA)

1. Dimeric and polymeric form of IgA

2. Neutralization and immune exclusion

3. Presence of high frequency of S-IgA$^+$ B cells in GALT and NALT

C. Mucosal internets and intranets

1. Formed by Th1 and Th2 cells, S-IgA$^+$ B cells, and epithelial cells for the production of S-IgA

2. Cross-regulation between epithelial cells and intraepithelial $\gamma\delta$ T cells

D. Essential cytokines for the induction of IgA responses

1. TGF-β: isotype switching.

2. IFN-γ: enhancement of the secretory component (SC) production.

3. IL-5 & IL-10: differentiation of S-IgA$^+$ B cells.

4. 1L-6: generation of IgA plasma cells.

Induction and Expression of Immunity

Overall, mucosal immune functions can be separated into induction and effector phases. For the induction of antigen-specific immune responses at the mucosal barrier of the GI tract, one must consider the common mucosal immune system (CMIS), which is composed of IgA inductive effector tissues (McGhee *et al*,1992). Oral immunization has been shown to be an effective way to induce mucosal immune responses, because this antigen deposition pathway can effectively prime and stimulate immunocompetent cells in the mucosal inductive sites in the GI tract (i.e. GALT, which consists of organised lymphoid follicles [**Peyer's patches** and lymphoid aggregates]). After antigen presentation, antigen-specific lymphocytes travel from the inductive site (i.e. GALT) and migrate to distant mucosal effector tissues (e.g. the lamina propria region of the intestinal tract) via the blood circulation using specific mucosal homing receptors (Picker, 1994;Phillips-Quagliata and Lamm, 1994). This dynamic cell trafficking between IgA inductive and effector tissues are essential for the generation of antigen specific immunity after oral immunization.

Inductive Tissues in the GI Tract

Peyer's patches (**PP**) are concentrated in the distal ileum in Man and contain all the necessary immunocompetent cells including $CD4^+$ T cells that become Th1 and Th2 type helper cells, $CD8^+$ T cells with cytotoxic function, IgA committed B cells (surface IgA^+; $SIgA^+$ B cells), macrophages and dendritic cells (DC). The epithelium overlying PP has been termed a follicle-associated epithelium (FAE), because it contains specialized antigen-sampling cells, termed microfold (**M**) cells. One of the major functions of **M** cells is to engulf and pass intact antigens from the apical membrane exposed to the lumen of the GI tract to the basolateral surface, where the antigen can interact with adjacent follicle-derived lymphoreticular cells including antigen-

presenting cells (APC), $CD4^+$ T cells and $SIgA^+$ B cells to give rise to an immune response (Kraehenbuhl *et al* 1992). **M** cells have been shown to be the portal of entry for a variety of bacterial and viral pathogens (Krahenbuhl *et al*,1992).

T cells present in the parafollicular regions of PP are mature and more than 95% express the $\alpha\beta$ T-cell receptor (TCR), while a small subset, which remains poorly characterized, exhibits a $\gamma\delta$ TCR (McGhee *et al*, 1992). The $CD3^+$ $CD4^+$ $CD8^-$ Th cells make up approximately 60% to 65% of $\alpha\beta$ T cells in PP, while the remaining are $CD3^+$ $CD4^-$ $CD8^+$ (cytotoxic /suppressor) T cells (25%) and $CD3^+CD4^-CD8^-$ T cells bearing $\gamma\delta$ TCR (5%) (Takahashi and kiyono, 1999).

PP have distinct B cell zones (follicles), which have a germinal centre containing about 8 to 15% of SigA + B cells (McGhee *et al,* 1992). The tissue also contains biologically active antigen-presenting cells (APC) including follicular dendritic cells within the germinal centre, interdigitating cells in close contact with the lymphocytes of parafollicular zones (McGhee et al, 1992; Kato and Owen, 1994). Taken together, mucosal inductive tissues, such as GALT or **PP,** consist of all the essential epithelial (i.e. **M** cells) and lymphoreticular cells for the induction and regulation of mucosal immune responses.

Effector Tissues in the GI Tract

As mentioned briefly earlier, after the initial stimulation of immunocompetent cells in the PP, antigen-specific $CD4^+$ Th1 and Th2 type cells, $CD8^+$ T cells and $SIgA^+$ B cells leave the tissue via the efferent lymphatics. They are transported to the systemic circulation through the thoracic duct, ultimately entering mucosal effector sites such as the lamina propria (LP) of the GI tract. This circular loop for dissemination of antigen-activated T and B cells from the inductive tissue to the mucosal effector sites is usually termed common mucosal immune system (CMIS) (Takahashi and Kiyono,1999). One of the hallmarks of the mucosal immune response is the presence of antigen-specific secretory IgA (SIgA) antibodies in external secretions (Ferguson *et al*,1994). At least 3 types of cells are required for the formation of SIgA, these are: IgA enhancing cytokines (e.g. IL-5, IL-6 and IL-10) producing Th2-type $CD4^+$ T cells, $SIgA^+$ B cells, and epithelial cells residing in the effector tissue (Takahashi and Kiyono, 1999). This will be discussed further in the next section of the review of mucosal antibodies.

The LP of the GI tract consists mainly of T cells (more than 60%) and B cells (≅ 20%, including plasma cells), with macrophages (≅ 5%) and other cell types (McGhee *et al*, 1992; Kiyono and McGhee,1994). Approximately 70% to 90% of these plasma cells produce IgA in Man and are responsible for antigen-specific SIgA found at mucosal surfaces of the GI tract (McGhee *et al*, 1989). As already mentioned, macrophages may represent the major APC in LP and eosinophils (1% to 2%), and mucosal mast cells (1% to 2%) may mediate hypersensitivity reactions (Takahashi and Kiyono, 1999). It has been shown recently that mast cells may also play an important role in bacterial immunity (Malaviva *et al*, 1996).

Intraepithelial Lymphocytes

The lymphocytes residing between intestinal epithelial cells (i.e. intraepithelial lymphocytes [IEL]) are the first T cells to contact luminal antigens that cross the mucosa via the columnar epithelial cells in the M cell-independent manner (Takahashi and Kiyono, 1999). Most gut IEL from humans, mice and rats are T lymphocytes, which are largely $CD8^+$ T cells (70% to 85%) (Kiyono and McGhee,1994). In addition, three other minor populations of $CD4^+$ $CD8^+$ (double positive) T cells, $CD4^+$ $CD8^-$ T cells (helper type; Th) and $CD4^-$ $CD8^-$ (double negative) T cells are also present in IELs. Murine IELs contain high numbers of $\gamma\delta$ T cells (40% to 60%) in addition to $\alpha\beta$ T cells. The frequency of $\gamma\delta$ T cells in IEL of humans is lower than that of murine IELs, but still approximately 15% T cells under normal conditions (Takahashi and Kiyono, 1999). The number of $\gamma\delta$ T cells significantly increases in pathological conditions such as coeliac disease (Halstensen *et al*, 1989)

The precise functions of IEL in vivo are not yet clear, however several functions for these cells have been described, including antigen-specific delayed-type hypersensitivity, and natural- killer cell-like function (Klein and Kagnoff, 1984; Cuff, 1994).

The actual function of $\gamma\delta$ T cell in the induction and regulation of mucosal immune responses is currently under extensive investigation. Recent observations suggest that IEL $\gamma\delta$ T cells and epithelial cells maintain interrelationships in order to regulate reciprocally growth and function of both cell types. For example, $\gamma\delta$ T cells have been shown to modulate growth and differentiation of epithelial cells via the keratinocyte growth factor (Boismenu and Harvan,1994). On the other hand, epithelial cells have

been shown to produce IL-7 and stem cell factor (SCF) that can provide activation signals via IL-7 R expressed on γδ T cells (Fujihashi *et al*,1996). These new findings suggest that an interdependence named as mucosal intranet ((Takahashi and Kiyono, 1999) exists between epithelial cells and intraepithelial T cells at the mucosal surface barrier (Kiyono and McGhee,1994; Fujihashi *et al*,1996), which is critical for the normal development of both γδ T cells and epithelial cells.

Cryptopatch

The GI mucosa may also provide a new nest for the development of thymus-independent T cell precursors. Cryptopatches (CPs) have been defined as discrete aggregates containing approximately 1000 $CD3^-$ $CD4^-$ $CD8^-$, Sig^-, $IL\text{-}7R^+$ $c\text{-}kit^+$ lymphoid cells enmeshed in a network of cells with the appearance of dendritic cells (Kanamori *et al*,1996). So far, they have only been found in mouse intestine. There are several studies ongoing to find similar structures in human small intestine. Obviously, it will solve some conceptual problems regarding T cell development as these novel lymphoid structures (CPs) are present in both severe combined immunodeficient (SCID) and recombinase activating gene (RAG) 1 knockout mice (Kanamori *et al*, 1996). Recently, it has been shown that CPs are a source of T lineage precursors that migrate to populate the intestinal epithelial layer to become IEL (Saito *et al*,1998). It seems that extrathymic T cells developed from CPs could be an important subset of mucosal T cells that contribute to the induction and regulation of unique immune responses associated with the GI tract.

Secretory IgA (SIgA): Basis of Specific Humoral Immunity in The Gut

SIgA is the most important humoral antibody/immune mechanism for the protection of the intestinal tract. Considering its enormous importance in mucosal humoral immunity, a whole section (section 2) of this thesis is devoted to a review of mucosal immunoglobulins and most details of SIgA are discussed in that section.

The Concept of Oral Tolerance

Ingestion of food protein or bacterial, viral and chemical antigens can result in subsequent inability to mount a systemic immune response to the same antigen following parenteral immunization (Challacombe & Tomasi,1980). This state of systemic unresponsiveness is known as 'oral tolerance'. While the phenomenon of oral tolerance has been extensively and exhaustively studied in animals, there is very limited information on this phenomenon in Man (So far, only two studies have been conducted in Man, one is from Alabama, Birmingham [Husby *et al*,1994], another is from the Edinburgh GI lab by Dr Robert Boulton-Jones [personal communication]).

The mechanisms by which oral tolerance is mediated include the generation of active cellular suppression (regulatory cells), clonal anergy, or clonal deletion; the determining factor is the dose of antigen fed (Strobel and Mowat,1998; Ferguson *et al*,1994). Low doses favour active suppression, whereas higher doses favour anergy and deletion (Weiner and Mayer, 1996). It has been proposed that a breakdown in oral tolerance may be involved in the pathogenesis of coeliac disease and inflammatory bowel disease (Brandtzaeg *et al*, 1989). It is vital therefore, to determine the role of oral tolerance in disease pathogenesis in Man.

Section 2.

Mucosal Immunoglobulins

2.1 Immunoglobulin A (IgA)

Introduction

A little more than 30 years ago IgA was identified as predominantly a mucosal immunoglobulin (Chodirker and Tomasi,1963). The interest in secretory immunity has broadened considerably since that time, but the study of the structure and functions of IgA and the role of other mucosally secreted immunoglobulins, is still under active research.

The mucosal surface acts as an interface between the external environment and the internal milieu of the body. This represents the largest area of exposure to pathogens, 400 m^2 compared to only 1.8 m^2 for the skin (Childers *et al*, 1989). IgA is the immunoglobulin class primarily responsible for humoral immune protection at this large exposed surface. On a daily basis, the body synthesises far more IgA, 66mg/kg body weight, than all other immunoglobulin classes combined (Mestecky and McGhee, 1987).

Biological properties of IgA

IgA has several unique properties which enable it to carry out its specialised function in mucosal defence. Firstly, IgA can associate intracellularly with **J** chain via a cysteine residue in the C terminal 'tail' to form dimeric IgA (dIgA) (Koshland,1985) which can bind to the polymeric immunoglobulin receptor (pIgAR). The complex is transported across mucosal epithelia (Mostov, and Blobel,1982; Mostov, 1994) to be released as secretory IgA (sIgA). The IgA thus transported acts in the first line of humoral defence at mucosal surfaces (Underdown and Sciff ; 1986). Accordingly, it is also the target of specific receptors and proteases produced by a number of pathogenic bacteria in an effort to escape from IgA mediated immunity (Kilan *et al*, 1988). Blood contains a large quantity (2mg/mL) (Mestecky and McGhee, 1987) of predominantly monomeric IgA; this circulatory pool is largely independent of the mucosal pool in Man (Jonard *et al* 1984). Secondly, IgA is known to bind to Fcα receptor (mFcαR,CD 89), which has been cloned recently (Shen *et al*, 1989, Monteiro, *et al* 1990, Maliezewski *et al*, 1990) on the surface of eosinophils (Monteiro *et al* 1993), neutrophils (Kurita *et al*, 1986) and monocytes/ macrophages (Shen *et al*,1989) thereby triggering effector responses. Thirdly, B lymphocytes and T lymphocytes possess surface receptors for Fcα through which immunoregulatory signals are thought to be transmitted (Weisbart *et al* 1988). Fourthly, a receptor specific for sIgA has been identified on eosinophils which may have a role in eosinophil activation and degranulation during the inflammatory response (Lamkhioued *et al*, 1995). Finally, IgA is thought to activate complement through the alternative pathway (Lucisano-Valim and Lachmann, 1991; Carayannopoulos *et al*,1994) . Owing to these functional properties, especially the ability to be transported through cells, IgA not only plays a role in host defence against viruses and bacteria, but is also potentially critical for

neutralisation of intracellular viruses in tissues expressing polymeric immunoglobulin A (pIgA) (Mazanec *et al*,1992), and destruction of the helminths, protozoa and other eukaryotic parasites (Grzych *et al*, 1993).

Structure and function of IgA

IgA is a tetrameric protein comprising of two identical light chains (κ or λ) and two identical heavy chains (α) which give IgA its specific biological properties. In man, there are two IgA isotypes, IgA1 and IgA2 - defined by the differences in the α chain, mostly due to a deletion in the hinge region at the mid point of the α2 chain (Kerr, 1990). Within the α chains of the IgA2 subclass there are two allotypic variants, IgA2m(1), and IgA2m(2). As immunological mediators, however, no major functional differences have been assigned to the two subclasses. However, their ratio can vary in the local population of plasma cells in different parts of the body, in different body fluids and secretions. There is also considerable variation in antibody response to different kinds of antigens and in certain diseases (Mestecky *et al*, 1989). The IgA1 subclass is uniquely susceptible to proteolysis by IgA proteases, released by certain pathogenic bacteria, that manifest unusual substrate specificity for the hinge region of the α1 chain, but not IgA2 (Plaut, 1983; Kilian *et al*, 1988). The IgA2 subclass thus appears to have some advantage in resisting infections by these pathogens. Moreover, IgA2 is more glycosylated than IgA1 suggesting a role of glycosylation in protection of this secreted antibody in the mucosal milieu (Tomana *et al*,1976).

In contrast, IgA in serum, is mostly monomeric IgA1, compared to the IgA produced by the mucosal plasma cells that enters the local secretions which is relatively enriched in

IgA2 (Kett *et al*, 1986) (although the magnitude varies in different mucous membranes). For example, the relative amount of IgA2 increases along the intestinal tract with distance from the stomach (Mestecky *et al,* 1989). As mentioned, mucosal IgA is mostly dimeric, i.e. composed of two of the basic four chain immunoglobulin subunits, so that the dimer possesses 4 α and 4 L polypeptide chains. The two subunits of the dimer are joined covalently by disulphide bridges from two of the α chains and by a disulphide bridge from the other two α-chains C termini to a third type of polypeptide chain, the 17-kDa **J** chain (Mestecky *et al*,1974; Garcia-Pardo *et al*,1981; Bastain *et al*, 1992). **J** chains, like immunoglobulin heavy chains and light chains, are synthesised by plasma cells. These **J** chains are present in all oligomeric immunoglobulins, i.e., all antibody molecules having more than one basic four-chain subunit, for example, dimeric IgA and pentameric IgM. The **J** chain is thought to play a role in initiating or stabilising the oligomeric structure and in providing a conformation for IgA that enhances binding to the pIgAR, also know as transmembrane secretory component (SC) (Brandtzaeg and Prydz ,1984) . The single most important distinguishing feature of secretory IgA with respect to serum IgA is the presence of SC. In addition to its crucial role in the transport of pIgA to mucosal secretions, SC has been shown to confer resistance to proteolysis on secretory IgA, presumably an added advantage in the gut mucosal environment (Brown *et al*,1970).

IgA in mucosal defence

It is generally agreed that the role of mucosal IgA antibodies is to protect against infectious micro-organisms and other foreign materials, which gain access through to the mucosal linings of the body. This concept of protection by IgA is supported by

studies of oral (Sabin) vaccination against poliomyelitis, which induces intestinal IgA antibodies (Ogra and Karzon , 1971), as well as by studies of resistance to a variety of other mucosal infections. In all these studies, it has been found that resistance best correlates with the content of IgA antibodies in the local secretions (Liew *et al,* 1984; Offit and Clark , 1985). In addition, it is a well established fact that patients with IgA deficiency manifest an increased incidence of mucosal infections as well as other disorders related to mucous membranes (Hanson *et al* , 1983). This background supported the modern efforts to develop oral immunisation for intestinal pathogens like *Vibrio cholerae* (Clemens *et al*, 1986). The observation that IgA can neutralise intracellular viruses (Mazanec *et al*, 1992) in the epithelial cells during transcytosis, generates a recently developed technique (Chen *et al,* 1994) of using intracellular antibodies to fight disease or inhibit intracellular mechanisms causing disease. In this method, a gene therapy approach has been used, where the antibody genes are introduce into the cell. This method has the strength that expression of the antibody can be directed to the appropriate subcellular compartment. Although there is a clear limitation that only pIgA-expressing cell types can be targeted it may still be worth pursuing for epithelial cell specific viruses and tumours. Finally, it is now recognised that mucosal IgA is functioning in three different tiers to protect the host (Mazanec *et al*, 1992). The innermost tier is the lamina propria, into which abundant plasma cells secrete dimeric IgA. If IgA antibody binds antigen in the lamina propria, the antigen-antibody complex can be directly excreted. The middle tier is the epithelial lining of the mucous membrane. If IgA antibody in transit to the secretion meets an infectious pathogen inside an epithelial cell, it can neutralise or excrete it. The outer tier, is the well known place for secretory IgA, e.g. intestinal lumen , where it can bind to microbes or their toxins and prevent attachment to and penetration through the mucosal epithelium.

2.2 Immunoglobulin M (IgM)

IgM accounts for approximately ten percent of the immunoglobulin pool. The molecule is a pentamer of the basic antibody four-chain structure. The individual heavy chains have a molecular weight of approximately 65 000 and the whole molecule has a molecular weight of 970 000 (Roit *et al*, 1996). It is a largely poly-reactive, natural antibody confined to the intravascular pool and considered as a 'primary' antibody as frequently seen in the immune response to antigenically complex infectious organisms. In 1970, it was pointed out that IgM also appears in exocrine fluids because of active secretion. Secretory IgM (SIgM) was later shown to be associated with SC (secretory component) and to follow the same intracellular route through glandular epithelia as SIgA (Brandtzaeg, 1970 & 1974 ; Brown *et al*, 1976). However, the concentration of secretory IgM is substantially lower than that of secretory IgA because of the lower proportion of IgM producing cells in the mucosa. It has been shown in a study that the number of IgA and IgM-producing cells in various segments of normal human gut mucosa varies considerably; these are as follows: in the upper small bowel, about 18% cells are IgM producing cells compared to 80% of IgA, and in ileum and large bowel , these are 11% *vs* 84% and 6% *vs* 90% respectively (Brandtzaeg, 1985). It may also be possible that IgM is not transported as efficiently as pIgA because of a molecular weight restriction in SC-dependent transport (Schiff *et al*,1984). Interestingly, a compensatory increase of IgM is observed in mucosal tissue and secretions of IgA-deficient individuals (Plebani *et al*,1983).

2.3 Immunoglobulin G (IgG)

Immunoglobulin G (IgG) is the major immunoglobulin in normal human serum, accounting for 70-75% of the total immunoglobulin pool (Roit *et al* ;1996). IgG consists of a single four-chain molecule with a sedimentation coefficient of 7S and a molecular weight of 146000. In Man, there are four subclasses of IgG, IgG1-IgG4. These subclasses differ only slightly in their amino acid sequences. Most of the differences are clustered in the hinge region and give rise to differing patterns of interchain disulphide bonds between the four proteins. The most significant structural difference is the elongated hinge region of IgG3 which confers on it a higher molecular weight and may possibly give some enhanced biological activity (Roit *et al*, 1996). Having two identical binding sites in a single IgG molecule allows the antibody to bind with high avidity to antigens with repeating epitopes or to aggregates of antigens (Janeway and Travers , 1996; Male *et a,l* 1996). The flexibility of the IgG molecule, inherent within the elbow bend of the Fab (between V and C domians), and particularly within the hinge region allows for the two binding sites to cope with antigenic epitopes with a range of spacing and orientations (Pumphrey, 1990). The binding of antigen can result in direct inactivation of infectious agents by blocking of functional sites with enzymic or receptor-binding activity. Other important properties of IgG is that even if it becomes aggregated by antigens it can still interact with other components of the immune systems by either activating the complement cascade or by binding to Fcγ receptors on various cell types (Janeway and Travers,1996). Both of these processes can assist in the opsonisation of antigen and in the triggering of inflammation and the enhancement of an immune response against infectious agents. However, any consideration of the interaction of IgG antibodies with Fc receptors needs to take account of the different types of receptor. In humans, there are three

different classes of Fc receptors for human IgG (FcγR). Human FcR1 (CD64) can bind monomeric IgG with high affinity and is expressed constitutively on macrophage and monocytes and can be induced on neutrophils and eosinophils.

Human FcγRII (CD32) binds IgG only in complexed or polymeric forms and is widely expressed on a range of cell types including monocytes, macrophages, basophils, eosinophils, Langerhans cells, B cells and platelets. Human Fc RIII (CD16) is also a medium to low affinity receptor and is expressed on macrophages, and some large granular lymphocytes, killer (K cells), some natural killer cells (NK cells), and neutrophils . This also can be induced on eosinophils and monocytes (van de Winkle and Capel ,1993).

Another important property of IgG that has been recognised for many years is that the biological half-life (3-4 weeks) is much longer when compared to other plasma proteins, including other immunoglobulins such as IgM and IgA (3-7 days) (Janeway and Travers, 1996; and Male *et al*,1996). It has also been noted that the half life is dependent upon the total concentration of IgG in the plasma. Thus if the concentration of IgG is raised as in a condition such as myeloma, the half life is greatly reduced while if the IgG concentration is lowered as in agammaglobulinaemia, the half-life of administered immunoglobulin is extended. This suggested a saturable receptor-driven mechanism for the control of the catabolic rate of IgG (Brambell *et al*, 1964). Thus a relatively constant plasma concentration of IgG can be maintained over a fairly wide range of synthetic rates. IgG antibody is also distributed evenly between the intravascular and extravascular pool, and notably in humans, all the subclasses of IgG can cross the placenta and thus maternal IgG confers immunity in neonates.

IgG concentration in mucosal secretions is approximately the same as or somewhat greater than that of IgM. IgG is thought to enter the mucosal secretions non-specifically via para-cellular transport or fluid phase endocytosis (Ogra *et al*, 1994). However, IgG-producing cells constitute 3% -5% of the immunocyte population in the normal intestinal mucosa but a considerably larger contribution (12%-14%) is found in the gastric mucosa (Brandtzaeg *et al*,1985). This gastric contribution may be affected by low grade gastritis even in a healthy subject (Valnes *et al*,1986). In certain diseases such as inflammatory bowel disease, an increased concentration of IgG is found in whole gut lavage fluid. It has been shown that increased IgG in lavage can be used as an objective measure of disease activity in IBD (Choudari *et al* ,1993). The source of the high IgG concentration in the gut lumen is currently a subject of debate. It has been postulated that this high concentration of IgG is serum-derived because there is no special mechanism for transportation of IgG in the gut lumen. It has also been shown in some other studies that there is evidence of local production of high IgG in the gut lumen suggested as a ' second line defence' in the mucosa. (Brandtzaeg *et al* 1985). Lately, a pathway involving FcRn within intestinal epithelia which may mediate the bi-directional transport of IgG across intestinal epithelia, has been reported (Christ *et al*,1997).

However, it is worth knowing the source of this increased concentration of IgG as it is not only beneficial as a second line defence in the mucosa but also has inflammatory potential.

2.4 Immunoglobulin E (IgE)

IgE is the immunoglobulin found in lowest levels. The concentration of IgE in the serum of a normal individual is of the order of 100-300ng/mL (Ogra *et al*, 1994). However, IgE antibodies have unique biological activities. The antibodies sensitise mast cells and basophilic granulocytes of homologous species and the reaction to antigen to cell bound IgE antibody induces the release of various mediators that cause allergic reactions. In fact, it is now known that there are two different receptors for IgE on cells. The high affinity receptor (FcεRI) is found on the mast cells and basophils and is the 'classical' IgE receptor. This receptor is part of the immunoglobulin supergene family and quite distinct from the low affinity receptor for IgE (FcεRII) found on leucocytes and lymphocytes. The importance of the high affinity IgE receptor for IgE is that although the serum half life of free IgE is only a few days, mast cells may remain sensitised by IgE for many months. This is due to the high affinity of binding to the IgE receptor FcεRI, which protects IgE from serum proteases.

Normally, allergic diseases are induced by exposure to extrinsic antigens such as allergen; therefore IgE mediated allergic diseases are induced most frequently in the respiratory tract, gastrointestinal tract, and the skin. In the gastrointestinal tract, the IgE positive mucosal mast cells are involved in the pathogenesis of certain gastrointestinal diseases (Brandtzaeg,1986).

Section 3.

Gut bacterial antigens

In this section, various antigens associated with bacteria are discussed. In particular, attention is given to the Gram-negative Enterobacteriaceae which are one of the major aerobic inhabitants of the human gut. The genus *Escherichia* includes *Escherchia coli* a typical member of Enterobacteriaceae and is closely related to a number of other members in the family. I will discuss this organism in detail as a model of Enterobacteriaceae.

It is generally agreed that micro-organisms are broadly divided into three groups: Archea, Prokaryota and Eukaryota. Prokaryotes have a simple structure and true bacteria come under this classification. The features of Prokaryotes include: a single chromosome, absence of nuclear membrane, mitochondria and other organelles; they divide by binary fission. On the other hand algae, protozoa and fungi are classified as Eukaryotes. The more sophisticated intracellular features which are essential for Eukaryotes are: multiple numbers of chromosomes, presence of organelles such as mitochondria and nuclear membrane, and multiplication by mitosis (Carlile and Skehl, 1974). The Archea represents very primitive bacteria that are found in extremes of environment: none of these is pathogenic.

3.1 General structural features of bacteria

Cell envelope

The prokaryotic envelope can be defined as the complex of membrane-associated macromolecules which together form the boundary between the inside and outside of the cell. Basically, the cell envelope has two components: an inner **cytoplasmic membrane** and outer layer :**cell wall / outer membrane.**

Essentially, the cell envelope is responsible for a number of important functions: it confers the cell shape, supports other structures of its own and gives rigidity; and thereby protects the cell from the external environment as well as from osmotic lysis. It also acts as a selective barrier and maintains ionic balance between inside and outside (Poxton, 1993). There are considerable differences in the structure of the cell envelope between Gram-positives and Gram-negatives. These are described later.

The cytoplasmic membrane:

The cytoplasmic membrane is the site of many major cellular functions (Jawetz *et al*, 1987), which include: a) selective permeability and transport of solutions, b) electron transport and oxidative phosphorylation, c) secretion of extracellular hydrolytic enzymes (exoenzymes) and d) biosynthesis of phospholipids and proteins.

Bacterial cell wall

Bacterial cells have high intracellular concentrations of inorganic ions and thus have high osmotic pressure (5-20 atmosphere) (Hancock and Poxton, 1988). This leads to

fluid influx into bacterial cell. Therefore, the cell walls need to resist cell lysis. Bacterial cell walls are described in more detail later in this section.

Bacterial capsule

It is a well known fact that many bacteria are surrounded with a large amount of extracellular polysaccharide. This polysaccharide layer is described under different names depending on its condensation; some times it is known as the 'slime layer'. When this layer is condensed and well defined, it is called a 'capsule' and when it is a loose meshwork, it is then called a 'glycocalyx'.

The capsule protects pathogenic bacteria against non-specific host defences, notably the action of complement and phagocytes. Thus, encapsulated bacteria are often virulent, and their capsules are the virulence factors. Some bacterial capsules mimic host macromolecules and thus camouflage the underlying bacterial antigens from the host immune systems.

The Nucleus

Bacteria lack true nuclei since there is no nuclear membrane or mitotic apparatus as found in eukaryotic cells. The DNA of the bacterial 'nucleus' is a single circle of double stranded DNA and it is thus regarded as a single chromosome of about 1 mm in circumference and 2-3x10 ^{9} molecular weight. Under the electron microscope this DNA can be seen as fibrils within the cytoplasm. The nuclear DNA is associated with a small amount of RNA and RNA polymerase. The DNA is folded around an RNA core

which seems to keep the DNA in its condensed form (PettiJohn, 1976; Kleppe *et al*, 1979).

Ribosomes

Ribosomes in a bacterial cell are distributed throughout the cytoplasm and are the principal site of protein synthesis. The cytoplasmic RNA of bacterial cells is of three types, separated according to function: ribosomal (rRNA), amino acid transfer or transfer RNA (tRNA) and messenger RNA (mRNA). Bacterial ribosomes have a sedimentation constant of 70S compared to that of the human (eukaryotic) ribosomes which is 80S. The aggregates of the 70S particle are attached to a common strand of mRNA to form polysomes. In these, the mRNA determines the order of amino acids in the peptide chains formed i.e. functions as the template for protein synthesis. Despite the fact that there are many similarities between bacterial ribosomes and those of human cellular tissues, differences still exist which allow the use of antibacterial agents such as streptomycin which interferes with bacterial metabolism at the ribosomal level without unduly affecting human cell ribosomal function (Jawetz *et al* ,1987).

Flagella and Fimbriae (Pili)

Flagella: These are thread like appendages, about 12-30 nm in diameter composed entirely of protein called 'flagellin'. They are the organ of locomotion of bacteria. If the flagella are mechanically removed, new flagella are formed and the motility is restored within 3-6 minutes (Macnab and Aizawa, 1984). Three types of arrangement are known: monotrichous (single polar flagellum), lophotrichous (tuft of polar flagella), and peritrichous (flagella distributed over the entire cell).

Bacteria respond to change in the environment by a mechanism called sensory transduction (Tayler, 1983). Sensory transduction may occur due to chemotaxis, phototaxis (movement towards light) or aerotaxis (movement towards optimum oxygen concentration) (Boyd and Siman, 1980).

Fimbriae (Pili)

Many Gram-negative bacteria possess rigid surface appendages called 'pili' (Latin 'hairs') or fimbriae (Latin 'fringes'). These are composed of a protein subunit called 'pilin'. There are two types of Pili : 1) sex pili which are responsible for transfer of genes from a donor to a recipient cell during bacterial conjugation; 2) ordinary fimbriae which are responsible for adherence of bacterial cells to host cells. In certain pathogenic bacteria such as enterotoxigenic *E.coli*, these are, known as colonisation factor antigens (Beachey, 1980).

Structure of bacterial cell walls

The bacterium owes its strength to a layer composed of a substance called mucopeptide or peptidoglycan. This layer will be described in detail later in the chapter. However, bacteria are classified as Gram-positive or Gram-negative according to their response to the Gram-staining procedure. This procedure, named after its inventor, was developed in an attempt to stain bacteria selectively in infected tissue. Bacteria that retain the crystal violet stain are called Gram-positive, while bacteria decolourized by washing with acetone or alcohol, which subsequently take up a red counterstain are called Gram-negative. The difference between Gram-positive and Gram-negative bacteria is in fact due to differences in the structure of the cell walls which determine whether the gram stain is lost or retained. Although, there are a

number of variations in bacterial cell structure, in a simplistic view this can divided into two groups:

1) Gram-positive bacteria: The cell wall is composed of a thick, cross linked peptidoglycan layer which is much thicker than that of the Gram-negative bacteria (20-30 nm vs 3-5 nm respectively). These bacteria also contain teichoic acid which consists of a water soluble polymer of ribitol phosphate or glycerol phosphate residue (Schleifer and Kandler, 1972). 2) Gram-negative bacteria: The cell wall is composed of a thin, cross linked, peptidoglycan layer (figure:1.1). The outer layer of the cell wall of Gram-negative bacteria contains, lipolpolysaccharide molecules which form 'endotoxins'.

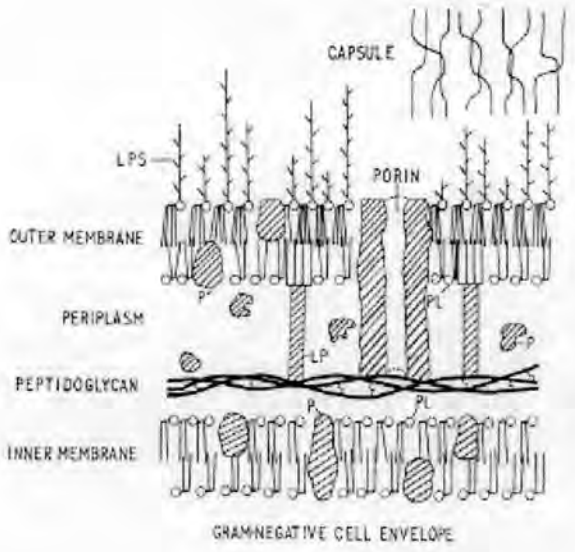


Figure 1.1: Gram -negative bacterial cell wall (Courtesy, Professor Poxton)

The peptidoglycans

The basic building block of peptidoglycan is a disaccharide of *N*-acetyl-D-glucosamine and *N*-acetyl-D-muramic acid, β linked (1-4) in long chains. A peptide side chain is attached through an amide linkage to the acyl group of *N*-acetyl muramic acid, which in turn is cross linked to side chains on adjacent glycan chains by peptide cross bridges. Variation in structure occurs in any of these three components. The glycan chain of alternating *N*-acetyl muramic acid and *N*-acetyl glucosamine is the least variable and found in almost all species of Gram-positive and Gram-negative bacteria, with a few exceptions such as *Mycobacterium* and *Nocardia* species in which N-glycolyl groups are found instead of N-acetyl groups (Poxton, 1993).

The peptide side chains of peptidoglycans are also well conserved. The main type consists of a tetrapeptide of alternating L and D form amino acids: usually L-alanine , D-glutamic acid, L- lysine (or another diamino acid) and D-alanine. The alternative to L-lysine, is either an isomer of di-aminopimelic acid (commonly *meso*-) or ornithine. This diamino acid is species-specific and often used in classification.

Chemical structure and special features of Gram-negative bacterial cell wall.

Gram-negative cell walls contain three polymers which lie outside the peptidoglycan layer: lipoprotein, outer membrane, and lipopolysaccharide (LPS).

Lipoprotein

Molecules of an unusual lipoprotein serve to cross-link the outer membrane and peptidoglycan layer . The protein component contains 57 amino-acids, representing repeats of a 15-amino-acid sequence ; it is peptide linked to di-aminopimelic acid residues of the peptidoglycan tetrapeptide side chains. The lipid component is noncovalently linked to the outer membrane (Jawetz *et al*,1987).

Outer membrane

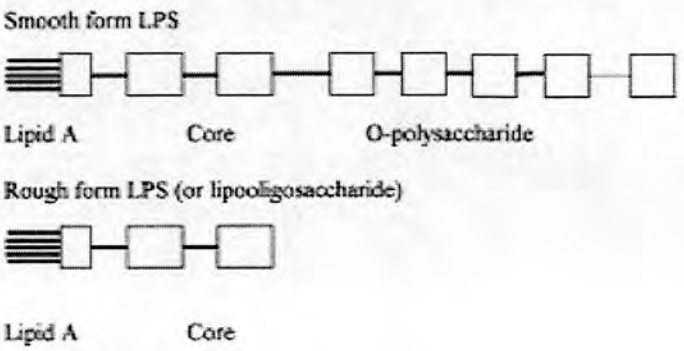
The outer membrane is a typical lipid bilayer with phospholipid in the inner leaflet and LPS in the outer leaflet. It is relatively permeable to small molecules, both hydrophilic and hydrophobic. Proteins of the outer membrane are individually named after the genes that encode them. In *E.coli* and *Salmonella typhimurium* the porin proteins OmpC, D and F penetrate both faces of the outer membrane and form relatively non-specific pores that allow diffusion of small hydrophilic solutes through the membrane. In addition to its barrier preventing the leakage of the periplasmic proteins, the outer membrane protects the bacterial cell from bile salts and hydrolytic enzymes especially in enteric bacteria. The protein pores render the outer membrane permeable to low-molecular weight solutes while higher molecular weight molecules, such as antibiotics, cannot enter easily. This may explain the higher antibiotic resistance of Gram-negative bacteria.

Lipopolysaccharide (LPS)

Lipopolysaccharide is an essential component of the outer membrane of all Gram-negative bacteria. Structurally, most types of LPS are composed of three distinct

regions: the lipid A, the core oligosaccharide and the O-polysaccharide consisting of long chains of repeating oligosaccharide units (figure: 1.2). This general

Figure 1.2 Diagrammatic representation of lipopolysaccharide



(courtesy Professor Poxton)

structure is typical of the LPS of *E.coli* and related enterobacteria and is often referred to as the smooth or S-form chemotype. Mutants of enterobacteria that have lost the O-polysaccharide region of their LPS molecule are described as rough and produce an LPS molecule of R-form chemotype (Poxton ,1995). The O-specific chain (O antigen) determines the O-serotype of the strain and in *E.coli* there are over 160 different known serotypes.

The core oligosaccharide structurally and serologically is divided into two regions: inner and outer core. In *E.coli*, there are five recognised core structures which show great similarity to each other and to that of *Salmonella* species. The outer cores consist of five hexoses (D-glucose, D-galactose and D-glucosamine in different arrangements and configurations), but showing an overall degree of similarity and cross-reactivity. These can be differentiated serologically by monoclonal antibodies (Gibb *et al* , 1992). The inner part of the core is much more conserved, and it contains the unique sugar called KDO (2-keto-3-deoxy-D-manno-octonate) and heptose trisaccharide as well as phosphate substituents . There is little variation between closely related species (figure:1.3).

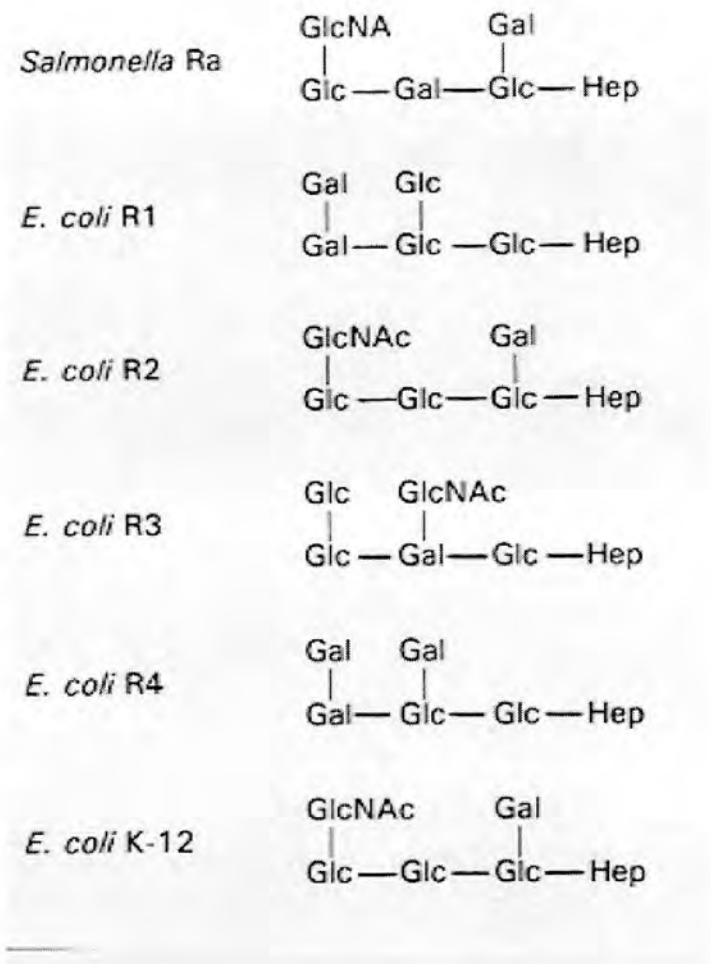


Figure 1.3: Chemical composition of various core types of LPS (courtesy Professor Poxton)

The lipid A region is much more conserved and has a unique fatty acid called β-hydroxymyristic acid. Lipid A is often highly toxic and it is the biologically active part of the lipopolysaccharide. The minimum structure of the LPS molecule, which is essential for the viability of the bacteria is equivalent to the Re-form LPS (Rietschel *et al*, 1990).

The role of lipid A and inner core regions of lipopolysaccharide in pathogenesis of sepsis

Different parts of the LPS molecule have got different biological activities and thus pathogenic potentials. The roles of these parts of LPS are described in detail.

The Endotoxin

The signs and symptoms seen in patients suffering from Gram-negative sepsis can be reproduced in animals by the injection of a suspension of killed *E.coli* or *Salmonella* bacteria; cultured suspension is less effective in reproducing the symptoms of shock (Raetz, 1991). This observation coined the term 'endotoxin' because it was found that a portion of the bacterium itself, rather than a secreted 'exotoxin' was responsible for the pathological process. Bacterial endotoxic shock (septic shock) is a serious medical condition associated with high mortality. Studies have identified the bacterial product responsible for producing fever and shock associated with Gram-negative bacteria. The lipid A-KDO portion of LPS is necessary to reproduce the signs and symptoms of sepsis (Young *et al* 1991). It was recognised that infections with a variety of gram-negative organisms produce very similar symptoms as they have very similar Lipid A . On the other hand, the action of an 'exotoxin' is quite specific.

The effects of endotoxin administration are numerous and include, fever, myocardial depression, coagulopathy, hypotension, acute respiratory distress and multi-organ failure (Shenep, 1992). It is well known that the capacity of LPS to produce such a variety of symptoms is due to release of cytokines and other mediators, which in turn are responsible for the activation of the acute phase response. In acute phase

responses there are a number of processes, e.g, haematological, metabolic and immunological, which become activated (Glauser *et al*, 1991). Interestingly, in health as well as disease, the host is constantly exposed to endotoxin; the symptoms related to the endotoxin insult depend upon the dose of LPS and the immune status of the host. In fact, several mechanisms have evolved for scavenging and detoxifying LPS in small amounts as well as responding to major insults of toxin. Great progress has been made in the last decade in the understanding of how endotoxins react with various components of the immune system.

Stimulation of mammalian cells and production of proinflammatory mediators by endotoxin

Endotoxin binds to a number of surface receptors on macrophages, monocytes and neutrophils, and also with plasma and intracellular proteins (Morrision, 1990; Lei and Chen, 1992). The binding of LPS to macrophages is greatly increased if the LPS forms a complex with a large acute phase protein; the LPS-binding protein (LBP) (Tobias *et al*, 1993). These LPS-LBS complexes then bind to the macrophage CD14 molecule (Wright *et al*,1990), which stimulates macrophages to produce the powerful proinflammatory mediators, like nitric oxide (NO), tumour necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6). Overstimulation of these cytokines, together with LPS induced effect on the clotting and complement cascades, results in the clinical symptoms of 'endotoxic shock' which is synonymous with 'sepsis'. Severe sepsis causes disseminated intravascular coagulation and multiple organ failure (MOF) leading to death (Bone, 1993).

Anti-LPS antibodies

Antibodies produced to the various domains of the LPS molecule have different specificity. For instance, antibodies to lipid A and the inner core are likely to be cross-reactive with LPS molecules from several different species of Gram-negative bacteria, because the lipid A-inner core region of LPS is conserved. Conversely, antibodies to the O-polysaccharide may only react with the LPS of specific serotypes (Poxton, 1995).

The major impetus of LPS antibody research, was to find an immunotherapeutic agent to treat Gram-negative sepsis or endotoxaemia. This was prompted by an elegant report of Zeigler and colleagues in 1982 showing that passive administration of plasma from donors immunised with the 'J5' mutant of *E. coli* (which possesses an LPS of Rc like chemotype) had a beneficial effect on patients with sepsis (Ziegler *et al*, 1982).

For the last decade, there has been considerable research on anti-LPS antibodies. Various methods and a vast range of immunisation protocols have been used to produce a wide range of antibodies. These vary from broadly cross-reactive to recognition of a single antigen. It has also been shown that it is possible to produce monoclonal antibodies of the IgM and IgG classes with differing proportions of all the isotypes of IgG (depending on the study). It has also been recognised that it is possible to demonstrate cross-species protection in a range of *in vitro* and *in vivo* assays (Di Padova *et al*,1993). Conversely, it has not been possible to demonstrate conclusively an antibody which binds to all LPS molecules and is also cross-reactive between species (Poxton, 1995). Several techniques have been tested to measure antibodies binding to LPS over the last couple of years. Also improvements have been

made in antibody production against LPS, by structural integration and thereby enhanced antigenicity.

Serology of anti-LPS antibodies

The 'Widal' reaction is a typical serological test for enteric fever caused by *Salmonella typhi* and *S. paratyphi*. The test measures antibodies to the *Salmonella* O-antigen, Vi antigen and H antigen by an agglutination technique. Many more serological studies for the detection of antibodies to LPS or O-antigens of a wide range of Gram-negative bacteria have been developed over the last two decades. Most of them employ the ELISA technique but few of them have been exploited commercially (Poxton, 1995).

It has been found that the measurement of 'anti-endotoxin antibodies' is important in various clinical settings. Some studies have suggested that high levels of antibodies to endotoxin are good prognostic indicators in sepsis patients and that levels of passively administered antibodies should be monitored (McCabe *et al*, 1972; Pollack *et al*, 1983). There has been a great deal of interest in Edinburgh in LPS antibody research, especially to find an anti-endotoxin antibody from blood donor plasma for therapeutic use (Barclay, 1990; Barclay and Scott, 1987).

Anti-LPS core (EndoCAb) ELISA - antibody to endotoxin

An ELISA technique for the measurement of cross-reactive, anti-LPS/endotoxin antibodies has been developed by Barclay and colleagues. This is used for screening blood donors for high levels of these antibodies, with a view to manufacture of therapeutic hyper- immune intravenous IgG. This technique has now been used in a

variety of situations for the indirect measurement of endotoxaemia (Barclay ,1990). Basically, the method employs a mixture of four 'rough' mutant LPS in a polymyxin ELISA. The hypothesis is that exposure to systemic endotoxin will result in a decrease in systemic antibody due to complexing of LPS with endotoxin and clearance. Subsequent to this decrease, levels can recover and rise to exceptionally high values. By observing the dynamics of the antibody levels it is possible to use them as a prognostic indicator e.g. depressed IgG and /or absence of the characteristic rise indicates a poor prognosis in septic patient. This method can also be used for monitoring levels during administration of therapeutic antibody (Poxton, 1995). Barclay and his group investigated this anti-endotoxin antibody technique in a variety of clinical settings. These include: patients with pancreatitis (Windsor *et al*, 1989), gut mucosal hypoperfusion in surgical patients (Mythen *et al*, 1993), patients with sepsis syndrome (Barclay *et al*, 1989), a patient with HUS (haemolytic uraemic syndrome; (Heyderman *et al,* 1994) and in victims of sudden infant death syndrome (Oppenheim *et al*, 1994). Poxton and his colleagues also attempted to measure antibody to LPS of a colonic *Bacteroides* species, Gram negative-anaerobic organisms making up a major part of the gut flora. The idea behind this approach was that *Bacteroides* LPS is more relevant than enterobacterial LPS, as much of the systemic endotoxin originating in the gut (large bowel rather than small bowel) has been translocated during periods of shock-induced gut ischaemia (Allan *et al*, 1995). This promising area needs further research.

Intestinal immune response to *E. coli*

As was mentioned earlier, the close relationship between *E.coli* and other Enterobacteriaceae, especially *Shigella* and *Salmonella* spp, suggests that the immune responses to these organisms may have much in common. It is worthwhile knowing the details of the host immune response to these organisms. Moreover, as *E.coli* is a major pathogen of neonates and young children, an understanding of these immune mechanisms may offer potential strategies for the protection of those at risk.

The nature of exposure to *E.coli* ranges from superficial gastro-intestinal colonisation to a pathological systemic infection. The range of immune responses is equally wide. It is important to know the antigenic and virulence factors of *E. coli* bacteria.

The antigens of *E.coli*

Like other Gram-negative bacteria, *E.coli* has antigens and virulence factors in the cell envelope, LPS and capsules. In *E.coli*, many of the strains produce polysaccharide capsules (capsular antigen K) that are important virulence factors which help the bacteria to evade or resist non-specific host defence during the early phase of infection. Specific anti-K antibodies are necessary for optimal opsonization of K antigen bearing strains. K-antigens, particularly K1, have relatively low immunogenicity, because they share partial homology with certain host structures (Silver *et al*, 1988).

O- polysaccharide (O-antigen)

As has already been mentioned the structure of the lipid A-inner core region of LPS is extremely constant while the outer core and O-polysaccharide chains vary

considerably. The variation may occur even within the same species. However, the combination of O-, H-(flagellar) and K- (capsular) antigens defines the serotype of *E.coli.* Apart from its five outer core structures, *E.coli* has 170 different O-antigens. These O-antigens can be differentiated by their reaction with polyclonal rabbit antisera (Orskov & Orskov, 1977). Precise chemical analysis of the O-polysaccharide from *E.coli* of the same O-serogroup shows heterogeneity. For example, three different chemotypes have been classified as O1 and the 170 different O-antigens may be an underestimate of the true variation (Gupta,1992; Jann *et al*, 1992).

There is a curious relationship between the pathogenicity of various *E.coli* strains and their O-serogroups. In spite of the large number of O-antigens, a few appear to predominate among isolates recovered from specific diseases, as compared with those isolated from the faeces of normal individuals (Robert *et al*,1975). Thus, a very limited number of O-serotypes are associated with *E.coli* that cause diarrhoea and these do not in general overlap with those frequently associated with urinary tract infection and sepsis (Orskov & Orskov, 1977; Orskov *et al*, 1982). It has been noted that the O-antigens of enteroinvasive *E.coli*, which cause a dysentery-like disease, are chemically similar to those of *Shigella*, classical agents of bacillary dysentery (Jann & Jann,1984).

It seems that there is a constant relationship between various O-antigens with particular diseases. These associations have given rise to the clonal theory of the pathogenicity of *E.coli.* This postulates that pathogenic strains of *E.coli* are the descendants of a few ancestral lines that somehow acquired pathogenic traits (Orskov & Orskov, 1977; Orskov *et al*, 1982). The ability of these strains of *E.coli* to cause certain diseases is not necessarily due to their particular O-antigens, but rather the

genes they carry for other traits that enhance their pathogenicity, such as enterotoxins and colonisation factors. Whether the specific O-antigens of these strains have a role in the disease process has not been determined.

Fimbrial antigens

There is a clear correlation between bacterial adhesion and pathogenesis (Krogfelt, 1991). About 70 per cent of wild *E. coli* express mannose-sensitive type-1 fimbriae. Fimbriae such as K88, K99 and colonising factor antigen (CFA) are associated with diarrhoeal diseases.

Other antigens

E.coli, like other Gram-negative bacteria has outer membrane proteins, enterobacterial common antigens, lipoprotein and α-haemolysin. α -haemolysin is secreted by the most haemolytic strains of *E.coli*. Production of α-haemolysin is an important virulence factor and anti-α-haemolysin antibody titre correlates closely with the degree of invasiveness of infection (Emody *et al*, 1982).

The antibody response in enteric *Escherichia coli* infections

Enterotoxin-producing *Escherichia coli*

Enterotoxin-producing *E.coli* (ETEC) is one of the major causes of infant and childhood diarrhoea in developing countries. Studies conducted in a large diarrhoeal diseases hospital in urban Bangladesh, showed that ETEC is responsible for diarrhoea in about 15 percent of cases of children under five (Hoque *et al*, 1994). This

organism is the commonest cause of traveller's diarrhoea. The diarrhoea is due to production of heat labile enterotoxin (LT) and/or heat stable enterotoxin (ST). The ST is poorly immunogenic. In order to produce diarrhoea, ETEC must also adhere to small intestinal mucosa and in humans various fimbrial (CFA) antigens have been described. Most patients with diarrhoea due to LT-producing ETEC can be shown to develop serum and local antibody response to LT, CFA and LPS, which confers homologous immunity, while asymptomatic acquisition of the organism is generally not associated with an immune response (Stoll *et al*, 1986). Studies in Bangladesh, showed that in paired samples from convalescents, an eight to nine fold rise in anti-CFA and a four fold rise in anti-LT can be demonstrated (Clemens *et al*, 1990). Immunity appears to be primarily directed towards CFA-type fimbrial antigens, and heterologous protection is not conferred where the only common antigen is LT (Levin *et al* 1979). Antibodies to LT and CFA appear to afford synergistic protection from diarrhoea.

Vero-cytotoxin-producing *Escherichia coli*

Vero-cytotoxin-producing *E.coli* (VTEC) are an important cause of diarrhoea in some areas and can cause severe life-threatening complications. Most important are: haemolytic-uraemic syndrome (HUS), and haemorrhagic colitis. At least two Vero toxins, VT1, and VT2, are associated with disease in humans and VTEC isolates may produce either or both of these toxins (Karmali,1989). Antibodies to bacterial components and to Vero cytotoxin can be detected in infection with VTEC. These antibodies, however, are either not found, or found in low titre, in healthy individuals (Kishore *et al*, 1992). The main immune response in VTEC infection is an IgM response to O157 LPS, and detection of such antibodies is useful in providing evidence of infection where faecal VTEC or Vero cytotoxin cannot be demonstrated

(Chart *et al*, 1989). Antibodies to both VT1 and VT2 can be detected by ELISA. However, most patients with *E.coli* O157:H7 infection develop a response to VT1.

Enteroinvasive *Escherichia coli*

Enteroinvasive *E.coli* (EIEC) causes dysentery similar to shigellosis by an invasive mechanism that depends on the presence of certain outer membrane proteins (Gross, 1991). There is a paucity of data in human studies. However, in animal models, the intestinal secretory IgA (SIgA) response is the principal component of the immune response which confers immunity to reinfection.

Enteropathogenic *Escherichia coli*

Enteropathogenic *E.coli* (EPEC) is one of the common causes of diarrhoea in the young and children in developing countries. The diarrhoea is due to the capacity of EPEC to adhere and efface the microvillous surface structure of intestinal epithelial cells. In a considerable number of strains localised adhesion is associated with the presence of a plasmid-encoded 94-kDa OMP, EPEC adherence factor (EAF). Studies show that recipients of EAF-positive strains develop both serum and breast milk antibody to this protein. The antibodies have been shown to inhibit bacterial attachment *in vitro*, and to protect individuals from diarrhoea after challenge with EAF-positive strains (Gray,1997).

Cell-mediated immunity in *Escherichia coli* infections

The humoral response to *E.coli* has been studied extensively while cell-mediated immunity has received less attention. However, the gastrointestinal tract contains numerous immune effector and regulatory cells of lymphoid and myeloid origin that are thought to play a critical role in host defence against enteric infection. Studies have shown that lysates of enteropathogenic *Escherichia coli* (EPEC) inhibit lymphokine production by mitogen-activated human peripheral blood and lamina propria mononuclear cells (Klapproth *et al,* 1995 and 1996). Recently, a further study by the same group has delineated the mechanisms of the alteration of the cytokine production (Malstrom and James, 1998). They showed that pre-exposure to EPEC lysates inhibited mitogen-stimulated interleukin-2 (IL-2), IL-4, and gamma interferon (IFN-γ) production by the murine spleen cells, but IL-10 production was increased. The inhibition was not due to increased apoptosis and was not also blocked by neutralizing antibodies against IL-10 or transforming growth factor β (TGF-β). They went on to do further experiments with other toxin-producing *E.coli* and related strains of bacteria and noted their effect on various human cell lines. They conclude that EPEC and certain related strains of bacteria have the potential to regulate cytokine production selectively by peripheral lymphocytes and gut-associated lymphoid tissue in both human and murine cells by mechanisms that appear to act directly on lymphocytes without increasing apoptosis.

Finally, infection and colonisation by *E.coli* is associated with a variety of host immune responses. These responses have broad significance for an understanding of the host -microbe interaction. There is a great deal of literature about *E.coli* and the antibody responses against it, but in most of the studies there is poor or no real quantitation of

the data. However, there are limited data on intestinal antibody responses against *E.coli* and other gut bacteria, mainly from Dhaka (Stoll *et al*, 1986), from Sweden (Holmgren & Svennerholm, 1992), and from Edinburgh (Ferguson, *et al*, 1994). This area will be discussed further later in the thesis and related to my research findings.

Section 3

Cell migration and Eosinophils

Cell migration

The circulatory and migratory properties of white blood cells have evolved to allow efficient surveillance of tissues of the body for infectious agents and rapid accumulation at sites of tissue injury and infection. Among the white blood cells, lymphocytes continually patrol the body for foreign antigens. They re-circulate from blood through tissue, into lymph, and back to blood. Granulocytes and monocytes, also emigrate from the blood stream in response to molecular changes on the surface of the blood vessels that signal injury or infection. However, unlike lymphocytes they cannot re-circulate. The nature of the stimulus determines whether lymphocytes, monocytes, neutrophils, or eosinophils will be recruited and predominate. The stimulus also exercises specificity in the molecular signals that control traffic of particular leukocyte classes (Springer, 1994).

General morphologic features of eosinophils

Eosinophils, like neutrophils and basophils are a type of granulocyte derived from bone marrow. This group of cells has some unique features which distinguish them from others by their morphological features, intracellular constituents, products, and association with specific diseases. They comprise 2-5% of blood leucocytes in healthy, non-allergic individuals. Eosinophils are similar in size to neutrophils but have bi-lobed nuclei and distinctive cytoplasmic granules. The

numerous specific granules, with their content and structured packaging of cationic proteins, are the distinguising marker of these cells. Although eosinophils in the blood are easily available for quantitation and investigative studies, they are in fact predominantly tissue-dwelling cells; several hundred times as many as in blood (Weller, 1991). These cells are abundant in the tissues with an epithelial interface with exterior, eg. G-I tract, respiratory tract etc. The actual life span of eosinophils is not known, but they live longer than neutrophils and may survive for weeks within tissues (Spry,1988).

Intracellular constituents

The specific granules of eosinophils contain lysosomal hydrolyses as well as most of the cationic proteins unique to eosinophils. The crystalloid core of the granule is composed of major basic proteins (MBP), and the non-core matrix contains eosinophil cationic protein (ECP), eosinphil-derived neurotoxin (EDN), and eosinophil peroxidase(EPO) (Weller,1991).

The MBP is the most abundant cationic protein in eosinophil granules; it is a 14,000-dalton protein rich in arginine residues. It has no recognised enzymatic activity, but is toxic to helminthic parasites, tumour cells and host cells (Butterworth *et al*, 1979; Gleich *et al,* 1986). The ECP is a markedly cationic polypeptide of between 18,000 and 21,000 daltons with bactericidal (Lehrer *et al,*1989), and helminthotoxic activities (Ackerman *et al*, 1985). Like MBP, it is also toxic to the host cells.
Eosinophil derived neurotoxin (EDN), a protein of 18,600 dalton, has some homology with the peptide sequence of ECP. As its name indicates, it is capable of damaging human nerves (Gleich *et al*,1986). Unlike, myeloperoxidase of neutrophils and

monocytes, eosinophil peroxidase (EPO) consists of two polypeptides of about 15,000 and 55,000 daltons. It is toxic to helminthic and protozoan parasites, bacteria, tumour cells and host cells (Jong & Klebanoff, 1980).

In addition to the preformed granule proteins, eosinophils synthesise and release bioactive mediators in response to stimulation. These include platelet-activating factors and eicosanoids, particularly leukotriene C4 (Weller,1991).

Cell surface receptors

Eosinophils, like other granulocytes, express receptors and ligands on their cell surfaces. The eosinophil receptors for immunoglobulin G (IgG) is the low affinity Fc receptor FcγRII (CD32) in contrast to neutrophils and monocytes which bear Fc receptor FcγRII and FcγRIII (CD 16), and FcγRI (CD64) and FcγRII respectively (Weller, 1991). Eosinophils also have receptors for IgE, which are of the low affinity type, quite different from the high affinity classical receptors (FcεRI) expressed on mast cells and basophiles (Capron *et al*, 1981). The eosinophil receptor for IgA binds secretory IgA preferentially and more potently rather than the monomeric form of IgA (Abu-Ghazaleh *et al*,1989). Because eosinophils localise to the mucosal surfaces of the respiratory and gastrointestinal and genito-urinary tract, their IgA receptors can engage secretory IgA at these sites.

Eosinophil receptors for complement components include those for C1q, C3b/C4b(CR1), iC3b(CR3), and C5a. The structure and affinity of the eosinophil C5a receptor differ from those of the C5a receptor on neutrophils (Gerard *et al*, 1989). There are receptors identified on eosinophils for cytokines which are very important for

eosinophil maturation and chemo-attraction. These are: interleukin-3, (IL-3), interleukin-5 (IL-5), and granulocyte-macrophage colony stimulating factors (GM-CSF). Recently, an eosinophil-specific chemokine was identified, this protein molecule not only causes chemotaxis but also is involved in eosinophil-mediated tumour killing (Rothenberg *et al* 1995).

Eosinophils also have receptors for lipid mediators like platelet-activating factors and leukotriene C4. The later are also chemoattractants for eosinophils and they can stimulate the degranulation of eosinophils, as well as the formation of superoxide anions and other oxygen derivatives. Eosinophils also have receptors for glucocorticoid and oestrogen (Weller,1991). Mature eosinophils retain the ability to synthesize proteins, including both the cell surface glycoprotein CD4 and class II protein of the major histocompatibility complex (HLA-DR) (Weller,1991). They have not only the capability to take part in immune responses but also to synthesize and elaborate cytokines (Wong *et al*,1990). In addition, eosinophils also express other cell surface molecules involved in cell-cell interactions, such as adhesion molecules; these integrins and cell adhesion molecules enable eosinophils to migrate from blood to inflamed tissues. Eosinophils express intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), $\alpha 4\beta 1$,$\alpha 4\beta 7$, and $\alpha 6\beta 1$ (Wardlaw *et al*,1996). These adhesion molecules play important roles in the migration and expression of functional responses of eosinophils. However, some of the adhesion molecules as yet have only been described in animal models. The presence and the importance of these adhesion molecules in eosinophil function in humans remains to be defined.

Production and activation of eosinophils

The development and differentiation of eosinophils occurs in bone marrow and is prompted by three important cytokines. These are GM-CSF, IL-3 and IL-5. The GM-CSF and IL-3 also stimulate other leukocytes whereas IL-5 acts more specifically on eosinophils. Studies showed that IL-5 is the principal cytokine responsible for increased eosinophil production (Weller,1991). Eosinophils, like lymphocytes are subject to processes of cellular activation. Several morphological, biochemical and functional studies demonstrated that there are significant differences in normal eosinophils from those from patients with eosinophil related disease. Eosinophils from patients with eosinophilia are morphologically distinct. They have cytoplasmic vacuoles, altered in size and number in granules and loss of granule core containing major basic proteins (Dvorak *et al*, 1990). They are also less dense than normal eosinophils when separated by density gradient centrifugation. These hypodense eosinophils have been found in the blood of patients with asthma, helminthic infections, neoplasia and idiopathic hypereosinophilia (Gleich and Adolphson,1986).

Role of eosinophils in health and disease

It is recognised that eosinophils have specialised roles in host defence against non-phagocytable multicellular parasites. They contribute to antiparasitic host defence by several means, including the release of their cationic granule protein which is highly toxic to mammalian cells. Some of the mechanisms employed in host defence, especially by releasing toxic proteins, are sometimes detrimental to the host's own cells.

The potential role of these cells in inflammation of the respiratory tract as well as in the gut is well recognised. The role of these cells in inflammatory bowel disease and coeliac disease is less clear (Sarin *et al*, 1987; Dvorak, 1980; Talley *et al*, 1992). The pathogenic role of eosinophils and their pro-inflammatory protein in disease has not been established firmly, because of the paucity of studies in this area. There are some questions still un-answered, for instance, how do eosinophils accumulate selectively in allergic diseases and how do they accumulate in high numbers despite being a minority cell type in the circulation? This area will be discussed later in the chapter in relation to my findings.

Section 4

An Appraisal of the Whole Gut Lavage (WGL)Procedure for Diagnosis of Mucosal Inflammation and Protein/Blood loss from the Gut

The WGL procedure which is routinely carried out as a bowel preparation prior to clinical investigations (e.g. colonoscopy, barium enema) has provided a novel opportunity for investigating gut mucosal inflammation and protein or blood loss from the gut. I have used this 'clinical tool' for my research work. It has already been shown from this Laboratory (GI Lab) that this procedure is highly relevant to patient management and has potential for further research in clinical gastroenterology. This procedure has many advantages when compared with the more traditional investigative methods, such as those involving invasive traumatic procedures (i.e. endoscopy and biopsy) or faecal collection, but there are also some disadvantages.

The WGL procedure involves the administration of an isotonic solution which is not absorbed from the gut. It is safe and well tolerated by patients and it clears the gut of faecal debris and leads to the production of a clear fluid per rectum within 3-4 hours. This procedure is non-invasive and in occasional cases involves passing a nasogastric tube for patients who cannot tolerate the oral lavage procedure. There are no gas-producing substrates in the lavage solution (previously mannitol solution was used for bowel preparation, which is fermented and produces hydrogen), so, subsequent patient investigations or therapeutic procedures such as diathermy are completely safe. Moreover, the WGL procedure is commonly used prior to colonic examination. The collection, processing and analysis of the resultant fluid does not require further patient involvement or inconvenience. The fluid can be processed, for example, as serum and urine are processed; thus filtration, centrifugation, or direct addition of

preservative into solution are all easily accomplished. These procedures are much less easily done with faecal specimens, which cannot be immediately dealt with in these ways. After adequate treatment with protease inhibitors, WGL fluid can yield valuable information in both healthy and diseased subjects by measurement of endogenous secretions present in the WGLF. Since the whole gut is perfused during the procedure, inflammatory lesions in areas which are difficult to investigate by direct access (for example, any pathology in the ileum) yield products that can be detected.

It has been confirmed by sequential analysis of WGL samples for different proteins that there is a steady state perfusion once the clear fluid is obtained (Brydon,1993; Sallam,1994, Ferguson *et al*, 1995 ;Croft, 1996). This however, does require monitoring of the patient by trained nursing staff to maintain the correct fluid intake. Measurement of fluid intake rate and concentration of analyte in WGLF can allow quantitative determination of the daily output. Moreover, additional knowledge of blood or serum concentration of the substance permits calculation of blood or serum clearance of the analyte. This eliminates the need for 24 hour faecal collections. Quantitative faecal measurements are not easily made, because the discipline required of the patient and staff and necessary facilities of an investigative metabolic unit are not generally available. Moreover, it has been shown very convincingly that data based on the analysis of faeces are highly misleading, and despite the apparent ease of specimen collection, such studies should be discouraged (Ferguson *et al*, 1995).

Furthermore, in the vast majority of studies where pathological faecal samples are compared with controls, the pathological samples may be of a different form (for example, diarrhoea) from the control and this itself may introduce a physiological difference as highlighted by Strygler *et al* (1990). Since a standard WGL procedure is

used for controls and patients any such sample differences are eliminated when WGLF samples are studied. A further advantage of WGLF compared with faeces in investigative work is the elimination of dietary interference by substances that may give false readings. For example, a dietary component such as red meat can make a significant contribution to the measurement of faecal haemoglobin, thus reducing the sensitivity and specificity of the assay.

The WGL procedure is very well tolerated and has been successfully used in a wide range of subjects from children to the elderly. However, the potential disadvantages of WGL procedure merit consideration:

a) Since the whole gut is perfused, pathological areas are not specifically located as in closed loop studies. Accordingly it is not possible to link protein measured in WGLF to intestinal cells or tissue type and so such investigations require to be combined with studies of biopsies or imaging. However, WGL here provides an extra dimension similar to the dual sugar permeability tests which give diagnostic information complementary to that of jejunal biopsy in patients with malabsorption.

b) Some proteins such as albumin are labile and still degraded to some extent during the lavage procedure, despite the prompt addition of protease inhibitors when the fluid is collected. The amount of degradation will depend on the site of protein secretion and activity of the patient's proteases, with more proximal protein secretion being more affected (Brydon,1993).

c) Patients on average pass samples every 10-20 minutes once clear lavage fluid is being produced per rectum, so gut losses are measured only during this time period; i.e. only continuous loss of protein or blood is measured by this procedure. Occasionally patients with active ulcerative colitis have been observed to pass fresh blood per rectum, have subsequently undergone the WGL procedure, and have been

found to have a normal WGL haemoglobin. This highlights the difference between continuous ooze and acute intermittent bleeding which may stop completely between episodes. Little is known about intermittency of protein loss although,reassuringly, sequential samples show little variation (Brydon,1993; Sallam, 1994; Croft, 1996).

Despite, certain limitations, the WGL procedure is a safe, non-invasive, well tolerated 'clinical tool' that has added a new dimension to the study of mucosal humoral immunity.

Chapter: 2
Materials and Methods

Section 1:

Patients

The patients were those who visited the GI unit who had had gut lavage for clinical diagnostic purpose. As a routine, their clinical materials and serum samples are stored and their lavage 'intake and output' were carefully recorded. For my research, I have taken three groups of patients, whose diagnosis and disease severity were confirmed by Professor Anne Ferguson.

2.1 A Cohort of Patients with a Spectrum of Diseases:

For my pilot study, which I will discuss in chapter 3, a total of 32 patients with a spectrum of diseases were studied. The clinical characteristics of these patients are presented in Table 2.1

Table 2.1 Clinical Characteristics of Patients Studied

Patient groups	No: of Patients (n)	Sex (M/F)	Age median (range)	Treatment received
IBD(UC+CD)*	10	4/6	46 (29-75)	Salazopyrine/ Prednisolone
Coeliac disease	2	1/1	41(40-42)	Gluten-free diet
Possible infection	10	2/8	30 (27-86)	Antibiotics
Miscellaneous (e.g. Polyp, anaemia, Ca** colon etc.)	10	8/2	76 (32-85)	Polypectomy/ Iron and vitamine supplement

*UC= Ulcerative colitis, CD= Crohn's disease, **Ca= Carcinoma

2.2. A Cohort of Patients with Inflammatory Bowel Disease:

A small cohort of patients with moderate to severe active inflammatory bowel disease (12 Crohn's disease and 11 Ulcerative colitis) were carefully selected and studied. The severity of the diseases were assessed by clinical and by total IgG in WGLF [IgG >10 μg/mL = moderate to severe disease (O'Mahony *et al* 1991; Chaudari *et al* 1993). Patients characteristics are given in table 2.2.

Table: 2.2: Clinical Characteristics of Patients with IBD

Patient group	n	Age median (range)	Sex M/F	Median Duration (yrs) of Diseases	Disease anatomy	Treatment received
UC	11	29 (19-66)	6/5	5	5 LS*, 2R, 3 LS+ Tr, 1 Pan C	4 S*,3,5asa +SE 3,5asa+OS,1az+ OS
CD	12	27 (18-70)	4/8	4.5	4 TI*,5 IC, 3 C	4 S+OS, 4, 5asa + OS, 2 az+ OS, 2 antibio**+ ED

* (LS= left sided colitis, R= rectum, Tr= transverse colon, Pan C= pancolitis, S=salazopyrin, 5asa= 5-amino salicylic acid, SE= steroid enema, OS= oral steroid, az= azathioprine, **antibio= antibacterial agents, especially metronidazole + Ciprofloxacin, ED= elemental diet)

2.3. A Group of Patients with Ileal Pouch- Anal Anastomosis (IPAA)

Ileal pouch anal-anastomosis (IPAA) is the operation of first choice following proctocolectomy in patients with ulcerative colitis. A small cohort of patients with IPAA who had had gut lavage for diagnosis of pouchitis were studied (Patients' clinical information and WGLF from these patient were a kind gift from Mr. Nikos Evganikos, a surgical colleague in the GI Unit). Patients' characteristics are provided in Table 2.3

Table 2.3: Clinical Characteristics of IPAA Patients

IPAA	n	Age median (range)	Sex (M/F)	Duration of disease before operation median (range)	Indication for operation
Normal	10	39 (28-73)	6/4	6 (1-28) years	Ulcerative colitis
Pouchitis	9	36 (28-74)	4/5	9 (3-16) years	Ulcerative colitis

2.4. Healthy Controls:

a. Edinburgh Controls:

Those people were immunologically normal and had no protein-losing enteropathy, no evidence of gut damage or bleeding, and were clinically normal, only functional bowel disease, e.g. irritable bowel disease or constipation came out as final diagnosis were studied. A cohort of such patients was considered as a healthy control group from Edinburgh (Table 2.4).

b. Dhaka Controls:

I have studied a group of healthy volunteers from Dhaka, Bangladesh. All the volunteers were local residents of Dhaka city. Characteristics of these healthy controls are given in Table 2.4.

Table 2.4: Characteristics of Healthy Controls.

Healthy Controls	n	Age median (range)	Sex (M/F)	Weight (kg) mean (±SD)	Height (cm) mean (±SD)
Dhaka	11	24 (18-32)	11 M	46 (± 5)	159 (± 7)
Edinburgh	12	39 (23-48)	4/8	60 (± 6.3)	166 (± 5.6)

General Standard Methods:

2.1 Whole gut Lavage (WGL) technique

This non-invasive clinical technique has been used throughout the world for bowel cleansing before barium enema, colonoscopy, and colorectal surgery. Recently it has been established as a clinical tool for studying various humoral immune functions and inflammatory parameters in the gut (Gaspari *et al* 1988; Brydon *et al* 1993). Briefly, after an overnight fast, patients or healthy volunteers drink the lavage solution (available commercially as Klean-prep from Norgine Ltd Oxford,UK. one sachet dissolved in one litre of tap water), supervised. They were monitored and 'intake/ output' were recorded by an experienced research nurse in Edinburgh (by myself for volunteer studies in Dhaka), aiming for a rate of 200 mL every 12 minutes. After a period ranging from 30 minutes to 3.5 h, several formed or semi-liquid stools are passed, followed by faecal-stained fluid. These are discarded until clear fluid resembling urine is being passed per rectum.

2.2. Processing of whole gut lavage fluid

Clear fluid (20 mL) is filtered through GF/A (Whatman) glass fibre filters. To 10 mL of the filtered fluid the following reagents are added (see details in appendix).

- Soya bean trypsin inhibitor in phosphate-buffered saline (PBS) (80µg/mL)

- Sodium ethylenediaminetetracetate(15 mM) in PBS (chelating agent for calcium and magnesium ions which are required for the activation of protease enzymes)
- Phenylmethylsulphonyl fluoride in 95% ethanol (2 mM) (protease inhibitor)
- Sodium azide (1mM) (bactericidal agent)
- New born calf serum (5% v/v) (to provide an alternative substrate for any remaining enzyme)

Aliquots of processed WGLF were stored at -70 °C for later analysis.

2.3 WGLF samples labelling:

It has been a routine practice in the G.I. Lab (also for the assay used in the thesis) that samples are labelled as follows to indicate different treatments of the samples.

UF/UP: Unfiltered and unprocessed (without any treatment)

UF/P: Unfiltered but processed (unfiltered but processing reagent added)

F/UP: Filtered but unprocessed (only filtered but no processing reagent added)

F/P : Filtered and Processed (both filtered and processing reagent added)

2.4 Analytical Methods

2.4.1 Enzyme linked Immunosorbent Assays (ELISA) for Total Immunoglobulins and antibodies.

Principle of the Method: Immunoglobulins are absorbed by class-specific antibodies bound to the solid phase (ELISA Plate). Then an enzyme-labelled antiglobulin conjugate (alkaline phosphate) is added and binds to the specific immunoglobulin required. A double antibody 'sandwich' technique was used for the total

immunoglobulin A, G, and M quantification using purified secondary standard material (Gaspari *et al*, 1988; O'Mahony *et al*, 1991, O'Mahony *et al*,1990).

An indirect technique was used for estimation of antigen specific immunoglobulins measured against a known high-titre human sample. Antibodies studied included those against ovalbumin and cholera-toxin B subunit (Gaspari *et al*, 1988).
Essentially the methodology of the assays is identical except for the initial coating of the solid plate.

General Procedure

- Class specific antihuman immunoglobulin or pure antigen is bound in excess to a solid phase overnight at 4°C in a moist box and then washed. Standard 96-well ELISA plates were used as solid phase. Different plate types were used as they have different binding characteristics.
- After washing, the plates are incubated with a protein- containing solution, usually the ELISA diluent, to 'block' non-specific binding sites.
- Standards and samples for total immunoglobulin and antigen-specific antibody quantitation are added to the plate in serial doubling dilutions, in order to cover the wide concentration range.
- Samples are incubated and then washed. Binding takes place between the antihuman globulin and the immunoglobulin quantified or between the specific antigen and corresponding anti-antigen immunoglobulin.
- A class-specific antihuman antibody conjugate with alkaline phosphatase is added to the plate, incubated then washed . This conjugate antibody binds to the complex bound to the solid phase.

- The substrate for the alkaline phosphatase is p-nitrophenyl phosphate in a diethanolamine buffer at pH 9.8. The substrate is added to the plate and the reaction is indicated by the change of colour (p-nitrophenyl phosphate which is colourless changes to p-nitrophenol which is yellow in colour).
- The colour development takes place usually within 30 minutes and the optical density at 405 nm is read on a dedicated ELISA reader (Dynatech MR 5000) when the top standard or reference standard has reached 1.0.
- For the total immunoglobulin determination a standard curve is plotted (using the 'Inplot 4 program' in computer) with the concentration on the x-axis against the optical density on the y-axis. A straight line section of the curve ($r>0.99$) is used as the standard line by including at least 4 consecutive dilutions of the standard.
- The concentration of the specimen is calculated by averaging two of the doubly diluted concentrations that lie within the standard line.
- Semi- quantitative antibody assays were performed by expressing the result as a percentage of the concentration of a single dilution of the standard (the same dilution as the top standard in the quantitative assays).
- Reagents and instrumentation are detailed in the appendix.

Test Procedure

1. Coat plates with appropriate antigens

Using an 8 channel multichannel pipette, 125 μl of appropriate coating solution including the antigen were dispensed to each well of the ELISA plate.

For total immunoglbulins

Immulon 1, flat bottomed 96 well plates (Dynatech) were used

Coating antibody	Dilution
anti-human IgA	1/2500
anti-human IgG	1/5000
anti-human IgM	1/5000

For specific Antibodies

Immulon 2 (which adsorbs coating antigens more strongly than 'Immulon 1' plates), flat bottomed 96 well plates were used.

The following antigens in coating buffer were made up:

for ovalbumin assay, 5 μg/mL of ovalbumin (sigma A-5503) was coated for anti-ova (IgA and IgG) and 10 μg/mL of ovalbumin for anti-ova IgM antibody.

2. Incubation

Each plate was covered with a plastic lid, placed in a moist box and incubated at 4°C overnight.

3. Blocking plate

The plate was washed x3 and then 250μL of ELISA diluent added (Appendix 1). It was left for a minimum of 1 hour.

4. Samples and standards

All were assayed in duplicate.

Standard Material

Standards were all made up in ELISA diluent.

1. Total Immunoglobulins:

Total IgG and IgM

- **SPS-O1** - standard material for quantifying IgG and IgM. It was purchased from the Dept. of Immunology, P O Box 894, Sheffield S5 7YT.

Total IgA

- Human IgA -purified immunoglobulin from colostrum (Sigma Chemical Co)

  Top standard for IgA assay = 1000 ng/mL.

2. Specific Antibodies:

Quantitative Assays: For **food antibodies** a serum sample from a patient (Main, Sample no. 1548 preserved in the GI Lab) with high levels of specific food antibodies was used . For calculation, arbitrary units have been allocated to this standard.

Standard no. 1548 diluted 1:264, which is equivalent to the 1:50 dilution of Main (patients serum) and this equals 2 units.

Samples

Total immunoglbulins A,M, and G

The samples were made up as follows in ELISA diluent for the top row to start at the dilution:

	WGF/ELISA diluent
Total IgA	1/100
Total IgM	1/25
Total IgG	1/25

Specific Antibodies

samples (125 μL) were added to 125 μLdiluent (1/2 dilution) in the top row.

The samples and standards were then serially diluted down the columns using a 12 channel pipette.

5. Incubation

The plates were incubated at 4° C overnight.

6. Conjugates

Alkaline phosphate conjugated anti-sera dilution were prepared in ELISA diluent following the dilution scheme in appendix 1.

The plates were washed as above, then using a multichannel pipette 125 μL conjugate were dispensed to each well of the ELISA plate.

7. Incubation

The plates were covered with a plastic lid, placed in a moist box and left at room temperature for 3 hours.

8. Colour development

Alkaline phosphate substrate was prepared 30 minutes before it was required.

The plate was washed as above, then using a 12 channel pipette 125 μL of substrate dispensed to each well of the ELISA plate. They were left on the bench for 5 minutes as the colour starts to develop, then shaken on a Denley Wellmix 1.

9. Reading

The colour development was monitored manually on the Dynatech MR5000 Microplate reader until the top standard reached its endpoint of approximately 1.00 (read with the filter set at 405 nm and the reference filter set to 630nm).

10. Calculation

The concentration of the samples was calculated as described above. The results were accepted if the co-efficient of variation (CV) was less than 15% and 5% in intra assay and inter assay respectively.

2.4.2 Haemoglobin in Whole gut lavage fluid

(Reference: Brydon & Ferguson 1992).

Principle of Method:

Haemoglobin is converted to porphyrin with the removal of ferrous iron by the commensal flora. Porphyrin is extracted with solvent and then detected by fluorescence spectrophotometry. A three step purification procedure eliminates other interfering fluorescent materials which may be present.

Gastrointestinal blood loss is routinely assessed in the GI laboratory using this method. The healthy volunteer samples from Dhaka were assessed by Mr. John Bode. Reference values for lavage haemoglobin in adults were obtained by studying healthy volunteers. The normal range is 0-5 μg/mL (Brydon & Ferguson, 1992).

2.4.3 Albumin and α-1 Anti-trypsin in Whole gut lavage fluid

Reference (Brydon *et al*,1993)

Principle of the method:

Albumin and α-1 anti-trypsin in WGLF are measured by simple manual procedures based on immunoturbidimetry. Turbidimetry involves the measurement of light-scattering particles in solution by means of the decrease in intensity of an incident beam as it passes through the sample. The light has been lost due to absorption, reflection and scatter. Turbidimetric measurement can be made with a spectrophotometer.

Albumin and α-1 anti-trypsin in WGLF are routinely tested as measures of gastro-intestinal protein loss. The test was done by Mr. John Bode in the GI lab. Reference normal values for lavage albumin and α-1 anti-trypsin were obtained by studying healthy controls. These values were <26 μg/mL and 19 μg/mL for albumin and α-1 anti-trypsin respectively.

3. Cytokines assays in WGLF and serum

The cytokine assays are commercially available ELISA kits that have been adapted for analysis of WGLF.

Principle

These kits use the 'sandwich' type ELISA similar in principle to that described for the total immunoglobulin and antibody assays. Monoclonal antibody to a particular cytokine [(for example, interleukin (IL) -1β and IL-8)] is coated onto the microtitre plate provided. Standards and samples are added and any particular cytokine that is present is bound to the solid phase. After washing, an enzyme -linked polyclonal antibody is added to the well to attach to the chemokine previously bound. Following a further wash a substrate solution is added to the wells and the colour develops in proportion to the amount of the substance bound initially. The colour development is stopped and the intensity of the colour is measured.

In order to attempt to replicate as closely as possible the environment for both standards and specimens the following solutions were used as diluents.

1. Standard diluent: PBS, 0.02% Tween 20, and 1% ABS
2. Processed Klean-Prep (Peg +) - Klean-prep (Norgine, Oxford UK) + processing agents for WGLF.

3.1 Interleukin - 8 (IL-8)

IL-8 concentrations in WGLF were assayed using a commercially available sandwich ELISA kit (Quantikine, R & D Systems, Minneapolis, USA).

Methods:

Samples and Standard

Standard:

Seven tubes were labelled: 240, 96, 38.4, 15.4, 6.1, 2.5, and 1 pg/mL.

Volume (610 μL) of 50:50 working strength diluting buffer : Peg+ was pipetted into the 240 pg/mL tube and 300 μl into each of the other tubes. IL-8 standard (15 μL of the 10ng/mL) was put into the 240 pg/mL tube, mixed and then transfered 200 μL into the 96pg/mL tube followed by , mixing. This procedure was repeated through to the last tube. Each standard (100 μL) was added to the appropriate duplicate wells.

WGLF samples

UF/P samples were used in cytokine assays. These samples were not diluted prior to addition to the microtitre plate where they were diluted 1/2 in the standard diluent (e.g. 100 μl samples added to 100 μl processed Klean-prep already in the wells).

Assay Procedure

Day 0

1. The plate and coating antibody was brought to room temperature
2. The coating antibody was centrifuged at 3000g for 1 minute
3. The coating buffer was prepared.
4. Coating antibody was diluted 1:100 in coating buffer, i.e. 120 μL of antibody was added to 12 mL of buffer.

5. 100 μL was added to all wells.

6. The plate was covered and incubated overnight at room temperature.

Day 1

1. All reagents (except streptavidin-HRP) were brought to room temperature. Vials were centrifused at 3000g for 1 minute.

2. Blocking buffer was prepared.

3. The plate was washed x5 with PBS alone.

4. (200 μL) blocking buffer was added to each well and incubated for 1 hour at room temperature.

5. Standards were prepared and samples were diluted.

6. The plates were washed x5 with washing buffer (PBS+Tween 20)

7. Each standard (100 μL) was added to appropriate duplicate wells.

8. Assay diluent (50 μL) was added to all sample wells. Blanks= 100 μ

l of 50:50 diluting buffer: Peg+

9. Pre-diluted samples (50 μL) were added to duplicate sample wells. The plates were covered and incubated at room temperature for 1 hour.

10. The biotinylated antibody 1:100 in dilution buffer was prepared just before use.

11. The plate was washed x5 with wash buffer.

12. Biotin conjugate (100 μL) was added to each well, the plate was covered and incubated (after tapping to mix) at room temperature for 1 hour.

13. Streptavidin-HRP conjugate was stored at -20^{0}C until use. The conjugate was centrifuged at 3000g for 1 minute before use. Then, just prior to use, 3 μL streptavidin was added to 30 mL working strength dilution buffer.

14. The plate was washed x5 with wash buffer.

15. Streptavidin-HRP (100 μL) was added to all wells, tap to mix, then covered and incubated for 30 minutes at room temperature.

16. K-blue substrate (15 μL) was brought to room temperature.

17. Plate was washed X5.

18. Substrate (100 μL) was added to all wells. Incubated for (although the kit suggest 30 minutes, I have found less than 15 minutes was necessary.

19. Stop solution (100 μL) was added to all wells, tapped and mixed.

20. The plate was read at 405nm within 30 minutes.

21. Calculation of results:

Using a curve fitting program in Revelation software installed in Dynex (The microtitre Company) ELISA reader, an ' r ' value of >0.99 including all points required in the standard curve. Concentration of the samples were then extrapolated from their optical densities.

Sensitivity: The sensitivity of this assay is 1-3 pg/mL.

3.2 Interleukin-1β (IL-1β)

High sensitivity Interleukin-1β ELISA kit -CISTRON BIOTECHNOLOGY, New Jersey, USA). The principle is the same as the 'sandwich' ELISA described for immunoglobulins, specific antibodies and IL-8.

Standard and Specimens

Standards: The standard was prepared in 50:50, Peg+ :PBS Tween 20, 0.02%

The kit standard was reconstitute with 500 μL of distilled water and left for 15 minutes. This gives a stock solution of 50,000 pg/mL; this was diluted then to gave 300, 150, 100, 50, 20, 10, 5 pg/mL

Samples:

UF/PP of WGLF have been used . Samples were diluted 1/2 by adding 50 μl of PBS/Tween 20 to each wells prior to adding 50 μl of lavage.

Assay procedure:

1. To a prepared coated plate 100 μL of each standard concentration was added to duplicate wells.
2. To sample wells 50 μL of PBS/Tween 20 was added followed by 50 μL of lavage samples. Blanks wells = 100 μL of 50:50.
3. The plates were covered with adhesive strip and incubated at 37 ^{0} C for 1 hour.
4. The contents were aspirated and washed x3 with wash buffer.
5. Polyclonal IL-1β (50 μL) of mature anti-serum (rabbit) was added to each well. The plate was covered and incubated at 37 ^{0}C for 20 minutes followed by washing x3 .
6. Diluted anti-rabbit IgG-HRP conjugate (100 μL) was added to each well.
7. The wells were covered and incubated at room temperature for 20 minutes
8. Substrate was prepared 5 minutes prior to use by mixing equal amounts of A (supplied) and B followed by washing x3
9. Substrate (100 μL) was added to all wells and incubated uncovered at room temperature (although the supplied kit suggests 20 minutes incubation time, I found that less than 10 minutes is necessary).
10. Sulphuric acid (50μL of 2M) was added to stop the reaction.
11. The plate was read within 15 minutes at 450nm.
12. A standard curve was constituted and calculation of values were by extrapolating optic density readings.

Sensitivity: The sensitivity of this test is 2 pg/mL.

Precision: The intra-assay coefficient of variation of this test as coated is <5%.

3.3 Interleukin- 5 (IL- 5)

High sensitivity Human IL-5 ELISA kit -R&D systems (D5000). The principles is the same as the 'sandwich' ELISA described for immunoglobulins, specific antibodies and IL-8 and IL-1β .

Standard and Specimens

Standards: Standard was prepared in 50:50, Peg+ :PBS Tween 20, 0.02%

The IL-5 stock standard was reconstituted by adding 5 mL of Peg+. The stock standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions of 500, 250, 125, 62.5, 31.2, 15.6,7.8 pg/mL

Samples:

Unfiltered and processed (UF/PP) of WGLF have been used . Samples were 1/2 by adding 50 µl of PBS/Tween 20 to each wells prior to adding 50 µl of lavage.

Assay procedure:

1. To a ready coated plate 50 µL of assay diluent was added to each wells
2. Each standard dilution (200 µL) was added to appropriate wells.
3. PBS/Tween 20/ABS (100 µL) was added to sample wells. Blanks contained 200 µL of 50:50.
4. Samples (100 µL) were added to duplicate wells. These were then tapped and mixed and cover with adhesive strip and incubated at room temperature for 2 hours.
5. The wells were aspirated and washed x4 with wash buffer.
6. Substrate solution (200 µL) was added to each well and incubated at room temperature for 20 minutes (we observed that only 10 minutes was required).

7. Stop solution (50 μL) was added to each well.

8. Results were read at 450 nm (with correction at 570).

9. The concentrations of the samples were then extrapolated from their optical densities, using the curve fitting program in Revelation software installed in Dynex (The microtitre company).

Sensitivity: The minimum detectable dose of IL-5 as mentioned in the kit was <3pg/mL.

Precision: According to the Kit the coefficient of variation in intra assay and inter assay was around 4% and 7 % respectively.

3.4 Interleukin-6 (IL-6)

High sensitivity IL-6 ELISA kit -Millennia (MKL61). The principle is the same as the 'sandwich' ELISA described for immunoglobulins, specific antibodies and other cytokines.

Standard and Specimens:

Standard: Six tubes were labelled: G (500 pg/mL), F (250 pg/mL), E (125 pg/mL), D (61.5 pg/mL), C (31.3pg/mL), B (15.6 pg/mL). samples of 0.5 mL of IL-6 Calibrator/Sample Diluent were pipetted into each tube, pipette 0.5 mL of the reconstituted Master Calibrator was pipetted in to the tube G (500 pg/mL) and mixed thoroughly. A volume of 0.5 mL was transferred from tube G to F (250 pg/mL) and mixed thoroughly. This process was repeated successively to complete the 2-fold dilution series. The reconstituted Master Calibrator H served as the highest calibrator (1,000 pg/mL). IL-6 calibrator/Sample diluent was used as the zero calibrator A (0 pg/mL).

Samples: 100 µl of serum sample (frozen at -20 ° C) was used for the assay.

Assay Procedure:

1. A single microplate was prepared with enough 8-well strips to accommodate calibrators (A to H), controls and patients' samples in duplicate.

2. Each standard, control and patients samples (100 µL) was pipetted in the wells prepared (pipetted directly to the bottom of the plate).

3. IL-6 Sample buffer (150 µL) was added to every well. The plate was covered and rotated for 2 hours on a plate mixer.

4. The plate was decanted and washed x4 with Micro Wash, each time with 300 µl Buffered Wash Solution

5. Enzyme-Labelled Anti-IL-6 antibody (200 µL) was added to every well.

6. The plate was covered and rotated for 2 hours on a plate mixer.

7. The plate was decanted and washed as described above.

8. TMB/Substrate (200 µL) was added to every well.

9. The plates were incubated without shaking for 30 minutes in the dark.

10. Stop solution (50 µL) was added to every well.

11. The plate was read at 450nm within 15 minutes of adding the stop solution.

12. The concentrations of samples were then extrapolated from their optical densities, using the curve fitting program in Revelation software installed in Dynex.

Sensitivity: The detection limit of the of the assay, according to the kit is 4 pg/mL.

Precision: The coefficient of variation within run is <4% and in run-to-run is <6.5%.

4. Total Granulocyte Elastase (GE) Assay:

Principle of Method: The elastase activity of a sample is determined by its amidolytic effect on the substrate pyroglu-pro-val-pNA.

< Glu-Pro-Val-pNA + H_2O------Granulocyte Elastase-------> <Glu-Pro-Val-OH +pNA

The rate at which p-nitroaniline (pNA) is released is measured photometrically at 405 nm. This can be read after stopping the reaction with acetic acid. The correlation between the absorbance and the granulocyte elastase activity is linear in the 0.1- 1.5 μkat/l. The amidolytic activity does not necessarily parallel the elastolytic activity for different elastase preparations.

Reference: (Kramps *et al*, 1983)

Reagents:

1. Triton X100 (Sigma Chemical Co.,)

2. Dimethyl sulphoxide (DMSO, Sigma Chemical Co,)

3. Substrate (S-2484, from KabiVitrum AB, Sweden): The substrate (25 mg) is dissolved in 7 mL of DMSO. One volume of this stock solution is diluted with 3 volumes of distilled water.

4. Buffer pH 8.3 (25° C): The composition of this elastase buffer is: Tris 12.1g (100 mmol/l), NaCl 56.2 g (960 mmo/l), Distilled water 800 mL. Adjust the pH to 8.3 at 25 °C by adding approximately 50 mL of 1 mol/l HCl. Fill up to 1000 mL with distilled water.

5. Acetic acid 20% : Acetic acid is used in the acid-stopped method.

Control Material

Positive control was a sonicated preparation of granulocytes isolated from the blood of a normal volunteer. The control concentration was adjusted to give a GE activity value of approximately 1.0-1.3 μkat/l.

Sample: Unfiltered and Unprocessed (UF/UP) lavage samples were used for this assay.

Assay procedure:

1. Trion X -100 (1μL) was added in a microcentrifuge tube for each sample to be tested

2. UF/UP lavage sample (600 μL) was added to each tube and mixed.

4. A 2 mmol/L working substrate (S-2484) solution was prepared and placed in a waterbath at 37°C.

5. Two plastic round bottom tubes were labelled for each sample to be tested one for test and one for the blank.

6. Elastase buffer (250 μL) was placed into each tube.

7. The rack of tubes was placed in waterbath and allowed to warm (2-3 minutes).

8. UF/UP sample (200 μL) was added to both the test and blank tubes, and mixed and allowed to warm.

9. Acetic acid (200 μL) was added to the blank tubes only and mixed.

10. The digital timer was set at 3 minutes and after the starting timer 200 μL of pre-warmed substrate was immediately into all tubes keeping to a regular rhythm while pipetting (test tubes should take priority). They were mixed well.

11. After exactly 3 minutes, 200 μL of acetic acid was added to all of the tubes in the same rhythm and mixed.

12. The tubes were read in a spectrophotometer at 450 nm.

Calculation of the results:

The amount of GE activity present in each sample was calculated as follows

(Average test 'A' reading - Average blank 'A' reading) X 2.31 μkat/l.

Acceptance of Results:

If positive controls gave an appropriate value (co-efficient of variation has always been less than 5%), samples results were acceptable, providing duplicate wells gave similar values and blanks were not exceptionally higher than test values.

Software Used in this Thesis

Data were stored using Access version 2.0 (Microsoft).

The word-processing package used in this thesis was Word for Windows V 6.0 (Microsoft). Statistics were calculated using Minitab for Windows V10.2 (minitab statistical software). Graphs were produced using Prism V 1.03 (GraphPad) or Excel v 5.0 (Microsoft).

Statistical Analyses Used in this Thesis:

Minitab for Windows was used for the statistical analysis. For non-parametric data, results were expressed as medians and interquartile ranges. Mann-Whitney U-test was used for independent sets of data. Spearman's rank correlation coefficient was used to study the relationship between two variables. However, where data were normally distributed, Student's 't'-test was used to compare two independent groups. The Pearson correlation coefficient 'r' was used to examine the relationship between two variables.

Ethics of WGL for Research in Volunteer:

The Lothian Health Board of Scotland approved the WGL study on volunteers in 1992. The volunteer study at Dhaka was approved by the ethical committee of the International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B).

SECTION 2

Methods for Quantification of Anti-bacterial antibodies Including Technical Developments:

1. Anti-LPS core (EndoCAb) ELISA:

As has already been discussed in the literature review, a semi-quatitative ELISA method to detect antibody to endotoxin has been developed by Dr. G.R. Barclay. I adapted Barclay's original method for the measurement of IgG antibody responses in blood donor sera (Barclay and Scott, 1987). The method has been modified and optimised for detecting the antibody response to endotoxin in WGLF (e.g. IgA antibody).

Principle of the method: The principle is the same as that discussed in the general method for 'Sandwich' ELISA. Microplates were coated with 'endotoxin' core antigen cocktail (Rough mutant LPS from *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Salmonella minnesota* complexed with polymyxin B, these plates were a kind gift from Dr. Barclay).

Reagent: Endotoxin-free buffer is needed for plate washing and sample dilution, composition of which is given in the appendix .

Standards and Samples:

Standard:

Human standard serum was used as a reference (Courtesy Dr. Barclay)

IgG, IgM and IgA values (MU) in the standard serum after reconstitution were:

IgG 7.84 IgG MU

IgM 3.30 IgM MU

IgA 9.04 IgA MU

Where MU= "median unit". The units are based on the median of the normal healthy Scottish adult range of EndoCAb IgG, IgM, and IgA antibodies, where the median of each immunoglobulin range is 100 MU. The units for the different immunoglobulin classes (G,M and A) are not equivalent. The "normal" range usually employed for each class is the 10 th to 90th percentile, which is approximately 35 (10th centile) to 250 (90thcentile) for each immunoglobulin class when median units are used.

Top Standard Dilution: (optimised and modified)

IgG 1:250

IgM 1: 50

IgA 1: 25

Sample: Filtered and Processed (F/P) WGLF was used for EndoCAb assay

Dilution: For lavage 1:1, for serum samples 1:100.

Assay Procedure:

1. All reagents were brought to room temperature before use.

2. The samples and standards were put onto ready coated plate and kept in a dry box.

3. The plates were incubated at 37° C for 90 minutes (instead of 1 hour suggested by Dr. Barclay)

4. After incubation the plates were washed x3 with ELISA wash.

5. The alkaline phospatase conjugate diluted 1: 1000 dilution (for A,G,and M) Sigma chemical Co.

6. The plates were incubated in a box at 22^0C for 3 hour. (instead of 90 minutes by Dr. Barclay).

7. After 3 hour incubation, the plate were washed x3 and substrate (DEA buffer + PNP) added in all well.

8. The colour development takes place usually within 3-5 minutes and the optical density at 405 nm was read on a dedicated ELISA reader (Dynatech MR 5000) when the top standard or reference standard had reached 1.0.

A standard curve was plotted (using Inplot 4 programme in computer) with concentration on the x-axis against the optical density on the y-axis. A straight line section of the curve ($r>0.99$) was used as the standard line by including at least 4 consecutive dilutions of the standards.

Precision: In between run and run-to-run coefficient of variation was observed as <4% and < 12% respectively.

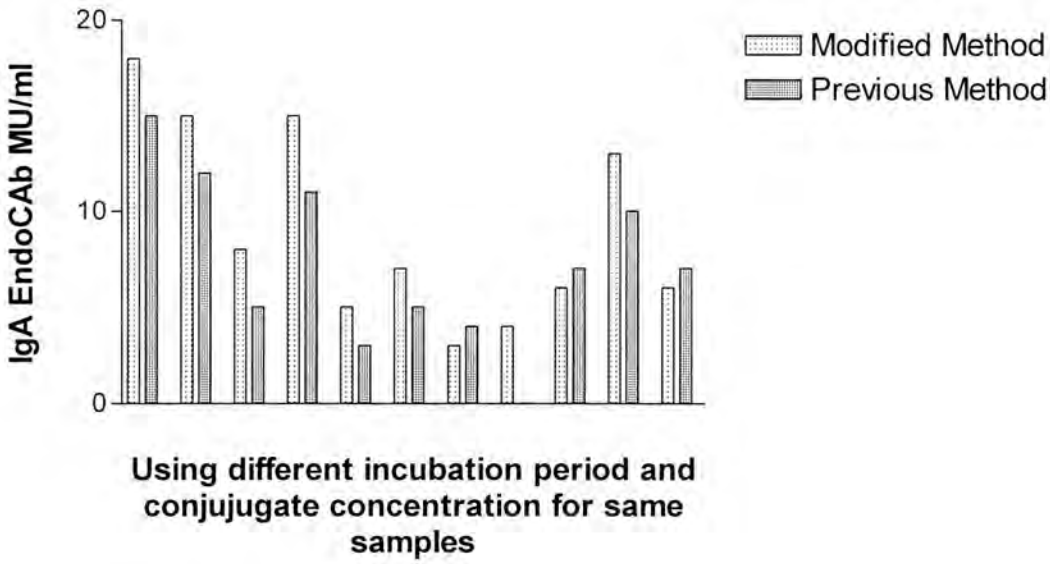


Figure 2.1: Comparison of Dr. Barclay's method and modified method

(Nine samples were analysed following Barclay's method and the optimised method to detect antibody in WGLF)

2. Anti-cholera toxin antibody-ELISA:

The principle and Methods was the same as already described in general methodology for detection of specific antibody by ELISA. However, for the **cholera toxin** assay there was a two stage coating phase, the first coats with monosialoganglioside (GM1, Sigma Chemical Co....G7641) to the well. This is a constituent of enterocyte cell walls to which cholera toxin (CT) [List Biological lab.CVX-20] binds strongly:

Stage 1 : Monosialoganglioside (GM_1), 10 μg/mL in coating buffer. Plates were coated with this solution of 100 μL/well follwed by incubating over night at 4°C.
The plates were washed x3

Stage 2: Cholera toxin, 5μg/mL in coating buffer. Plates were coated with this solution (100 μL/ well) followed by incubation overnight at 4° C.
the plates were washed X3, followed by blocking with blocking solution (200 μL/well) and incubation at room temperature for 2 hours.
For the cholera toxin antibodies, the standards were used from volunteers. Lavage sample was donated by Martha Falconer for CT-A with serum sample from Dr Croft for CT-M and CT-G.

Top Standard Dilution

Arbitrary Units (Units/mL of **Undiluted** standard)		Dilution of Top Standard		Conc. of Top Standard (units/mL)
CT-A	100,000	Falconer	1/100	1000
CT-M	100,000	Croft	1/100	1000
CT-G	100,000	Croft	1/100	1000

Standards and samples diluted as necessary in diluent solution were applied and then incubated overnight at 4°C

The plates were washed x3

Conjugate (1/1,000 dilution for IgA, IgG and IgM) was applied and then incubated for 3 hours at room temperature.

plates were wash x3.

Other steps of ELISA development and calculation of the results were as for the specific antibody ELISA already discussed in the general methodology section.

3. An ELISA for quantification of mucosal IgA response against LPS core types (R1, R2, R3 and R4) of *Escherichia coli* and surface antigens of *Bacteroides fragilis*.

I adapted the methods described by Dr. A.P. Gibb (Gibb *et al*, 1992) and Dr. I.R. Poxton (Poxton *et al*, 1995). However, I have optimised the method for studying antibody in the specimen of WGLF. This ELISA method is similar to EndoCAb ELISA except for the preparation of LPS and coating on ELISA strips.

LPS preparation and coating onto ELISA strips

a) Growth of the organisms

four strains of *E.coli* were taken.

E.coli (R1) MPRL 2316

E.coli (R2) MPRL 2317

E.coli (R3) MPRL 2318

E.coli (R4) MPRL 2431

The above strains were provided lyophilised in ampoules. Each was reconstituted with nutrient broth (NB) and used to inoculate nutrient agar (NA) plates as a purity check. The following method was used for each of the above strains:

Day 1. 20 mL NB was incubated overnight (ON) at 37°C as a sterility check.

Day 2. The broth was then inoculated from the stock culture and incubated statically ON at 37°C (inoculum). 10x1 litre of NB was incubated ON at 37°C (sterility check)

Day 3. Each of 10x1 litre of NB was inoculated with 2 mL of the inoculum culture and incubated aerobically ON at 37°C in an orbital shaker.

Day 4. The cells were harvested by centrifugation at 14,000 g (9K rpm) for 10 minutes (4° C). The pellets were pooled and washed twice in PBS pH 7.4 (9K rpm for 10 minutes at 4° C). The final pellet was frozen. The frozen pellet of harvested cells from all of the above strains were then lyophilised and ready for LPS extraction.

b) Extraction of LPS with phenol/chloroform/petroleum (PCP).

(Reagents detail in appendix)

1. One part by weight of freeze-dried bacterial cell was suspended in 4 parts by volume of PCP reagent and stired for 2 minutes at room temperature using a glass rod.
2. The mixture was transferred to DuPont 'Corex' glass centrifuge tubes and centrifuged in an enclosed rotor (Sorvall SS34) at 10,000 g for 15 minutes at 4° C.
3. The supernate was filtered through Whatman No.1 paper into a round-bottom flask.
4. The pellet was re-extracted with another volume of PCP (equal to the original volume used), and the filter supernate collected as in step 1 to 3 above. The pooled supernates were filtered.
5. The chloroform and petroleum was removed by rotary evaporation.
6. The dry material was taken up in 3-5 mL of water using a 26-gauge needle and syringe (the aim was to solubilize all the LPS thus making a translucent solution).
7. LPS was sedimented by ultracentrifugation 100,000g (35k rpm) for 3 h (4°C).
8. The LPS was taken up in pyrogen-free water and lyophilised.

[From step 5, LPS can be precipitated by another method (e.g. aqueous phenol method), the choice of which depends on the organism being extracted (e.g. *Bacteroides*)].

c. Coating on to ELISA plates:

Lyophilised LPS of *Bacteriodes fragilis* (MPRL no 1669) [prepared by the aqueous phenol method of Westphal and Luderitz (Westphal and Luderitz, 1954)] as described by Hancock and Poxton (Hancock and Poxton, 1988)] and *E.coli* R1, R2, R3 and R4 were complexed with polymyxin B after the modification of the method developed by Scott and Barclay (Scott and Barclay,1987). LPS (1mL of a 1mg/mL solution) was mixed with polymyxin B sulphate (1mg/mL of solution) in a Reactivial (Pierce), and sonicated in a short burst for 30 second in an ultrasonicator (MSE Soniprep) set at 10μ.It was stirred at room temperature for 2 hours, sonicated and then dialysed in 2000 MWCO Spectrapor membrane overnight against distilled water to remove excess polymyxin. After removal from dialysis bags, it was resonicated , diluted 1 in 50 in the ELISA coating buffer (0.05 M carbonate /bicarbonate, pH 9.6, containing 0.02% sodium azide). ELISA strips (Immunomodule polysorp F8 Nunc, Intermed) were coated at 100 μl/well and incubated at room temperature overnight. LPS-coated strips were washed four times with PBS containing 0.05% Tween 20 and 0.05% sodium azide, and then post coated with 100 μl volumes of PBS containing 5% w/v bovine serum albumin (ICN-Flow) and 0.02% sodium azide and incubated overnight followed by washing and stored at -20 $^{\circ}$ C until use.

Standards and samples:

Standard: A top standard was identified by studying healthy and patient populations. An arbitrary unit was assigned to that standard to study the different populations by antibody profiles.

Top standard dilution:

Serum sample no 2215/95 diluted 1in 50 would give 1000 SAMI Unit (arbitrary).

Samples: WGLF was diluted 1:1 with ELISA diluent (must be endotoxin-free).

Assay procedure:

All the reagent should be at room temperature.

1. The sample and standard were added in duplicate on the ELISA strips.
2. They were incubated in a dry box at 37 °C for 90 minutes
3. The plates were washed x3 in ELISA wash
4. Alkaline phosphatase conjugate was added (for IgA 1: 1000, Sigma chemical Co).
5 They were re-incubate at 22 °C for 3 hours
6. The plates were washed x3 after incubation
7. The substrate (DEA buffer +PNP) was added.
8. The colour development takes place usually within 10 -15 minutes and the optical density at 405 nm was read on a dedicated ELISA reader (Dynatech MR 5000) when the top standard or reference standard has reached 1.0.

A standard curve was plotted (using Inplot 4 program in computer) with concentration on the x-axis against the optical density on the y-axis. A straight line section of the curve ($r>0.99$) is used as the standard line by including at least 4 consecutive dilution of the standards.

Precision: In between run and run-to-run coefficient of variation was observed as 4.5% and 14% respectively.

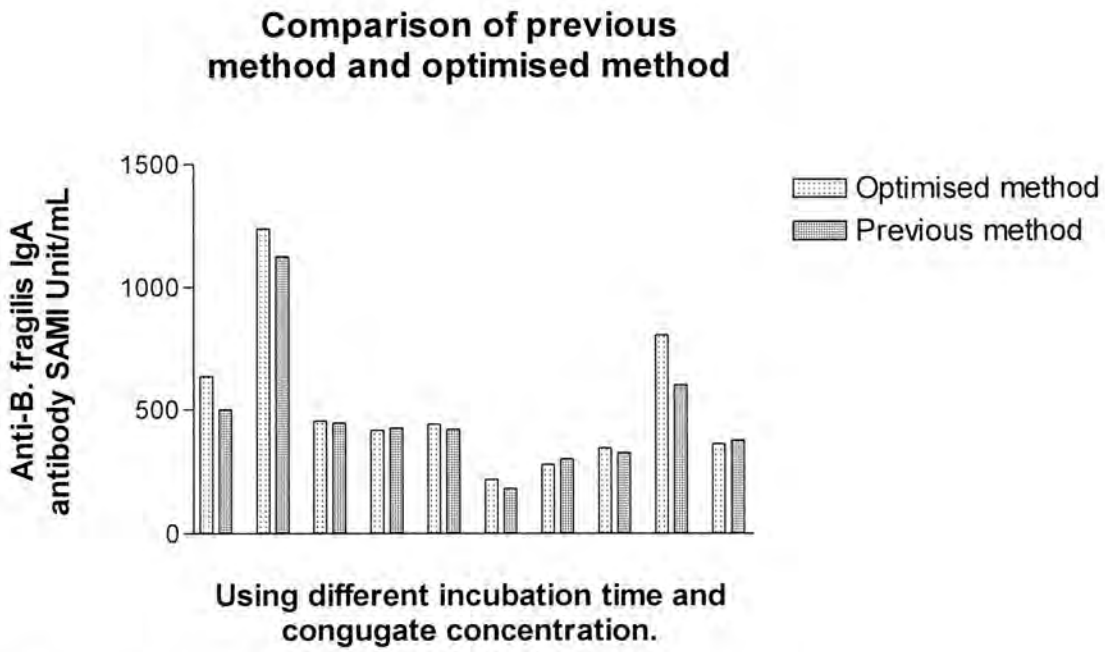


Fig: 2.2 Comparison between previous and optimised method.

4. An ELISA to measure mucosal IgA response against LPS of *E.coli* O157:H7

The method for the estimation of IgA antibody against LPS of *E.coli* O157:H7 is similar as described earlier for EndoCAb and various core types of *E.coli*, except for coating ELISA plates with the LPS of *E.coli* O157:H7 (MPRL No: 4252). The coating of ELISA plates was done by Carol Currie (a research associate of Professor Poxton), who is currently studying mucosal and serum antibody responses against *E.coli* O157:H7.

5. Polyacrylamide Gel Electrophoresis (PAGE)

(Reagents detail in appendix)

1. Glass plates, spacers and comb were degreased with Mediwipes wetted with methylated spirits.

2. Plates were wiped with distilled water and gently wiped dry.

3. Vaseline was melted in a universal bottle, in a boiling bath.

4. The cassette was constructed, without touching any surfaces that will make contact with the gel. The bottom (long) spacer was placed onto the bottom edge and a side spacer onto each side edge of one of cleaned surfaces on one of the plates, and then covered with the other plate, ensuring that the cleaned surfaces face each other. The 2 plates were lined up and the spacers pushed up to leave a 1- 2 mm gap from the edge. The cassette assembly was clamped together using two Bulldog clips per spacer, leaving a gap between the clip and the edges of the plates. The comb was inserted and checked that it is a good sliding fit. Lines 1cm and 8.5 cm from the base of the comb were marked on to the glass plates. The comb was removed and then the

cassette sealed with molten Vaseline by running it (via a Pasteur pipette) into the small trough between the glass edges and the spacers.

5. The cassette was placed upright in a tray or on a section of 'Benchcote' with the plain glass facing front and notched glass to the rear. The cassette was pushed down, so that the base edge was in contact with the clips, in order to level it.

6. The separating gel solution and 4% stacking gel solution were prepared using SDS-containing or SDS-free buffers as required, in clean, filtration flask as follows:

Ingredient	**Separating gel concentration**			**Stacking gel**
Distilled water	6.95 mL (10%)	5.2 mL (12%)	3.45 mL(14%)	3.5 mL(4%)
Separating gel buffer	17.5 mL for all concentration			
Stacking gel buffer	-	-	-	5 mL
Acrylamide (40%)	8.75 mL (10%)	10.5 mL (12%)	12.25 mL (14%)	1mL

7. The solution was de-gassed by applying a controlled negative pressure to the flask via vacuum pump.

8. A syringe was loaded with 1mL water-saturated butan-2- ol, and pipettors adjusted to deliver 875 μL and 50 μL. 1.75 mL of ammonium persulphate solution (15 mg/mL APS) and 50 μL TEMED were added to the separating gel solution and mixed gently but thoroughly. The gel solution was injected into the cassette up to the mark (i.e. 1 cm below the comb level), then air sucked up into the syringe and placed into the flask that contained the gel solution. The gel in the cassette was carefully overlayed with about 1 mL of butanol. The gel was left to polymerize (about 1-2 minutes).

9. A 10 mL syringe (plunger removed) fitted with a wide bore needle, and adjust pipettors to 500 μL and 20 μL were got ready. When the separating gel had polymerized, the butanol was removed and washed away with tap water. The gel

assembly was replaced into the tray and the comb inserted so that one end was fully inserted and the other end slightly raised. The syringe barrel was inserted into the cassette. 500 μL APS and 20 μL TEMED were added to stacking solution and mixed gently but thoroughly. Whist holding the syringe to keep the whole cassette assembly upright, the gel solution was poured into the syringe barrel and the plunger replaced. The gel solution was run into the cassette as an overlay onto the separating gel ensuring that no bubbles were trapped on the comb. The gel was left to polymerize (about 2 minutes).

10. When polymerized, the cassette was layed horizontally and the wells numbered (i.e. 1-20), writing the number in the centre of each well and about halfway up the well. The 2 clamps at the base of the cassette and the bottom spacer were removed.

11. The "U"-shaped seal on the electrophoresis tank was lightly smeared with Vaseline. The final 2 clamps were removed and used to fix the cassette into the tank, making sure that there was a gap between the base of the cassette and floor of the gel apparatus. The comb was slowly removed and placed it into the discard tray. The PAGE electrode buffer was poured into the top tank to 2-3 mm above the tops of the wells and into the bottom tank to 2-3 mm above the base of the gel. Any bubble was removed from the base of the gel via syringe and curved needle.

12. Dry samples were dissolved in single-strength sample buffer to selected concentration. Liquid samples were mixed with an equal volume of double strength sample buffer. The sample (s) in SDS PAGE sample buffer were held in sealed Eppendorf tubes in a boiling bath for 3 minutes, and then allowed to cool to room temperature. The samples were mixed thoroughly without frothing and appropriate volumes added to appropriate wells in the gel. Single strength sample buffer was added to the outer wells and to any wells that did not contain samples.

13. The gel was run at constant 60 volts until samples had just entered the separating gel (about 1.5 h) and then increased to 150 volts. The gel was run until the dye front reached the mark 7.5 cm below the stacking gel (about 1.5 h).

14. The power pack was switched off, the lid removed from the electrophoresis tank, and the gel cassette removed, allowing buffer to drain into the gel tank.

15. The cassette was layed horizontally, numbered side up, on a piece of Benchcote or a paper towel, then the 2 side spacers were slid out. A spatula was inserted about 10-12 mm into the bottom right hand corner between the two glass plates. By levering upwards the plates could be separated.

16. If the gel required cutting, a sharp scalpel was used and vertical cutting motion (rather than a slicing action), and the line previously drawn on the glass plates used as a guide. The gel was then ready for the next process (e.g. silver staining, immunoblotting etc.)

6. Silver Stain for LPS

(Reagents are listed in the appendix)

1. The gel was transfered to a box and fixed in 200 mL fixative overnight.

2. The fixative was discarded and the gel oxidised in 154 mL oxidizer solution for 5-15 minutes.

3. The oxidizer was discarded and washed 4 times in distilled water, 200 mL/wash and 1 hour/wash.

4. Fresh ammonical silver nitrate solution was prepared. The final wash water was discarded and the silver stain added and left for 15 minutes (recipe in the appendix).

5. Silver stain was discarded and washed in at least 4x 200mL changes of distilled water and 10 minutes /wash

6. The freshly prepared developer was warmed to 25° C. The water discarded and 200 mL developer added, maintaining the 25° C temperature. The gel was left until required staining intensity was achieved.

7. The developer was discarded and the gel briefly washed (2-3 minutes/ wash) in several changes of 200 mL of distilled water.

8. The gel was stored in distilled water in the dark.

7. Comassie blue stain for PAGE gel

(Reagents are listed in the appendix)

1. The gel was tranferred to a box or staining tray containing solution 1 and left overnight at room temperature on a flat surface.

2. Solution 1 was poured off and solution 2 added and agitated on a rocker platform at 10 to 20 tilts/minutes for 1 hour.

3. As 2, but solution 3 added

4. As 3, but solution 4 added

5. As 4, but solution 5 added. Gel should be stored in solution 5 until discarding.

[Molecular weight marker can be added to the gel prior to running and then stained up using the above technique]

8. Immunoblot (Dot Blot)

1. The Nitrocellulose (NC) was pre-soaked in Tween TBS (Tris-buffer saline) and dried.

2. Samples (2-5 μL) were loaded and dried.

3. Monoclonal antibody (mAb) reagent was prepared in 1:10 dilution and added.

4. Allow to incubate for 3 hours

5. The conjugate (I in 1000 dilution) was prepared.

6. The conjugate was added and incubate for 31/2 hour

7. The NC was washed x2 in TTBS

8. The NC was rinsed briefly in 3 changes of pyrogen-free water.

9. Colour was developed with the following solutions.

Solution A (in a glass universal). 30 mg of 4 chloro-1-naphthol and 10 mL methanol (Analar grade)

Solution B (100mL flask). 50mL of TBS and 30 μL hydrogen peroxide (30% solution)

10. Solution A was added to solution B just prior to using

11. The developer was poured onto the NC and development of colour was observed over 15-30 minutes, Stopping the reaction by washing in tap water.

Section 3

Methods of Studying Eosinophil Migration.

Prompted by a case, I looked into the importance and mechanisms of eosinophils causing disease in gastro-intestinal mucosa. I have set up some assays in WGLF which had not previously been done. Dr.Croft (Croft,1996) had done the pioneer work in setting up an ECP assay in this lab. I have reproduced his method to detect ECP in WGLF as well as set up the eotaxin assay in WGLF.

1. Eosinophil Cationic Protein (ECP) Assay:

ECP radioimmunoassay (RIA), Kabi Pharmacia

Principle

This kit is a double antibody radio-immunoassay where ECP in the sample competes with a fixed amount of ^{125}I labelled ECP for the binding sites of specific antibodies. Bound and free ECP are separated by addition of a second antibody immunosorbent followed by centrifugation and decanting. The radioactivity in the pellet is then measured and is inversely proportional to the quantity of ECP in the sample.

Methods:

Standards samples

Standards and samples were all assayed in duplicate.

Standard:

As included in the kit in a range of dilution

Samples:

UF/P WGLF, no pre-dilution necessary.

Procedure:

1. Standards and samples (50 μL) were added to polystyrene centrifuge tubes.
2. 50 μl ECP ^{125}I (blue in colour) was added.
3. 50 μl anti-ECP (yellow in colour) was added.

Now (all the tubes were then green).

4. Tubes were mixed, covered and incubated for 3 hours at room temperature.
5. 2 mL of decanting fluid (well mix the decanting fluid before use) was added.
6. Incubation was for 30 minutes at room temperature.
7. All tubes were centrifuged for 10 minutes at 1500g. Tubes were carefully replaced into rack and immediately decanted and let stand for 30 seconds upside down on absorbent paper.
8. The radioactivity in the tubes were determined in Gamma counter..
9. Calculation of the results was follows:

- The mean count (B) for the standards and samples expressed as a percentage of the mean counts of the zero standard (B_0)

$\Rightarrow$ % activity bound= $100xB/B_0$.

- The percentage values obtained were plotted for the standards against the concentration on a linear-log paper and a standard curve was constructed.
- The concentration of the unknown samples was read from the standard curve

Precision: According to the kit, the detection limit is <2 μg/l and recovery 103±3% (mean±2SD). The recovery experiment in WGLF done by Croft found 100% recovery (Croft,1996)

2. An Eosinophil Specific Chemokine (eotaxin) Assay:

High sensitive human eotaxin immunoassay kit - R&D systems (DTX00).

Principle is the same as the 'sandwich' ELISA described for immunoglobulins, specific antibodies and other cytokines.

Standard and Specimens

Standards: Standard is prepared in PBS Tween 20 (0.02%)/ABS (1%)

Reconstitute Eotaxin standard with 1 mL of distilled water=10,000 pg/mL. Place 900 µl of PBS/ Tween 20/ ABS into 1000 pg/mL tube. place 500 µl into remaining tubes. Carry out dilution series which would give 1000,500, 250, 125, 62.5, 31.2, 15.6 pg/mL.

To produce standard and samples in same final matrix it is necessary to prepare a 'standard plate diluent' and a 'sample plate diluent'.

Sample Diluent:

10 mL (75% PBS/Tween 20/ABS) by 5 mL PBS/Tween 20/ABS + 5mL 50:50, (PBS/Tween 20/ABS : PEG+) was prepared.

Standard Diluent:

4 mL (75% PEG+) by adding 2 mL PEG+ to 2 mLs 50:50 (PBS/Tween 20/ABS : PEG+ was prepared.

Blanks= PBS/Tween 20/ABS 50 µl + 100 µl of Standard diluent.

Procedure

1. All reagents, working standards, and samples were prepared.
2. 100 µL of 'standard plate diluent' was added to standard wells
3. 100 µL of 'sample diluent' was added to sample well

4. Standard or Sample (50 μL) was added to appropriate wells and cover the microplate at room temperature for 2 hours

5. The plates were aspirated and washedx3 with wash buffer.

6. 200 μL of eotaxin conjugate was added to each well, cover the plate and hold at room temperature for 1 hour. Substrate was prepared.

7. The plate was aspirated and washed x3

8. 200 μl of substrate solution was added to each well and hold for 30 minutes at room temperature.

9. 50 μl of stop solution was added to each well.

10. Read within 30 minutes at 450 nm wave length correction at 540 or 570nm.

Precision : According to the kit, the minimal detectable dose of Eotaxin is <5 pg/mL. The intra assay and inter assay co-efficient of variation is <6 and <12 % respectively.

Effect of filtering on eotaxin assay in WGLF.

I looked at the effect of filtering on the level of Eotaxin in WGLF by analysing filtered and unfiltered specimens.

Figure 2.3, shows that there is no significant variation in the measured eotaxin in the filtered and unfiltered specimen.

It is possible that eotaxin, which is an 8.3 KDa, 74 amino acid residue, non-glycosylated polypeptide is easily filtered out through the 1 micron pore size of the filter paper.

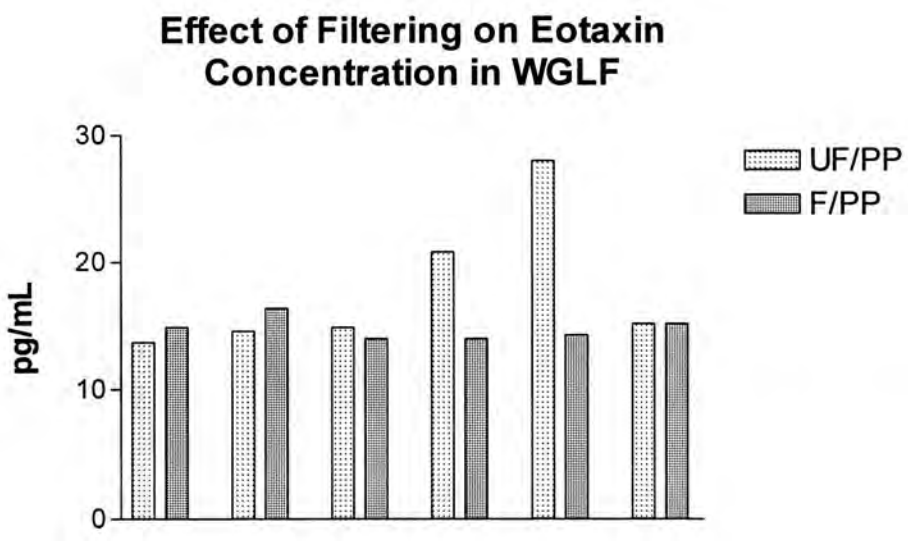


Figure 2.3: Eotaxin concentration in filtered and unfiltered samples of WGLF

Section 4:

Methods for studying biological potentials (nitric oxide and cytokine production) of LPS, using mouse and human colonic carcinoma cell lines.

1. Determination of TNF- α secretion by L929 bioassay.

1. The L929 mouse fibroblast cell line, which is sensitive to TNF, was cultured in growth medium; DMEM [Dulbecco Modified Eagle's Medium (Sigma, D-5546)] containing 5% FCS (Foetal calf serum) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 1 mM L-glutamine, and maintained by splitting 1:10 twice weekly. Cells were subcultured once weekly by splitting 1:20.

2. Cells were dislodged by 0.005% trypsin/0.02% EDTA to avoid cell clumping, washed and resuspended in DMEM to $2x10^5$ cells/mL.

3. Cells were dispensed in flat bottomed microplate (Cellstar, Greiner) at 100 μL/well and incubated at 37°C, 5% CO_2 overnight.

4. The growth medium was then aspirated, discarded and replaced with 100 μL/well of assay medium: DMEM containing 5% FCS supplemented with 1mM glutamine and 2 μg/mL actinomycin-D (to stop further cell growth without killing the cells).

5. To experimental wells, 100 μL of test supernatants which had been diluted 1:5 in assay medium, was added.

6. A standard of recombinant TNF (National Institute for Biological Standards and Controls, UK) diluted serially 1:5 at a starting concentration 1000 IU/mL and a well without TNF were included. Plates were covered and incubated at 37°C, 5% CO_2 over night. The medium was then discarded and replaced with 100 μL/well of filtered (0.22 μL) crystal violet solution [0.5% crystal violet in 20% (v/v) methanol in distilled water) which stains surviving cells. After 2 minutes the plates were washed vigorously under

tap water and dried. The crystal violet was dissolved by addition of 100 μL of 20% (v/v) acetic acid to each well.

7. The plate was read at 590 nm.

2. Nitric oxide (NO) assay

Mouse monocyte cell line (J774) and human colonic carcinoma cell line (CaCo2) were used.

1. Cells were plated (24 well tissue culture plate) at $1X10^{6}$ cells/well

2. Plates were left for 1 hour.

3. Different concentration of various core types of LPS were added.

2. The cells were centrifuged at 300g for 7 minutes.

3. 100 μL of supernatant were added in triplicate.

4. 25 μL 1mM $NaNO_2$ to 175 μL RPMI medium (Sigma, R-5886) were added to first well.

5. Greiss reagent (100 μL) was added to each well.

6. Plates were left for 10 minutes.

7. The plates were read at 540 nm.

Results Section

Chapter: 3

Pilot Study

Introduction

Before starting well designed in-depth studies I did a small study using specimens collected from patients with a spectrum of diseases. The aim of the study was two-fold: (I) To establish whether the research findings using the WGL technique from the GI lab were reproducible. (II) to test my competence at reproducing those findings using the same, as well as some new techniques.

Patients

A small cohort of patients is presented in Table 2.1 in chapter 2.

The patients studied were heterogeneous in disease distribution and their previous and current treatment. Among the 10 inflammatory bowel disease (IBD) cases, 7 had Crohn's disease (3 active, 4 inactive disease on the basis of lavage total IgG) and 3 had inactive ulcerative colitis. The IBD cases were relatively older compared to other groups. The possible infection group, on the other hand, is relatively younger (Table2.1, chapter 2). This group comprises Idiopathic diarrhoea (n=2), Travellers' diarrhoea (n=1), giardiasis (n=2), diverticulosis (n=1) and transient diarrhoea (n=4). From the miscellaneous group, two patients (1 Ca Colon, 1 anaemia/cirrhosis) have been excluded from the analysis as they have extremely high total lavage IgA and IgG values respectively.

Assays Performed

- Total immunoglobulin in WGLF (IgA, IgM and IgG)

- Specific antibody in WGLF(IgA, IgM and IgG EndoCAb and anti-Ova IgA). Methods are described in chapter 2.

Results:

1. Technical:

Five WGLF samples were analysed independently, in parallel by two technically expert research technicians (KH and NA) in the GI Lab and by myself (SAMI).

Sample no.	SAMI	KH	NA
94/062	117	114	109
94/256	63	52	56
94/375	97	86	82
94/802	122	113	127
94/802	87	91	85

Table 3.1: Comparison of Total IgA μg/mL estimation in WGLF by different personnel

Table 3.1 shows that there is an excellent agreement (correlation coefficient r= 0.96) between my results (SAMI) and the results from others (KH & NA), who are technically expert in ELISA method and doing routine ELISA in this laboratory.

Table 3.2: Estimation of different antibodies in WGLF in a patient on 3 different occasions

Lavage no/ Date	Total IgA μg/mL	Total IgG μg/mL	IgA Endo MU/mL	IgA Ova Unit/mL	IgM Endo MU/mL
1702:3/6/93	25	1	7.7	0.71	0.04
1733:9/8/93	30	5	4.6	0.91	0.00
1763:2/9/93	26	2	3.8	0.50	0.02

Antibody levels in WGLF taken on three different occasions from a patient with constipation (otherwise healthy) showed very consistent results (albeit with a slight scatter, Table3. 2).

2. Laboratory Methods

Lavage total IgG

Figure 3.1 records the total IgG in WGLF from the different patient groups. In the miscellaneous group, an 85 year old female patient diagnosed with anaemia/cirrhosis had a very high lavage total IgG (excluded from the analysis). Another 84 year old female patient who had been diagnosed with a rectal adenoma, also had a very high IgG value (67μg/mL). In the IBD group, 3 Crohn's patients had lavage IgG >10 μg/mL. These patients had active disease and were on therapy consisting of Prednisolone and Salazopyrin. One case of giardiasis also had a high lavage IgG level(16 μg/mL).

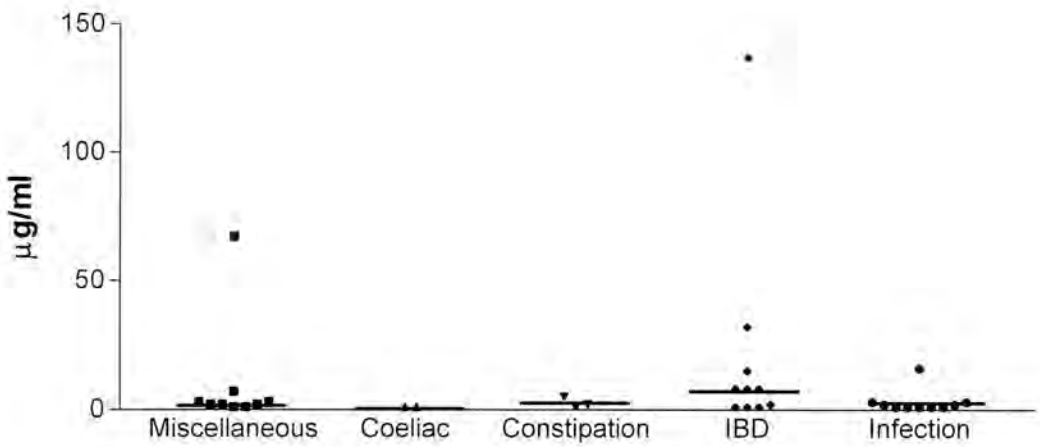


Figure: 3.1 Total IgG in WGLF from different groups of patients.

Lavage Total IgA

Figure 3.2 shows the total IgA in WGLF . There were no significant differences in lavage total IgA between patient groups. Total IgA values ranged from 9-175 μg/mL However, higher values of IgA were observed in coeliacs. A 72 year old male, who was admitted with unexplained anaemia and altered bowel habit, and was diagnosed with colonic carcinoma, had high IgA values. Another patient, a 29 year old female, diagnosed with Travellers diarrhoea had a very low total IgA in lavage. This may be a case of IgA deficiency. It would merit checking her serum level of IgA antibody.

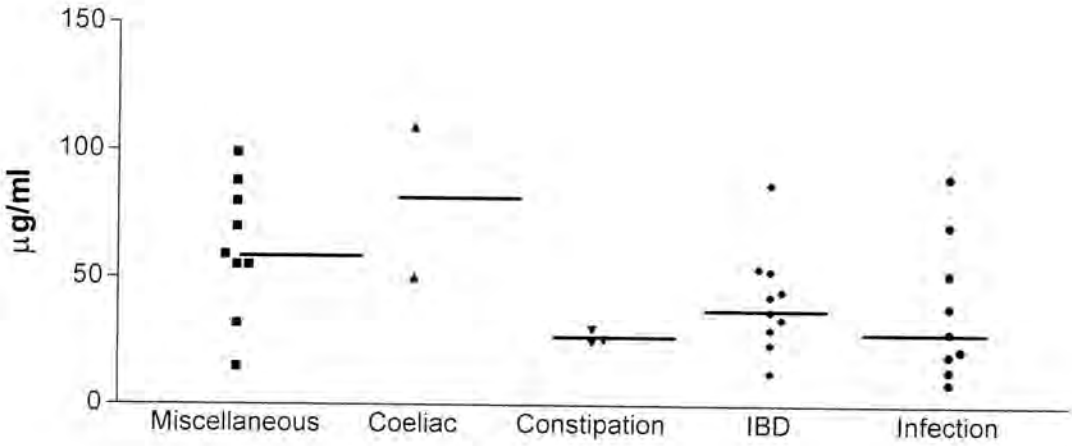


Figure: 3.2 Total IgA in WGLF from different groups of patients

IgA EndoCAb

Specific secretory IgA antibody against bacterial antigens was detected in all 35 cases. The values ranged from 1.3 -19.9 MU/mL and are shown in figure 3.3.

There were no significant differences between the groups. However, again there was increased values observed in patients with coeliac disease. It is noteworthy that the lowest value of IgA EndoCAb (1.3 MU/mL) was in the patient who was suspected to have IgA deficiency. This finding lends support to her diagnosis.

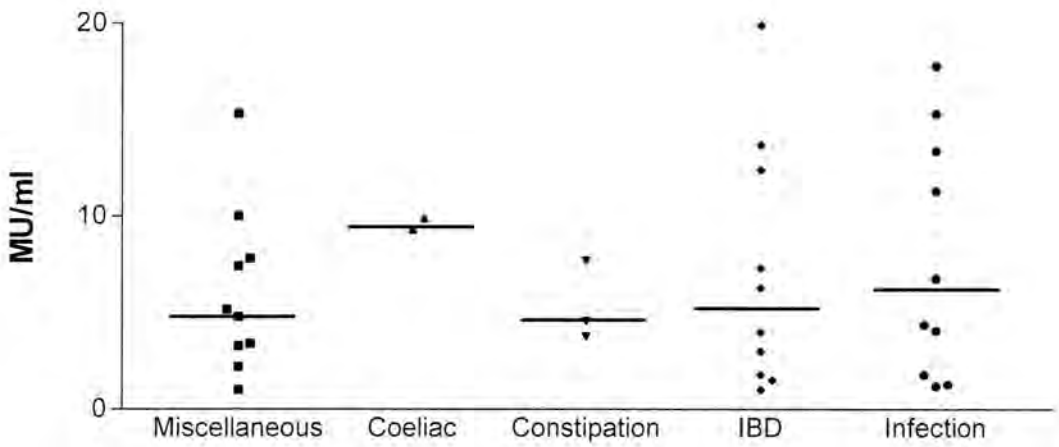


Figure: 3.3 IgA EndoCAb in WGLF from different groups of patients.

One interesting phenomenon was observed in the IBD cases. There was a significant correlation between the total IgA and IgA EndoCAb titre in lavage (figure:3.4).

There were also increased IgA EndoCab responses observed in two cases, one with giardiasis and another with diverticulosis.

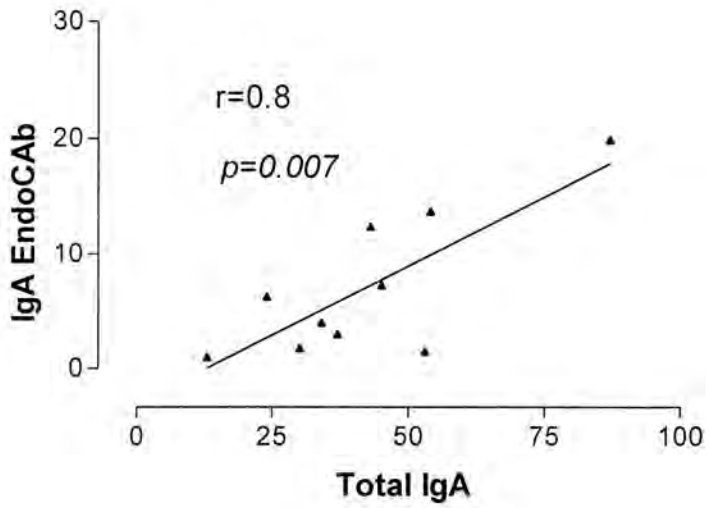


Figure: 3.4 Correlation between total IgA and IgA EndoCAb in WGLF from patients with IBD

IgM EndoCAb

Figure 3.5, illustrates the IgM antibodies against 'Endotoxin' core antigens in different patient groups. There were significant differences in the expression of IgM EndoCAb response in coeliacs compared with IBD ($p<0.05$), possible infection ($P<0.05$), and various ($p<0.04$) groups.

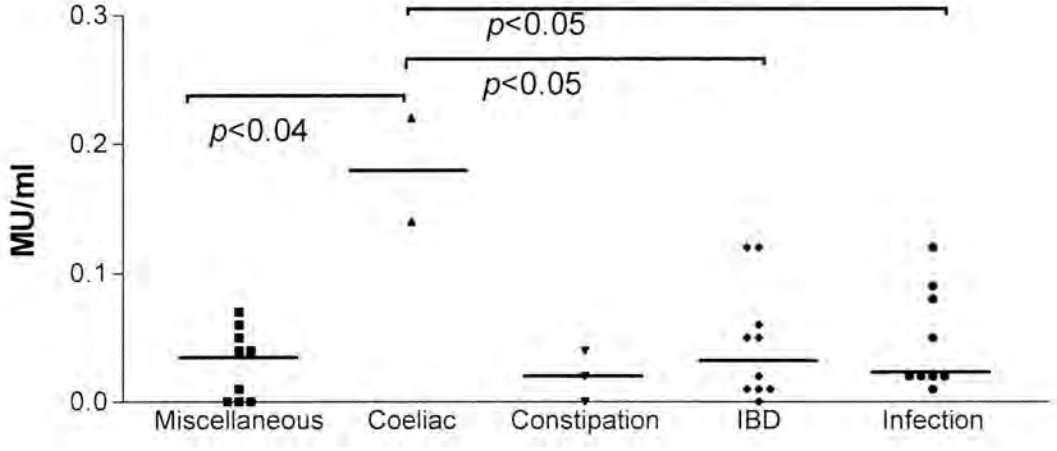


Figure: 3.5 IgM EndoCAb in WGLF from different groups of patients

Discussion

By comparing my results with the results from others it shows that I am technically competent in this laboratory work. Information from the patient with constipation confirms that antibody tests in WGLF provide a relatively reproducible test. Therefore, it is reasonable to expect to find a meaningful difference between patients groups as there really are differences in gut immunopathogenicity. This was also shown previously by other investigators from this laboratory (e.g. Sallam, 1995).

As discussed in chapter 2. Total IgG in WGLF has been shown to be a useful objective parameter of GI protein loss as well as disease activity in IBD (Chouduri *et al,*1993). Higher values of total IgG in WGLF were also observed in this study. However, statistical significance was not reached due to small groups of patients.

In this study, high total IgA values were observed in patients with coeliac disease (although only in 2 cases). This finding is in agreement with those of others (Colombel *et al*, 1990; Wood *et al*, 1987; Crabtree *et al*, 1989) who showed enhanced local secretion of IgA and IgM antibodies and increased Ig- synthesising plasma cells in the lamina propria.

IgA-specific antibody responses against 'Endotoxin' core antigens were also higher in patients with coeliac diseases. In a similar fashion, to total IgA, this increased response may be due to generalised immune up-regulation in the gut in coeliac disease. Alternatively, these results may suggest a selective contribution by antibacterial antibody responses to the increase in intestinal Ig content.

The increased levels of IgA EndoCAb paralleled the increase in total IgA in patients with IBD probably due to increased non-specific response to gut bacterial antigens. This may indicate a breakdown of gut immune tolerance to resident intestinal flora. This result may reinforce the finding of Dunhmann and colleagues (Dunhmann *et al*, 1995) who showed that tolerance exists towards resident intestinal flora but is broken down in active IBD.

Over-expression of total IgM antibody levels in intestinal secretions from coeliacs has been previously shown in this laboratory (O'Mahony *et al*, 1991). It was also postulated that plasma leakage (coeliac disease is also a protein-losing enteropathy) could be partially responsible for this high IgM value. However, an increase in the intestinal fluid immunoglobulin content was not accompanied by equivalent changes in serum immunoglobulins . In addition, several *in vitro* studies (Falchuk *et al*, 1974; Ciclitera *et al*, 1986) assessed the relative contribution of specific antigliadin antibodies to the increase in total intestinal immunoglobulin content in coeliac disease. They found a variable contribution (from 12 to almost 50%) of antigliadin IgM antibody to the total concentration of IgM antibody.

I am not aware of any *in vivo* studies that have assessed the contribution of antibodies against gut bacterial antigens to the increased total immunoglobulin in intestinal secretions in coeliac disease. These immunoglobulins may have a significant contribution to the enhanced intestinal antibody production in this disease.

Conclusions:

From this pilot work, a number of useful pieces of information have already emerged. However, further studies will be needed using carefully selected patients along with healthy controls to confirm and expand on these findings. Similar range assays could be done on lavage and paired sera from the patients which may yield important results. However, a colleague in the GI lab, who has interests in coeliac disease wanted to take this issue further.

Chapter:4

Studies of Healthy Controls from Dhaka and Edinburgh

Section 1 Studies of Gut Damage Parameters

Introduction

Gastrointestinal infections and parasitic diseases are highly prevalent in developing countries, like Bangladesh (Baqui *et al*, 1992). This is not the case in Western societies. The resultant question is therefore whether any alteration or fundamental differences exist in gut mucosal immunity between these two heterogeneous population groups.

Various immunological parameters in the gut mucosa of patients and healthy volunteers have been well studied in Edinburgh (Mahony *et al*,1990, Croft, 1996, Sallam, 1994). The significance of these immunological parameters in relation to diseases (for example, Coeliac disease, Inflammatory bowel disease) has been described in publications originating from this gastrointestinal unit (Mahony *et al*, 1990 & 1991; Brydon *et al,* 1992 & 1993, Choudari *et al*,1993, Ferguson *et al*, 1992 & 1994, Croft,1996). For example, in all of these studies WGLF total IgG has been shown to be an excellent objective marker of gut inflammation or damage.

In the following two sections, I have compared various immunological and gut damage parameters in WGLF from healthy volunteers and controls from Dhaka and Edinburgh.

Results:

Table 4.1 summarises the findings in the two control groups of various factors that are indicators of mucosal damage and inflammation in the gastrointestinal mucosal system.

Table 4.1: Comparison of Gut Damage Parameters among Healthy Controls from Dhaka and Edinburgh

Gut damage parameters	**Substances analysed**	**Dhaka (N=11) Median (range)**	**Edinburgh (N=12) Median (range)**	*p* value
GI Bleeding	Haemoglobin	4 (2-11) μg/mL	3 (1-9)	0.37
Mucosal Inflammation and Protein loss	Total IgG μg/mL	0.9 (0.2- 4)	1 (0-1)	0.47
	Albumin μg/mL	15 (11-22)	1.5 (0-13)	0.0001
	α-1 Anti-trypsin μg/mL	4 (2-10)	2 (0-7)	0.123

Gastrointestinal Blood Loss

Gastrointestinal blood loss is indicated by measurement of haemoglobin concentrations in WGLF. The normal range for adults (between 0 and 5 μg/mL) has been established in Scotland (Brydon & Ferguson, 1992). All the controls from Dhaka

have normal lavage haemoglobin, except one volunteer. This was a 32 year old (Mr. N), healthy man whose lavage haemoglobin was 11. No obvious reason was found for this high value. He might have had a parasitic infestation. Parasitic infestation is quite common in developing countries. Similarly, all the controls from Edinburgh had values within the normal range, apart from one: a 29 year old female (PL) who had a lavage haemoglobin of 9 . She was constipated and occasionally had problems with haemorrhoids. Thus, with the exception of these two cases there was no evidence of gastrointestinal blood loss in either control group.

Mucosal Inflammation and Protein loss

Total IgG in WGLF

The normal range for healthy control adults was studied in this unit (Sallam,1995). All had total IgG of less than 5 μg/mL. However, the normal range in adults is considered to be up to 10 μg/mL (Brydon *et al*, 1993). All the healthy controls both from Dhaka and Edinburgh had lavage IgG < 5 μg/mL . Thus there was no evidence of mucosal inflammation.

Albumin

The normal range for adults is up to 26 μg/mL (Brydon *et al*, 1993). The median and range in both control groups were within the normal range. However, there were significant differences in lavage albumin values between the control groups. Healthy volunteers from Dhaka have a significantly higher selective (no evidences of other protein loss) albumin loss through the gut compared with Edinburgh controls (P<0.001).

α-1 Anti- trypsin

The normal range in Scottish adults is up to 19 μg/mL (Brydon *et al*, 1993). The healthy controls both from Dhaka and Edinburgh both had values <10 μg/mL.

Section 2 Studies of Gut Mucosal Immunity Parameters

I will now discuss the results of different immunological parameters for the healthy volunteers from Dhaka. These results will be compared with the normal ranges previously established in adults from Edinburgh (summarised in table 4.2)

Table 4.2: Comparison of Gut Immunity Parameters Among Healthy Controls from Dhaka and Edinburgh

Immunity Parameters	Substances Analysed	Dhaka (N=11) Median (range)	Edinburgh (N=12) Median (range)	*P* values
Secretory Immunoglobulins	Total IgA μg/mL	51 (33-138)	36.5 (18-55)	0.0242
	Total IgM μg/mL	9 (4- 34)	7 (0.34-23)	0.23
Cytokines	IL-1β pg/mL	0 (0- 20)	0(0-13)	0.83
	IL-8 pg/mL	0(0- 18)	0(0-23)	0.503
Cellular Mediators	ECP ng/mL	70(12- 98)	11.4(4-29.2)	0.0023
	GE μkat/mL	0 (0- 0.194)	0 (0- 9)	0.14

Secretory Immunoglobulins

Immunoglobulin A (IgA)

In a previous study of healthy Scottish adult volunteers, the total IgA in WGLF was found to have a median concentration of 73 μg/mL (10-173 μg/mL) (Sallam,1995) The median (and range) concentration of total IgA in WGLF from healthy volunteers from Dhaka and Edinburgh were 51 μg/mL (33-138) and 36.5 μg/mL (18-55) respectively. It is noticeable that the concentration of total IgA in WGLF from these controls was less than that found in the previous study. The reason for this apparent discrepancy in concentration of total IgA in healthy adult Scottish controls is not clear. However, there was a problem with the IgA standard in the previous study (Mr. Norman Anderson, personal communication) which may have caused higher values in comparison to my studies (which have been verified and confirmed by two independent expert research technicians in the GI lab)

There is a significant difference in total IgA in WGLF between healthy controls of Dhaka and Edinburgh (p<0.0243). In the Edinburgh control group, some patients were constipated, which leads to the question of whether constipation can cause lower levels of IgA in WGLF. I will discuss this more fully later in the chapter.

Immunoglobulin M (IgM)

The median concentration of total IgM in WGLF from both Dhaka and Edinburgh control are 9 μg/mL (4-34 μg) and 7 μg/mL (0.34- 23 μg) respectively. These values are higher than total IgM in healthy adult volunteers studied previously (Sallam, 1995).

Cytokines

Interleukin-1β

Healthy adult controls have concentrations up to 18 pg/mL. Virtually no IL-1β was detected in WGLF in either control group. However, there was one exception in each group: a 26 year old man (Mr.S) from Dhaka group had an IL-1β level of 20 pg/mL. Similarly, a 38 year old female (CS) from Edinburgh group had an IL-1β level of 13 pg/mL.

Interleukin-8 (IL-8)

The normal adult range for IL-8 is less than 4 pg/mL. The median value and range in Dhaka and Edinburgh groups were 0 pg/mL (0-18 pg/mL) and 0 pg/mL (0-23 pg/mL) respectively. Three out of eleven volunteers from the Dhaka group had IL-8 levels of more than 10 pg/mL in WGLF no IL-8 was detected in the remainder . No IL-8 in WGLF was detected in the Edinburgh control group with the exception of one case: a 29 year old female (PL, her lavage Hb was 9) whose WGLF IL-8 was 23 pg/mL. No obvious reason was found for the slight increase in IL-8 concentration in these 4 cases

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Cellular mediators

Granulocyte Elastase (GE)

The normal range in adults is <100 μkat/mL (Ferguson *et al*,1994). There was no GE detected in either of the control groups, except in three cases, whose values were <10 μkat/mL. This suggests there was no evidence of active inflammation in either group.

Eosinophil Cationic protein (ECP)

There is no available adult reference range for ECP in WGLF. The median value and range in healthy adults from Edinburgh was 11.4 ng/mL (4 - 29.2 ng/mL). The ECP concentration in WGLF in healthy controls from Dhaka was higher, with a median value and range of 70 ng/mL (12 - 98 ng/mL). This value is significantly different from the Edinburgh control group ($p<0.0024$). Higher incidence of parasitic disease in developing countries, like Bangladesh (Baqui *et al*, 1992) may explain this difference.

Total serum IgG and IL-6

In view of the higher concentration of total IgA in WGLF in controls from Dhaka, I decided to investigate the possibility that the higher IgA response could be due to non-specific immune activation resulting from a high bacterial antigenic load from the contaminated environment (Stoll *et al* 1986; Baqui *et al*, 1992). I measured total serum IgG and IL-6. These are good indicator of non-specific and recurrent infection The values are significantly different from those for the Edinburgh control groups, $p<0.0006$ and $p<0.0026$ respectively (Figures 4.1 & 4.2). It is worth mentioning that due to unknown reasons several cytokines, e.g. IL-6, IL-5, IL-10, and TNFα could not be measured in WGLF (Hazel Drummond, personal communication).

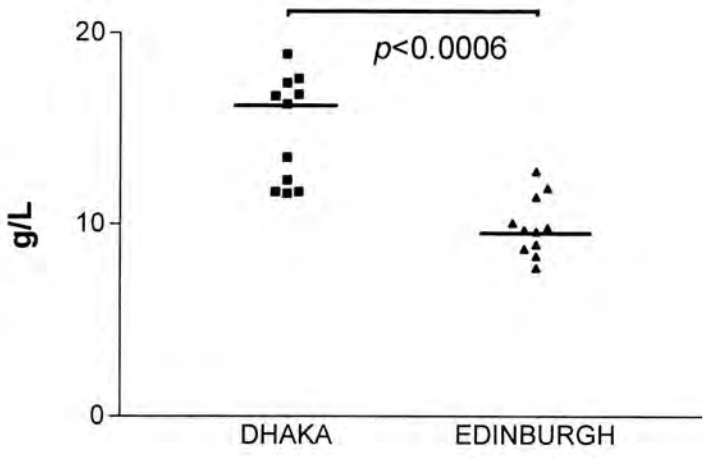

Figure: 4.1 IgG concentration in serum from healthy controls from Dhaka and Edinburgh.

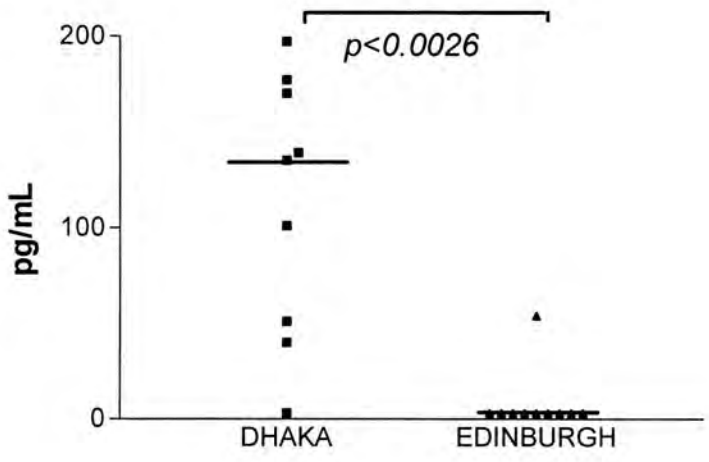

Figure: 4.2 IL-6 concentration in serum from healthy controls from Dhaka and Edinburgh

Discussion

Despite significant environmental and geographical differences, there is no evidence of gut damage or inflammation (as assessed by WGLF total IgG) in either of these two healthy control groups. As far as lavage albumin is concerned, all the values from both control groups were within normal adult ranges, according to a previous study (Brydon,1993). However, the WGL albumin level was significantly higher in the control group from Dhaka compared with Edinburgh. It is quite plausible that repeated enteric infections in controls from Dhaka may lead to increased permeability, which results in significant enteric protein loss. A study conducted at the International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B) has clearly shown that enteric infection can lead to significant protein loss which could occur even in the recovery period of the disease. Alteration in intestinal permeability is the cause of the protein loss following enteric infection (Alam *et al*,1994). However, the serum protein loss is quite selective (albumin but not α-1 antitrypsin or IgG) . In spite of there being significantly higher serum IgG levels in the Dhaka controls (Figure 4.1), an insignificant amount of total IgG was detected in WGLF. This result may contradict the currently held belief that total IgG in WGLF, which has a good correlation with disease activity in IBD, is merely a 'seepage' from serum (Choudari *et al*, 1993). However, I will discuss the origin of WGLF IgG further in chapter 7.

The total IgA result in WGLF is interesting. The significant difference between the Dhaka and Edinburgh control groups needs further clarification. It could be due to the incidence of constipation in the Edinburgh control groups which may result in lower values of total IgA in WGLF, due to slower intestinal transit time in constipation. This may result in intraluminal degradation of total IgA or dilution of intestinal secretion by

the WGL solution. However, Croft (1996) examined these issues in his study as causes for lower detection of total IgA in constipated children. He clearly showed that intraluminal degradation or dilution of intestinal secretions by lavage solution are not the cause of low IgA levels in constipation. The reason for the increased total IgA in WGLF in the Dhaka control may be due to non-specific immune activation. In view of the high bacterial antigenic load from a highly contaminated environment (Albert *et al*,1991), it is probable that there may be an increased drive for the production of secretory IgA to prevent inflammation and bacterial translocation. It has been shown that T cells 'armed' with secretory IgA antibodies are capable of direct killing of bacteria (Tagliabue *et al*, 1986). In addition, a study conducted by Kett *et al* showed that enteric bacteria enhance secretion of the protease-resistant form of IgA i.e. IgA2 (Kett *et al*, 1995). Furthermore, significantly higher non-specific IgG and IL-6 responses in serum (Figures 4.1 and 4.2) from the Dhaka controls lend further support to the hypothesis of increased non-specific immune activation in the gut mucosa. It should be noted here that I have measured neither the specific IgA2 levels in WGLF nor the activity of serum IL-6. The activity of serum IL-6 by bio-assay and measurement of specific IgA2 in WGLF would be helpful to reveal the mechanisms of this non-specific immune activation. It would be worth doing further study in this area. Cellular mediators, especially, ECP, (which is significantly higher in Dhaka controls than Edinburgh controls), will be discused further in relation to 'cell migration' in Chapter 9.

Chapter 5

Studies of anti-Endotoxin and specific antibacterial antibodies in healthy controls from Edinburgh and Dhaka

Section 1 : Anti-Endotoxin and other antibacterial antibodies

Introduction

In a previous chapter, evidence has been presented to suggest that higher IgA responses in healthy controls from Dhaka are due to non-defined gut mucosal immune activation. This immune activation may be due to a higher microbial antigen load in the gut from the contaminated environment. Therefore, I decided to look at anti-endotoxin (using 'cocktail antigens, which have been described in the method development section of chapter 2) antibody responses as well as specific anti bacterial antibodies in these two population groups. I will describe these findings in the following two sections of this chapter.

Results

The concentrations of IgA [median and (range)] in WGLF against endotoxin core 'cocktail' antigens in Dhaka and Edinburgh were detected as 7 (3-18) MU and 1.02 (0.51-4.14) MU respectively. The Dhaka control group had a significantly higher IgA response against these antigens compared to the Edinburgh control group, $p<0.0001$ (figure:5.1).

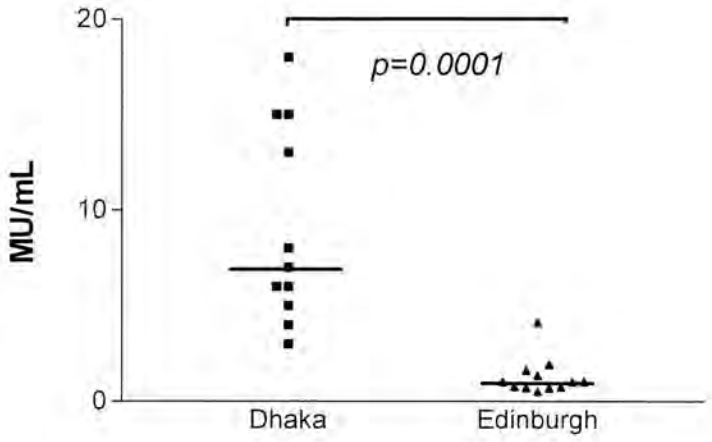

Figure: 5.1 IgA EndoCAb in WGLF from healthy controls

The higher IgA EndoCAb response in the Dhaka control group is well correlated with their total IgA in WGLF, r=0.78, $p<0.02$ (figure:5.2). Conversely, no correlation was found between IgA EndoCAb and total IgA in WGLF in the Edinburgh group (data not shown).

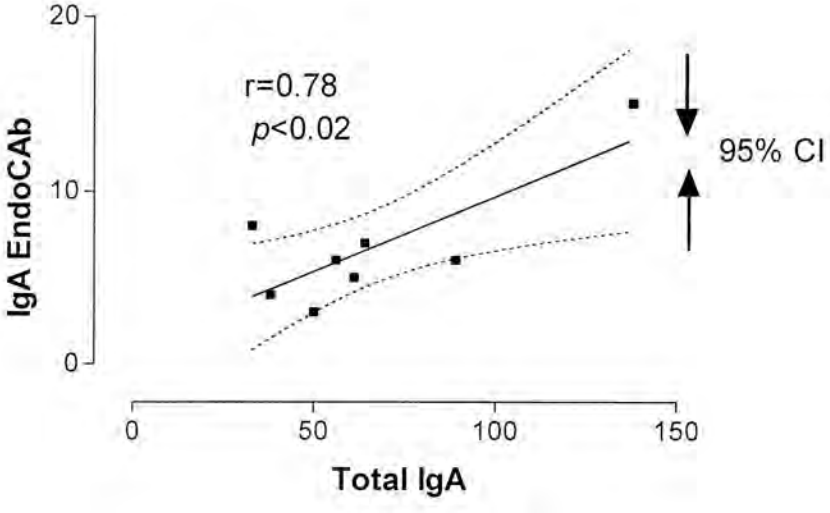

Figure: 5.2 Correlation between total IgA and IgA EndoCAb in WGLF from Dhaka control groups.

The IgM EndoCAb in WGLF in both Dhaka and Edinburgh groups are similar, these values are 0.18 (0.04 - 3.67) MU, and 0.18 (0.02 - 0.29) MU respectively.

No significant differences were found in serum EndoCAb antibodies (IgG, IgM and IgA) in these two population groups (figure:5.3). Their serum concentrations were similar. These values were (median and range): for IgG, 201.5 (103 - 523) MU, and 205 (105 - 999) MU; for IgM, 132 (70 - 251) MU and 104 (58 -234) MU; and for IgA, 170 (95 - 537) MU and 199 (125-522) MU in Edinburgh and Dhaka control groups respectively.

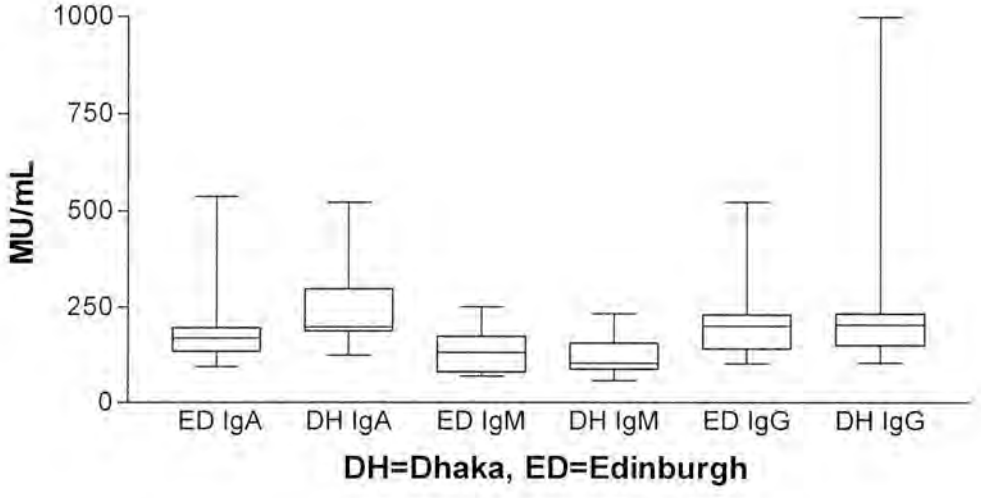

Figure: 5.3 Serum EndoCAb antibodies (IgG,IgM and IgA) in two control groups

Anti-cholera antibodies

The results of the specific anti-bacteria antibodies were quite surprising. There were no significant differences observed in anti-cholera toxin (CT) antibodies (IgA and IgM) in WGLF between Dhaka and Edinburgh control groups (figure:5.4).

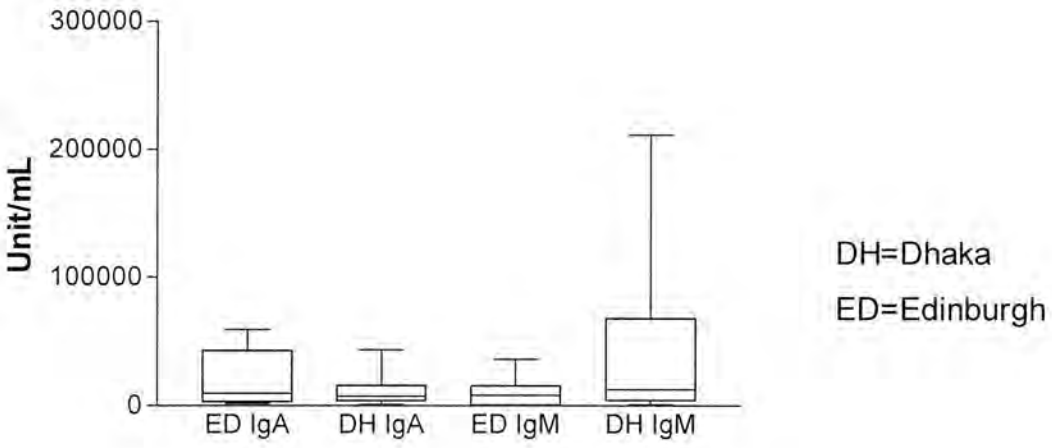

Figure: 5.4 Anti-cholera toxin antibodies (IgA and IgM) in WGLF in two control groups.

The median (range) values of these antibodies were : IgA, 7488 (1200 - 43456) units/mL and 9570.5 (1870 -59243) units/mL; and IgM, 12604 (836 -211488) units/mL and 8177.5 (0 - 36088) units/mL for Dhaka and Edinburgh control groups respectively. The anti-cholera toxin antibody responses in serum were also similar in these control groups with an exception of anti-CT IgG antibody. The serum anti-CT IgG response was significantly higher in Dhaka control compared to the Edinburgh control group, $p<0.001$ (figure:5.5).

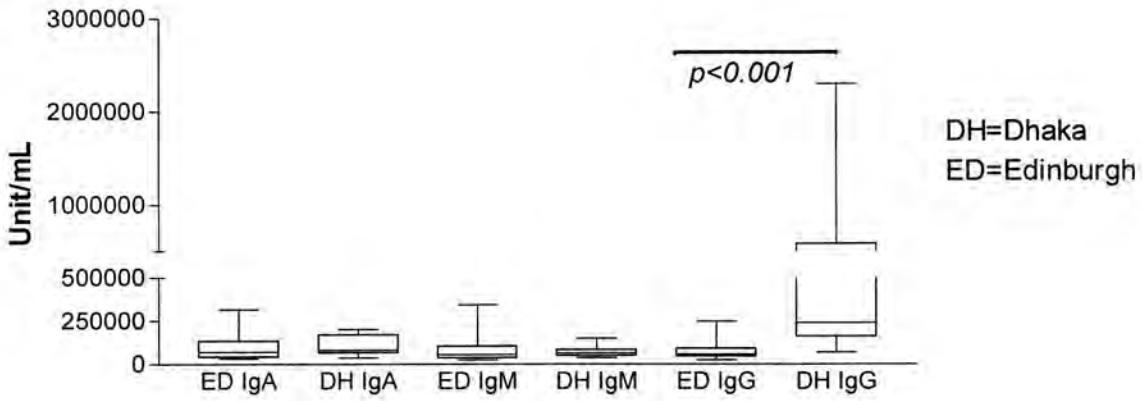

Figure: 5.5 Anti-cholera toxin antibodies (igG, IgM and IgA) in serum from two control groups.

To check the purity of cholera toxin antigen, I have run the cholera toxin B in SDS PAGE gel, which was also stained with Comassie blue to rule out any other protein contamination (figure: 5.6)

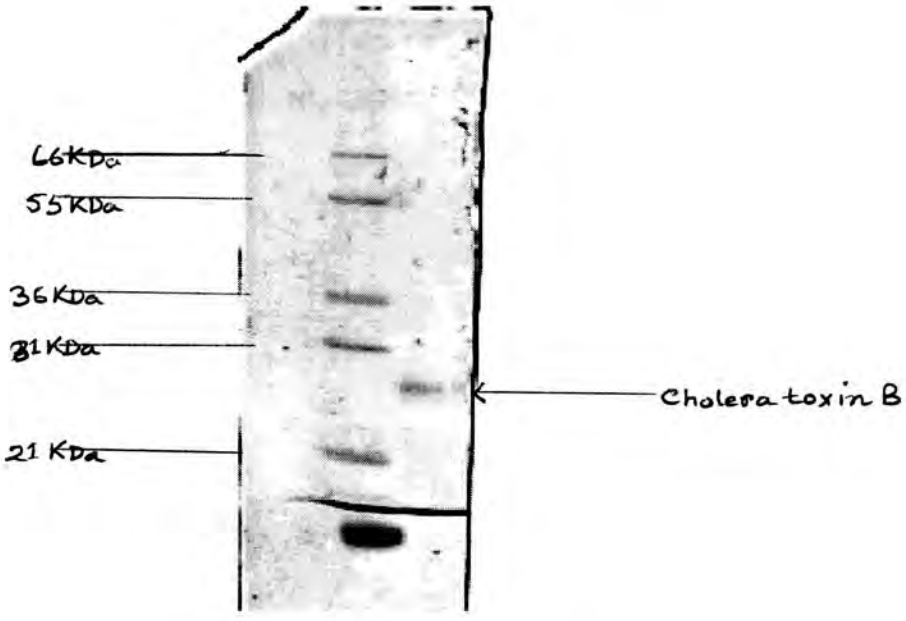

Figure 5.6: SDS PAGE of cholera toxin B with Comassie blue stain

Discussion

These data demonstrate that the higher IgA response in WGLF in Dhaka control groups is likely to be due to a higher level of Gram-negative enteric bacterial antigens in the gut. These enteric bacterial antigens may be responsible for the non-specific immune activation seen in these control groups. The specific IgA antibody responses against a representative number of Gram-negative enteric bacteria ('cocktail' antigens) is well correlated with their total IgA antibody in the WGL. This was not observed in the Edinburgh control group.

As far as serum antibodies are concerned, no real difference exists in specific antibody responses against the 'cocktail' antigens. This result suggests that differences exist between the systemic and mucosal immune compartments and support the statement that 'these two compartments are completely distinct' (Ferguson *et al*, 1994). Furthermore, this result also supports the recommendation that for the study of intestinal immunopathology, direct investigation of the gut is mandatory (O'Mahony *et al*, 1991).

The results of anti-cholera toxin (CT) antibodies were surprising for at least for two reasons: why should these occur in Edinburgh controls who have never had clinical cholera or been exposed to cholera vaccine? In some cases, primary antibody IgM is not detectable. However, some of the healthy volunteers from Edinburgh who said that they have had a history of a 'tummy upset' while they were on holiday or at home appear to have high levels of specific intestinal antibodies. Secondly, why should Dhaka controls have relatively lower levels of anti-CT intestinal antibodies ? A possible explanation for the first query is that these are antibodies to the heat-labile (LT)

enterotoxin of *E.coli*, which is antigenically (81% sequence homology) very similar to cholera toxin (Clements and Fikelstein, 1978; Takeda *et al*, 1983; O' Mahony *et al*, 1991 Ferguson *et al*, 1996). It has been shown that the LT from *E.coli* is immunologically related to both subunits, A and B, of cholera enterotoxin as demonstrated by neutralization and immunodiffusion (Clements and Finkelstein, 1978; Takeda *et al*, 1983). In addition, It has also been shown that cholera like-enterotoxin can be produced by some strains of *Camplybacter jejuni*. The toxin activity was blocked by cholera anti-toxin (Ruiz-Palacios *et al*, 1983). However, the possibility of Edinburgh people having recent exposure to LT is unlikely.

From previous studies in the GI lab, it has been shown that anti-cholera toxin antibody could be apparently detected in some patients from Edinburgh (O'Mahony *et al*,1991). The antigen used by O'Mahony was from the List Biological Laboratories, Inc. USA (Batch No: 103). I used antigen from a different batch (No: 569B) but from the same company. According to the company, the purity of the antigen was checked by disc gel electrophoresis (non denaturing condition), which gives a sharp immunoprecipitin band against cholera toxin antiserum. I have checked the purity of the antigen by running in SDS PAGE (figure 5.6). As it can be seen, there is also a sharp band in the gel which clearly suggests a single molecule. Cholera toxin is a protein molecule, composed of two distinct subunits. The A subunit (28,000 Da) is noncovalently bound to an aggregate of five B subunits (11,600 Da) (Reid *et al* ,1995). Ideally, in a SDS PAGE the B subunit should come out as either 11,6 00 Da or 57,500 Da. After comparing with molecular weight markers, it shows that the antigen did not reach 57,500 Da or 11,600 Da but was at around 28,000 Da. This is the correct molecular weight of the A subunit. Following inquiry, the company mentioned that B subunit came up as single molecule but they quoted the published molecular weight. It is therefore, reasonable to

conclude that the anti-cholera toxin results should be interpreted against all these backgrounds and obviously warrants further study before any firm conclusion can be made..

In explanation of the second query, it seems reasonable to postulate that the down-regulation of anti-CT antibody production could be due to 'oral tolerance' (Strobel & Mowat, 1998). This is possibly due to repeated exposure of individuals in a 'cholera endemic' area, like Dhaka. However, this statement contradicts the previous anti-endotoxin results and hypothesis. Nevertheless, it would seem unreasonable to postulate that 'oral tolerance' may be developed against generalised Gram-negative enteric bacteria.

In a recent murine study it was shown that mice orally immunised with killed *Salmonella dublin* in conjunction with a suitable adjuvant were protected against a lethal oral challenge compared to mice orally immunised with killed *S.dublin* alone, which were not protected. The protected group showed a four-fold increase in serum anti-LPS IgG but not in faecal anti-LPS IgA. There was a correlation between serum anti-LPS IgG antibody response and adjuvant-induced protection in orally induced mice (Chong, 1998). The significantly higher anti-CT IgG response in the Dhaka control groups compared to the Edinburgh groups may lend support to the theory that Dhaka controls are relatively immunised. A study done in the late sixties showed that the degree of protection against cholera is directly related to the level of serum vibriocidal IgG antibody (McCormack et al, 1969).

Section 2: Anti-Lipolysaccharide (LPS) Core Types (R1,R2,R3 and R4) of *Escherichia coli,* IgA antibody

Introduction

As described in the literature review section of chapter 1 (figure: 1.1), lipopolysaccharide (LPS) is an essential component of the outer membrane of all Gram-negative bacteria. Structurally, most types of LPS are composed of three distinct regions: the lipid A, the core oligosaccharide and the O-polysaccharide (O antigen) consisting of long chains of repeating oligosaccharide units (figure:1.2). The LPS core region of *E.coli* has a conserved overall structure, with an inner 2-keto-3-deoxy-octonate-heptose region and an outer hexose/hexosamine region. Most of the variation that does occur is in the outer part of the core, furthest from lipid A. In wild-type smooth *E.coli*, the core region can be typed into at least five different chemotypes, known as R1,R2, R3, R4 and K-12, the geneticists strain (Schmidt *et al,*1969; 1970).

The O antigen of smooth LPS may mask some of the epitopes and phage attachment sites in the core region. The chemical, serological and phage methods that have been used to define the different core types of *E.coli* are therefore not directly applicable to wild-type smooth strains (Gibb *et al*,1992). However, it is now well recognised that certain O-serotypes are associated with certain diseases, especially within *E.coli* (Orskov and Orskov,1992), and can be useful in epidemiology. Dr Gibb demonstrated that O serotypes of *E.coli* can be further analysed by core typing using monoclonal antibodies (Gibb *et al*, 1992). Certain O-serotypes appear to be always associated with

a single core type, while others may be associated with more than one core type. Very little is known about the frequency with which these different core types occur in wild-type *E.coli* serotypes. Similarly, the relationship of core type with various diseases caused by *E.coli* is unknown.

E.coli diarrhoea is common in Bangladesh (Hoque *et al*, 1994). In view of the recent enterohaemorrhagic *E.coli* outbreak in Scotland (Douglas and Kurien, 1997), I was prompted to look at whether there is a gut mucosal immune response against the different chemo-types of *E.coli* in these two population groups. It is a well known fact that gut mucosa is an interface between 'exterior' and 'interior', and acts as a barrier against infectious organisms or their products gaining access to the systemic circulation. In the field of sepsis, the source of LPS/ Endotoxin, whether of gut origin or due to bacteraemia, is debated (Poxton,1995). It is recognised that mucosal immunity is quite separate from systemic immunity. It is, therefore, essential to examine the mucosal antibody responses to core LPS types of *E.coli*. Knowledge of this area would have potential in the development of the oral *E.coli* vaccines. The comparative distribution of responses to core types of *E.coli* in these two geographically different population group was an important aim of this study.

Results

LPS extracts were run in PAGE gel and subsequently silver stained to check the purity of the LPS. Figure 5.7 shows the silver-stained PAGE profile of R1-R4 core types of *E.coli*. The purity of LPS were also checked by immunoblot (figure, 5.8) with core specific monoclonal antibodies [VN2 483.10 (R1 specific), H4361.23 (R2 specific), W4 434.07 (R3 specific) and WN1 222-5 (cross-reactive with all core types)], as described by Gibb (Gibb *et al*, 1992). These monoclonal antibodies were supernatants of hybridoma cell cultures grown in RPMI 1640 supplemented with 5% FCS. (These antibodies were a kind gift from Dr.Barclay). The R4 core LPS was not totally pure, some 'ladder pattern' of bands is visible corresponding to O- polysaccharides (track 4 in figure 5.7). This is typical of the R4 core type LPS. It is rather heterogeneous and has been observed previously (Professor Poxton and Robert Brown, personal communication).

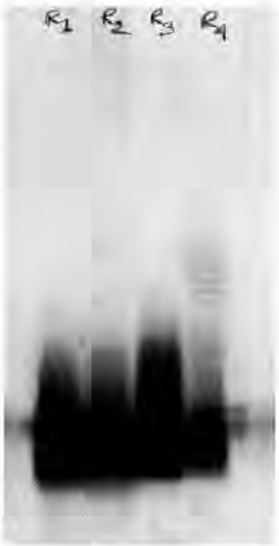

Figure 5.7 Silver stained PAGE profiles of core types of LPS *E.coli*
Track 1= R1, Track 2= R2, Track 3= R3 and Track 4= R4.

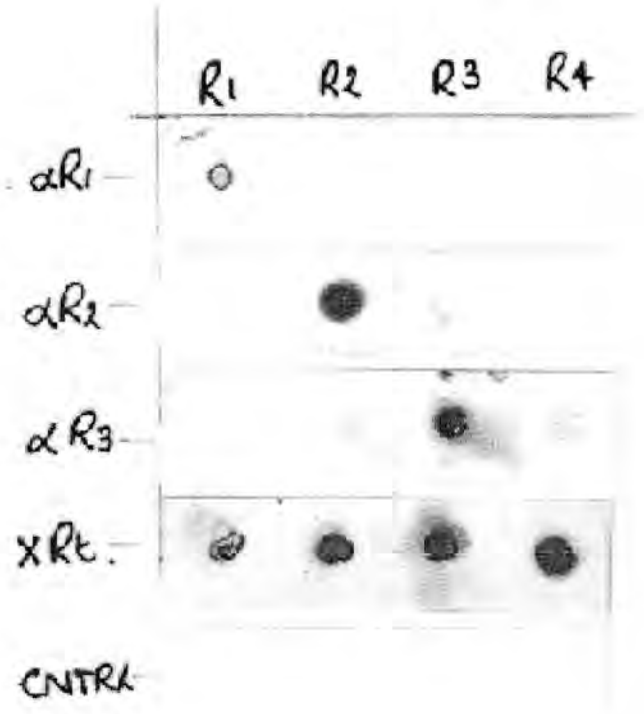

Figure 5.8 'Dot blot' of various core types of LPS with specific monoclonal antibodies αR1, αR2 and αR3= monoclonal antibodies specific for R1, R2, and R3 respectively. Xrt= cross-reactive with all *E.coli* R cores. CNTRl= control.

Figure 5.9 depicts the findings of WGLF IgA responses against LPS core types of *E.coli.* As can be seen in the figure, there were IgA antibody responses against the whole range of available core types (R1,R2,R3 & R4) of *E.coli.* In the Edinburgh control groups, the antibody responses were in descending order of magnitude: R1>R2>R3>R4. On the other hand, in the Dhaka control groups, the order was: R1>R3>R2>R4.

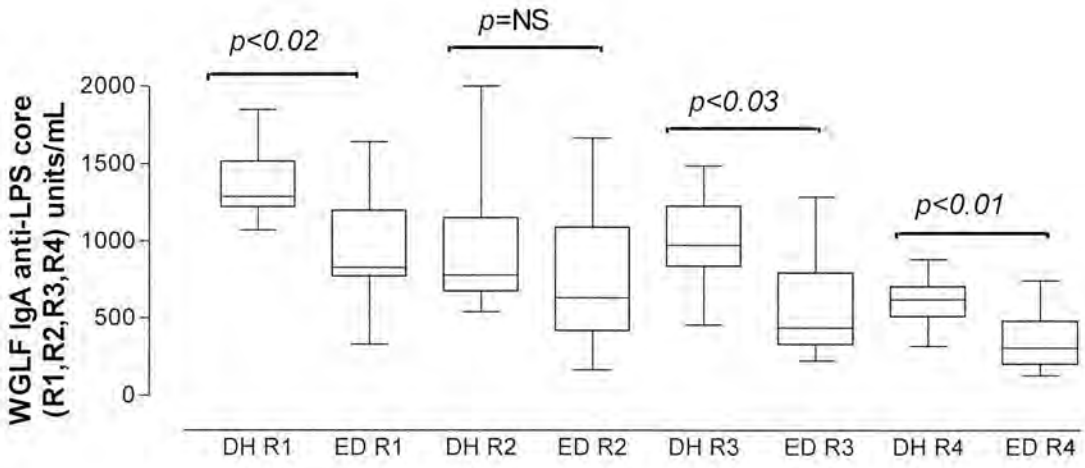

Figure: 5.9 Anti-LPS core types (R1,R2,R3 & R4 of *E.coli*) IgA antibody in WGLF in healthy controls.

There were significant differences in the IgA antibody response against core types of *E.coli* between Dhaka and Edinburgh. The IgA antibody responses against R1, R3 and R4 in Dhaka controls were higher and significantly different from the Edinburgh controls, $p<0.02$, $p<0.03$ and $p<0.01$ respectively. IgA antibody response against R2 in the Dhaka controls were also higher but not significantly different from the Edinburgh controls ($p=0.30$).

Discussion

This study has shown for the first time that there are mucosal IgA antibody responses present against the specific LPS core types of *E.coli* in the gut. In view of the fact that the core epitopes may not be readily accessible to antibodies, the mechanism of antigen expression and induction of antibody response is brought into question. In an excellent study done by Gibb *et al*, it has been shown that the LPS core types can be differentiated serologically by monoclonal antibodies (Gibb *et al*, 1992). These antibodies are selective for the LPS core of *E.coli.* They do not bind to a range of other Gram-negative bacilli, indicating that their binding observed is specific. However, most of the antibodies bind to *Salmonella* and *Shigella* species as these share *E.coli* core structure, and are in fact closely related to *E.coli* in many other respects..

Unsubstituted LPS core is present in smooth bacterial cells but may not be easily accessible to monoclonal antibodies in standard laboratory cultures (Nelson *et al*,1990). However, core epitopes are more accessible on bacterial cells grown in serum or magnesium-depleted medium, which is thought to mimic conditions in the infected host (Nelson *et al*, 1991). However, the environment of the gut lumen for the growth of *E.coli* and other members of the Enterobacteriaceae is completely different. We do not know the mechanisms of expressivity and induction of IgA mucosal immune responses to core epitopes within the GI tract. It is known that enterocytes (the absorptive cells in the intestinal mucosa) can function as antigen-presenting cells (**APC**s), i.e. are able to take up and complete endosomal degradation, processing and presentation of antigen to immunocompetent cells (such as T cells and macrophages) (Kato & Owen,1994). It is probable that the LPS core types of *E.coli*

may exploit the efficient antigen-processing system operated by enterocytes for the following reasons: firstly, enterocytes are the first point of contact for enteric bacterial LPS like other luminal constituents; secondly, the transcellular route through enterocytes has been recognised as the route of uptake of non-nutrients (Kato & Owen,1994). The route of uptake of non-nutrients was believed previously to be only paracellular. Moreover, normal absorptive cells in humans express MHC class II proteins on their surfaces (Mayer *et al*,1991) which is a prerequisite for a **APC** to initiate the process of antigen presentation.

Uptake mechanisms are separated into receptor-mediated and non-receptor-mediated pathways (Kato & Owen,1994). The core epitopes of LPS may use either or both pathways. Another important route of transportation of antigens is via **M** cells. **M** cells play an important role in antigen sampling, taking up particles from the intestinal lumen and transporting them to lymphocytes and macrophages enfolded in pockets in the basolateral surfaces of **M** cells (Trier, 1991). In general, antigen uptake and transport are determined by the nature, polarity and capacity of the intracellular pathways to take up antigens. The core types of LPS of *E.coli* may use any of these antigen transport systems to reach the inductive arm of the mucosal immune system.

Gibb *et al* found in their study of 180 wild-type *E.coli* isolated from clinical samples (blood and urine specimens) and from the faeces of asymtomatic individuals, that 123 were assigned to R1 core type, 14 to R2 and 18 to R3. Due to lack of suitable specific monoclonal antibody, they could not positively identify the R4 groups (heterogeneous, not purely extracted as seen in figure 5.7). They did have a group of 25 strains, assigned as RNC (R not classified), which may include isolates with R4 core with other as-yet unidentified core types (Gibb *et al,* 1992). Our data suggest a similar

pattern of distribution of the core types viewed by the mucosal IgA response in both Edinburgh and Dhaka control groups. However, we do not know the incidence of R cores in the Dhaka population. The mucosal IgA data suggest that R3 may be more common in Dhaka compared to Edinburgh.

As far as O-serotypes are concerned, Gibb *et al* found R1 core type was associated with O types 1,4, 6,8, 18 and 75 whereas R2 with only O75. R3 core type was associated with O15. There were some apparently constant associations between O types and core types: all 26 O6 isolates in his study were R1 and all 3 O15 isolates were R3. In contrast, O75 isolates were found among both R1 and R2 core types (Gibb *et al*,1992). They also found that the proportion of R1 that belongs to one of the common O types was greater among blood culture isolates. The relationship between core types and O serotypes of *E.coli* and their pathogenicity in man is not yet established. Significantly higher IgA responses against R1 core types in the Dhaka control groups, may be due to the fact that *E.coli* is not only a common diarrhoeal pathogen (Hoque *et al*, 1994), but also a common isolate in Gram-negative septicaemia in Bangladesh (Strulence *et al*,1985). It is possible that there may be a relationship between core types and pathogenicity.

The IgA response against R3 core types in the gut is fascinating, in view of the fact that all isolates of *E.coli* O157:H7 as well as other vero-toxin producing *E. coli* (VTEC) strains investigated in Scotland are R3 positive (Curie and Poxton,1999). Significantly higher IgA responses against R3 in the Dhaka control groups may be due to higher incidence of infections caused by *Shigella* and *E.coli* in Bangladesh (Hoque *et al*, 1992;1994). These organisms share closely related LPS cores (Gibb *et al*,1992; Heinrichs et al,1998). Gibb *et al* showed that *Shigella sonnei* strains reacted with the

R1-specific monoclonal antibody (mAb) while *Shigella flexneri* strains reacted with the R3-specific mAb. It is difficult to comment on R4 core types because this group is not yet well characterised. *Salmonella* shows homology with the R2 group.

This study suggests that the LPS core types of *E.coli* are expressed and capable of producing IgA antibody responses in the intestinal mucosa. Information concerning the LPS core epitopes that evoke protective immunity could be useful in the future design of oral vaccines. However, further studies are needed to investigate how these core type antigens are presented to the inductive arm of mucosal immune systems, especially the role of T cells and immunological memory. It is also necessary to establish the relationship between the LPS core types and O types and pathogenicity. It would be interesting to investigate specific monoclonal IgA antibodies and their relative protective capabilities, especially whether antibodies recognising a single epitope on the target organism can provide protection against colonisation or invasion of the intestinal mucosa.

Section 3: Biological potentials of core types of LPS of *E.coli*

Introduction

Septic shock syndrome is a serious problem associated with high morbidity and mortality. It is well recognised (already discussed in the literature review section) that the effect of excessive release of proinflammatory mediators -including reactive oxygen intermediates and nitrogen compound, such as nitric oxide (NO) and cytokines (notably,TNF α and IL-1β) induced by the LPS of gram-negative bacteria is the mechanism of this syndrome. It is also possible that inflammatory responses in the GI tract may be caused or exacerbated by LPS and some LPSs may be more biologically active than others. In an earlier section, it has been found that the IgA antibody response against core types of LPS of *E.coli* is quite variable. Furthermore, it is also known that several virulent pathogenic enteric bacteria (eg. *Shigella*, *E.coli* O157) possess R3 core types in their LPS. The following section tested the hypothesis that some core types, e.g. R1 or R3, are more active in terms of inducing proinflammatory mediators than that of other core types (R4 was omitted as it was not purely isolated and it is uncommon) Accordingly, I have measured induction of NO, TNF-α and IL-1β using the mouse monocyte cell line (J774) and human colonic carcinoma cell lines (Caco2).

Results:

Figure 5.10 depicts the effect of various core types of LPS on mouse monocyte cell line (J774) NO secretion. It shows that R1 is more potent in inducing NO secretion than other core types (R1>R2 & R3).

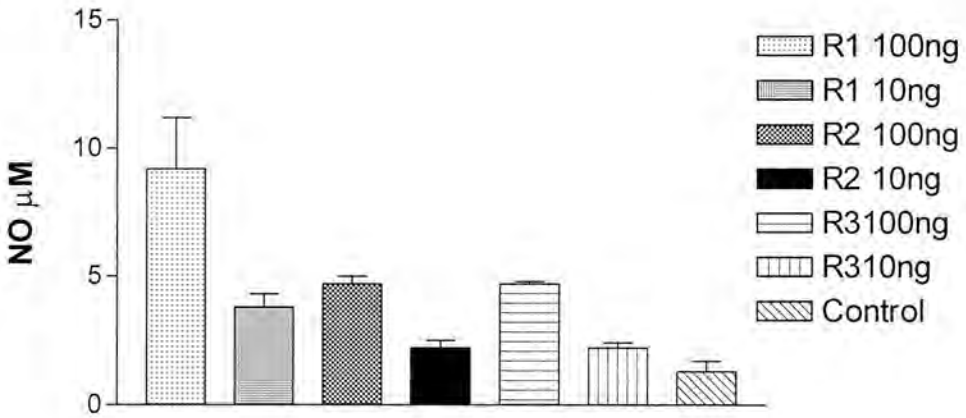

Figure 5.10: Effect of various LPS R core types in induction of NO secretion in mouse monocytes

Figure 5.11 illustrates the TNF-α secretion in response to different concentration of various core types of LPS. Apparently it shows that the R3 core type is much more potent in inducing TNF-α secretion than other core types (R3>R1>R2). Statistical analysis was not done in view of the small sample size.

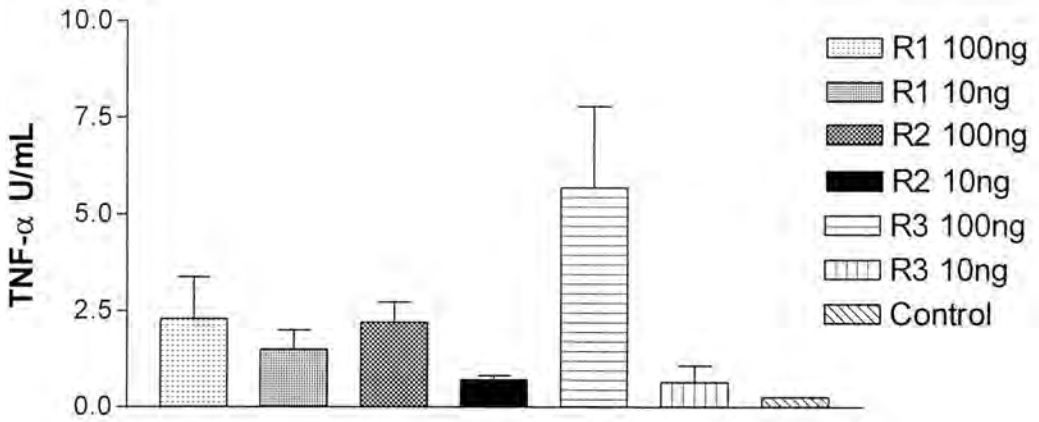

Figure:10.11 Effect of LPS R core types on J774 mouse monocyte cells in induction of TNF-α secretion.

No IL-1β was produced by the mouse cells J774 in response to LPS core types (in different concentration, upto 100 ng/mL)

Core types of LPS in concentrations up to 100 ng/mL used had no effect on human colonic carcinoma cell line in producing NO, TNF-α or IL-1β.

Discussion:

The single layer of epithelial cells that lines the intestinal mucosa is the initial site of interaction between host and microbial pathogens. Intestinal epithelial cells respond to bacterial invasion by up-regulating the expression of an inflammatory gene programme (Kagnoff and Eckman, 1997). Characteristic features of this programme include the increased production of chemokines that can act as early signals to activate an acute mucosal inflammatory responses (Eckmann *et al*, 1993; Jung *et al,* 1995; Yang *et al*, 1997).

Nitric oxide (NO) is generated by the conversion of L-arginine to L-citrulline by NO synthase (NOS), which exists in three isoforms, each encoded by a separate gene (Nathan and Xie, 1994). The expression of inducible NOS (iNOS) is regulated in various cell types and can be increased by stimulation of cells with several cytokines or with bacterial LPS (Nathan and Xie, 1994; Witthoft *et al*, 1998). NO can mediate an array of physiological effects in the intestine. It has been shown that blocking endogenous nitric oxide synthesis led to increased intestinal permeability; i.e. endogenous production of NO is essential for maintenance of the normal barrier function of the gut (Fink, 1999). On the other hand, it has been shown that excessive release of NO compromises the viability of intestinal and colonic epithelial cells in endotoxin-challenged rats (Tepperman *et al*, 1994). Thus, under some conditions, NO does not protect the gut,but rather injures the mucosa.

Initial experiments with the mouse monocyte cell line J774 reveal that LPS at concentrations as low as 0.01 ng/mL can induce NO production. However, the 100 ng/mL concentration was found to induce maximum NO production in a dose-

dependent manner but without killing the cells (C. Erridge, unpublished). In the present study, R1 core was found to be more potent in inducing NO production compared to other core types. Gibb *et al* found that most of the blood or urinary isolates of pathogenic *E.coli* are R1 core positive(Gibb *et al,* 1992). It may be possible that the LPS which induces much more NO has a predilection for systemic pathogenicity. It should be noted that some *Shigella* spp have an R core.

Tumour necrosis factor-α (TNF-α) and IL-1β are produced primarily by macrophages and fibroblasts in inflamed tissue. IL-1β and TNF-α appear to contribute directly to tissue damage through induction of the release of tissue-damaging enzymes (e.g. matrix metalloproteinase) (Tissi *et al*, 1999; Monteleone *et al*, 1999). In this study, R3 core produced huge amounts of TNF-α compared to other core types. Several enteroinvasive pathogens (e.g. other *Shigella* spp and *E.coli* O157) are R3 positive. It is quite possible that mucosal pathogens induce more TNF-α production compared to others who have predilection towards systemic pathogenicity. No core types (up to 100 ng/mL concentration) were able to induce IL-1β production from either J774 or Caco2 monolayer cells upto 24 hour post challenge. The reasons for not detecting IL-1β could have been due to the fact that IL-1β takes a longer time to synthesize or a larger dosage of LPS might have been needed for stimulation. Alternatively, J774 is not a suitable model at all for studying IL-1β. Similarly, Caco2 monolayer cells were found to be fairly resistant to the core types LPS (up to to 100 ng/mL) challenge. Normally, colon (large bowel) harbours the highest concentration of bacterial load in the human body (10^{12} bacteria/gm of colonic content). This may suggest that the colonic cell line is constitutively resistant to smaller dosages of LPS (inadequate

dosage for stimulation). Alternatively, interspecies difference may have been responsible for the discrepancy observed.

The further assessment of the biological potential of LPS in inducing inflammatory mediators is essential, especially for the development of a vaccine against septic shock syndrome or for the prevention or management of diarrhoea.

Section 4: Mucosal IgA antibody against LPS of *E.coli* O157

Introduction

Vero cytotoxin (VT)-producing *Escherichia coli* isolates (VTEC isolates, also called Shiga-like toxin [SLT]- producing *E.coli* isolates) are associated with a spectrum of illnesses that include diarrhoea (Pai *et al*, 1988), haemorrhagic colitis (Riley *et al*, 1983), and haemolytic-uraemic syndrome (Karmali, 1985; 1992). Whereas VTEC isolates belong to many different serotypes, O157:H7 is the most common serotype associated with human illness (Griffin and Tauxe, 1991).

The recent *E.coli* outbreak in Scotland caused major public concern regarding food safety and sparked several studies in this field (Douglas and Kurien, 1997). Despite the enormous burden of diarrhoeal disease with a wide range of infectious agents (including multiple agents) in Bangladesh, the isolation of *E.coli* O157:H7 is virtually non-existent there. (Hoque *et al*,1994;1998). All *E.coli* O157:H7 strains possess the R3 core type in their LPS (Currie and Poxton, 1999) and in an earlier section I noted that IgA antibody in intestinal secretions against R3 core type was significantly higher in healthy volunteers from Dhaka compared to the Edinburgh control groups. I wanted to test the hypothesis that the Dhaka population has higher IgA antibody against O157: H7 LPS in the gut compared to the Edinburgh population and hence has protection against O157:H7 infection, possibly through cross-reactive R3 core-reactive IgA.

Result

Figure 5.12 illustrates the concentrations of IgA antibody against LPS of O157:H7 in WGLF from healthy volunteers from Edinburgh(n=12) and Dhaka (n=11). The median concentration of IgA antibody in WGLF in the Edinburgh and Dhaka groups were 656 SAMI Unit/mL and 559 SAMI Unit/mL respectively. The levels thus tended to be higher in the Dhaka groups but this was not statisctically significant.

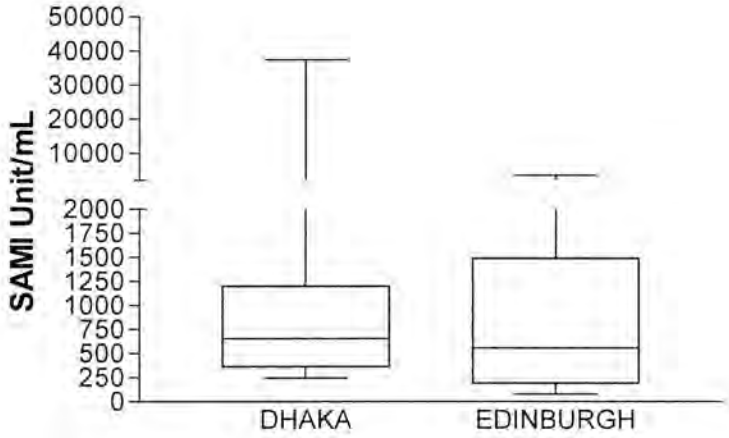

Figure 5.12 Anti-LPS IgA antibody against *E. coli* O157:H7 in WGLF between two healthy control groups

Discussion

In the present study, IgA antibody against *E.coli* O157:H7 was detected in healthy control groups both from Dhaka and Edinburgh. There was no significant difference in antibody concentration between the groups. This may be due to small sample size in both the groups. Despite the virtual absence of a problem of *E.coli* O157:H7 infection in Bangladesh, anti-LPS IgA antibody against *E.coli* O157:H7 was found in healthy volunteers from Dhaka. This may have been due to the fact that R3 core (present in *E.coli* O157:H7) which is present in *Shigella* might have stimulated the production of cross-reactive antibody to the LPS of *E.coli* O157:H7. Alternatively, there is evidence that sera from patients with *E.coli* O157:H7 infection react with the EPEC O55:H7 (an *E.coli* strain associated world-wide with outbreaks of infantile diarrhoea) (Whittam *et al*, 1993) but these strains may also have the R3 core. There is a close genetic relationship between VTEC O157:H7 and EPEC O55:H7 (Ludwig *et al*,1996). Both *Shigella* and EPEC infection are quite common in Bangladesh (Hoque *et al* 1992;1994).

The presence of anti-LPS IgA antibody against *E.coli* O157:H7 in the Edinburgh population is quite interesting. This finding has recently been replicated in an ongoing study of *E.coli* O157:H7 infection in Scotalnd (conducted in the Department of Medical Microbiology by Professor Poxton and colleagues). The most logical explanation for the presence of antibody against *E.coli* O157:H7 in the gut of healthy Scottish people is the presence of VT negative O157 strains. This organism is widespread in Nature and can easily be harboured by healthy people (Professor Poxton, personal communication).

This preliminary observation is fascinating and obviously warrants future studies. It is to be hoped that an effective oral *E.coli* vaccine may be developed targeting a wide range of pathogenic *E.coli* including *E.coli* O157:H7 by using LPS core as an antigen.

Chapter 6

Disease influences on intestinal humoral immunity

Section 1: Inflammatory Bowel Disease

Introduction

The term inflammatory bowel disease (IBD) encompasses two major pathological processes involving the human intestine, Crohn's disease (CD) and ulcerative colitis (UC). Both of these diseases are characterized by a chronic relapsing course in which there is recurrent tissue injury within the intestine mediated by the host's immune response.

In UC, the inflammatory process almost always involves the rectum and then extends proximally to a variable extent. Histologically, the abnormality is limited to the mucosa and superficial submucosa, and is characterized by a dense infiltrate of both acute and chronic inflammatory cells in the lamina propria. These cells can also localize to crypts forming micro-abscesses. The deeper layers of the bowel wall are characteristically not involved.

CD can present in a number of different ways, a reflection of its ability to involve any part of the intestine from mouth to anus and the extensive nature of the inflammatory process. This can include the deeper layers of the intestinal wall, with transmural inflammation being common. The cellular infiltrate is predominantly made up of lymphocytes and macrophages, and later can coalesce to form non-caseating granulomas in 40-60% of patients. These pathological features can produce superficial

and deep ulceration and can also lead to collagen deposition and fibrosis (Smith & Jewell, 1996).

The aetiologies of UC and CD remain an enigma. Despite much research over decades, no infectious agents have yet convincingly been associated with IBD (Brandtzaeg,1995). Furthermore, the mechanisms of the spontaneous exacerbations and remissions are also undefined.

There are a number of suggestions of possible environmental or genetic causes including persistent infections, vasculitis, and immunological abnormalities, such as autoimmunity. It is thought that UC and CD have different aetiologies on the basis of differences in location, and the macroscopic and microscopic appearance of the disorders. In addition, different cytokine and immunoglobulin isotype profiles are thought to result from differences in mucosal immune activation (Sawyer and Ferguson, 1994). Despite all of this the disorders may have very similar presentations and precipitants of clinical relapse (Fiocchi,1998).

Much recent research has indicated the importance of the interaction between luminal antigens and the mucosal immune system as the mechanism of relapse in IBD. The suggestions were based on the findings: firstly, in postoperative recurrence of CD, the histological and immunological evidence of mucosal inflammation occurs within 8 days after exposing the neoterminal ileum to autologous luminal content (D'Haens *et al*, 1998); secondly, increased intestinal permeability heralds relapse in CD, while normal permeability predicts a long-term remission (Arnott, 1998, Wyatt *et al*, 1993). Intestinal infection, non-steroidal anti-inflammatory drug use, and dietary indiscretions are known causes of relapse in CD. All of these cause increase in intestinal

permeability across the epithelial cell layer or have the potential to do so (Bjarnason *et al*,1995). Thirdly, normal resident luminal bacteria provide the dominant stimulus for experimental colitis, as demonstrated by absence of colonic inflammation in genetically engineered rodents raised in germ-free conditions (Sadlack *et al*, 1993; Rath *et al*, 1996; Dianda *et al*, 1997). Further evidence to support this hypothesis was seen with the rapid induction of colitis when adult HLA-B 27 transgenic rats (Rath *et al*, 1996) or IL-10 deficient mice (Sator, 1998) were exposed to specific pathogen-free enteric bacteria. Defined flora studies show that *Bacteriodes* species preferentially induce colitis in HLA- B 27 transgenic rats (Rath *et al*, 1996); and finally, in some patients with IBD, antibiotic treatment may be effective (Sator, 1998).

An earlier study of circulating antibodies in serum of IBD patients initially proposed that there were high titres against enterobacteria. These antibodies were also cross-reactive with colonic mucins (Thymer *et al* , 1969). Other studies showed that serum titres of these antibodies were not significantly different from those of the controls (Heddle & Shearman, 1979; Lanngercrantz *et al*, 1966; Carlsson *et al*,1977). Similarly, the theory of enterobacterial antigen specific activation of peripheral blood lymphocytes (Bull & Ignaczak, 1973) was not confirmed (Bartnik *et al*, 1974). It is also recognised that circulating immunoglobulin specificities may not be representative of locally produced mucosal immunoglobulins. The main immunoglobulin produced in the intestinal mucosa of normal subjects is IgA. It has been confirmed by immunohistochemistry and cultured mucosal lymphocytes as well as with intestinal lavage that in patients with active UC or CD, IgG production is dramatically increased (O'Mahony, 1990; Brandtzaeg *et al*, 1974; Scott *et al*, 1986; Ruthlein *et al*, 1992) reflecting an abnormal mucosal response.

In this chapter I wanted to test the hypothesis that there is an abnormally increased mucosal immune response against non-pathogenic commensal intestinal bacteria in active CD and UC. This was to determine whether the increased IgG response was specific for commensal bacteria hence, EndoCAb antibody was measured.

Furthermore, in contrast to IgG, the results of studies of IgA in IBD are conflicting. It has been found in several studies that there is decreased synthesis of IgA in IBD patients (McDermott *et al,* 1986; Verspaget *et al*, 1988). Conversely, Badr-el-Din *et al* (1988) found a four-fold increase in IgA production by cultured colonic biopsies from patients with UC compared to controls.

I therefore decided to study the humoral immunity in IBD. There is great deal of literature to suggest that groups of cytokines play a key role in controlling local and systemic immunity. Proinflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) are preferentially produced by monocyte-macrophages and production increases in gut inflammation (Fiocchi, 1996). Accordingly, I tried to measure two cytokines (viz. IL-1β, IL-6) as well as cellular mediators [e.g. Granulocyte elastase (GE), a proxy marker of luminal neutrophilia] in IBD with carefully selected, well-matched groups of patients with active disease.

Results

Immunoglobulins and anti-bacterial antibodies in WGLF

Figure 6.1 depicts the total IgA concentration in WGLF from patients with moderate to severe IBD. The results showed that there was a significant difference in total IgA concentration in WGLF between patients with active UC and CD. The median and (range) values of total IgA in controls were 39 (18-82) μg/mL. In active CD, the median value of total IgA was 28 (12-50) μg/mL, which was significantly lower than that of controls (p<0.03). On the other hand, in active UC, the median value was 52 (29-100) μg/mL, which was higher than the controls but not significantly different (p=0.15). When compared with active CD, the total IgA in WGLF was significantly higher in active UC (p<0.003).

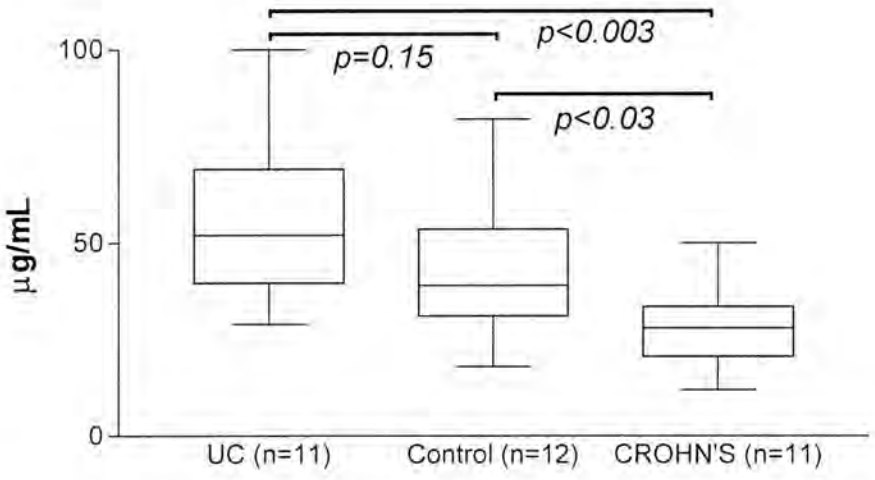

Figure: 6.1 Total IgA in WGLF from patients with active IBD and controls

The results of IgA antibody determinations against endotoxin core 'cocktail' antigens are shown in Figure 6.2. There were significant differences in IgA EndoCAb levels between active IBD and controls (Figure 6.2). In the control group, the median (and range) concentration of IgA EndoCAb in WGLF was 1 (0.5-4) MU. The median values of IgA EndoCAb were significantly higher in both UC and CD than that of controls. These values were 3 (1-8) MU (p<0.004), and 7 (1-23) MU (p<0.005) respectively. As can be seen from figure 6.2, the median value of IgA EndoCAb in CD is higher than that in UC, but they are not statistically different (p=0.21). However, there was good correlation observed between total IgA and IgA EndoCAb in CD (r=0.68, p<0.021) (Figure 6.3), but not in UC (r=0.5, p<0.13) (data not shown).

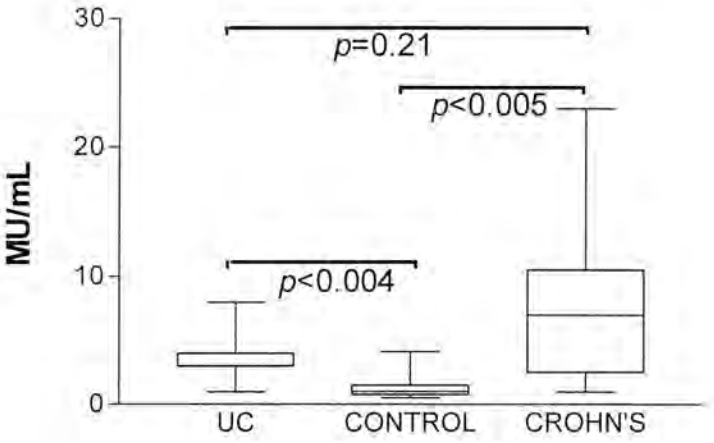

Figure: 6.2 IgA EndoCAb in WGLF from patients with active IBD and healthy controls

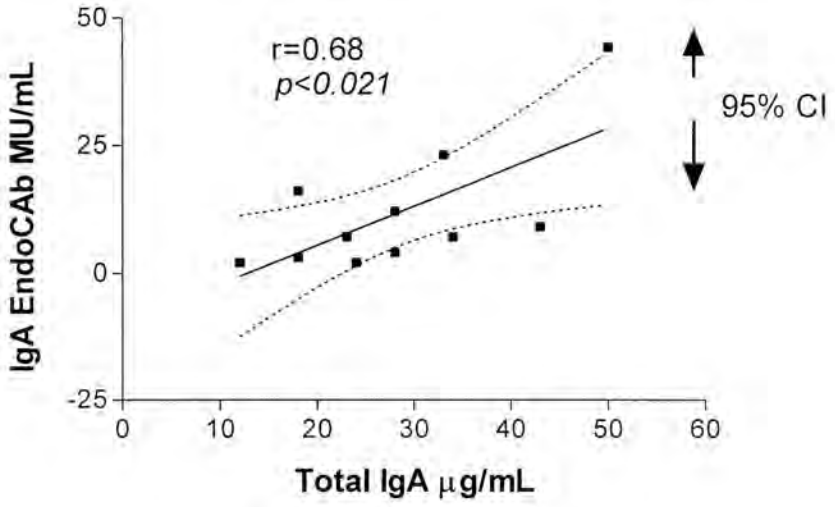

Figure:6.3 Correlation between IgA EndoCAb and total IgA in WGLF from patients with active Crohn's disease.

The levels of IgG antibody against endotoxin core 'cocktail' antigens in WGLF were similar to those for IgA EndoCAb. Unlike IgA EndoCAb, there was a significant difference in IgG EndoCAb levels between active UC and CD (Figure 6.4). The median (and range) concentration of IgG EndoCAb in WGLF in controls was 0.045 (0.019-0.2) MU/mL. In active UC and CD, the levels of IgG EndoCAb significantly increased (p<0.007 and p<0.001) when compared with controls, which were 0.14 (0.07- 0.47) MU and 0.29 (0.02-0.79) MU/mL respectively.

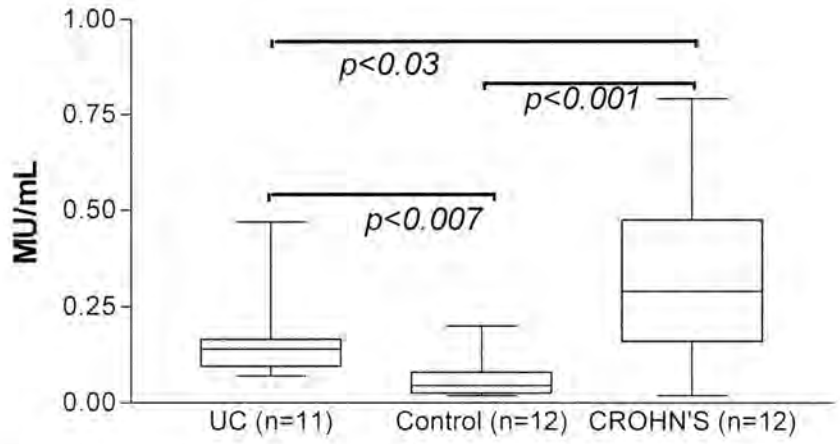

Figure: 6.4 IgG EndoCAb in WGLF from patients with active IBD and controls

A positive correlation was found between total IgG and IgG EndoCAb in WGLF from patients with both UC (r=0.83, p<0.003) and CD (r=0.7, p<0.03) (Figures: 6.5 and 6.6).

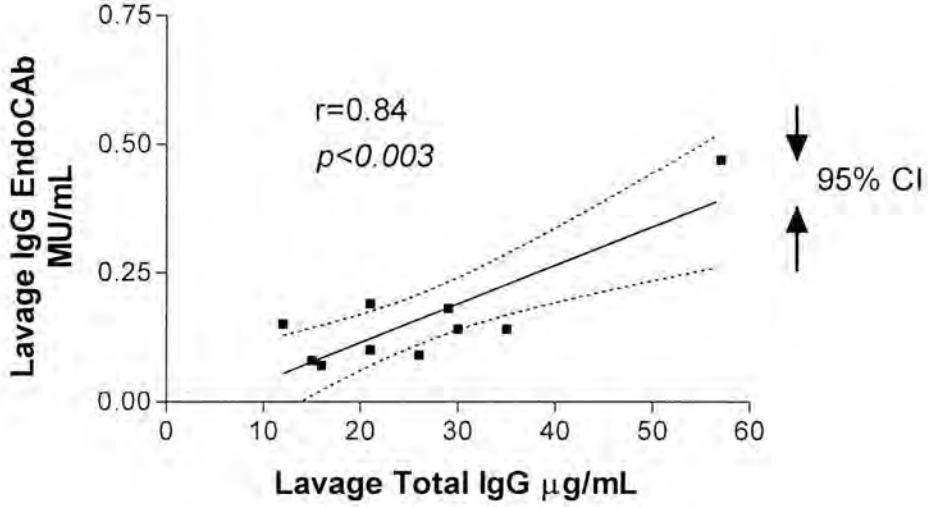

Figure: 6.5 Correlation between total IgG and IgG EndoCAb in WGLF from patients with UC.

Food antibodies

IgG antibody against ovalbumin in WGLF is undetectable in controls (Figure 6.7). Levels were higher in patients with active IBD. There were significant differences in anti-Ova IgG antibody between patients with active UC and CD ($p<0.02$).

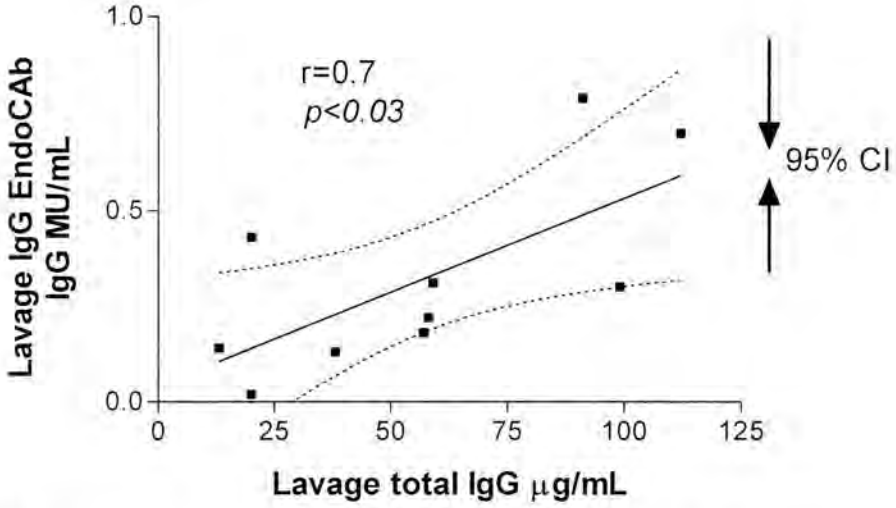

Figure:6.6 Correlation between total IgG and IgG EndoCAb in WGLF from patients with Crohn's disease.

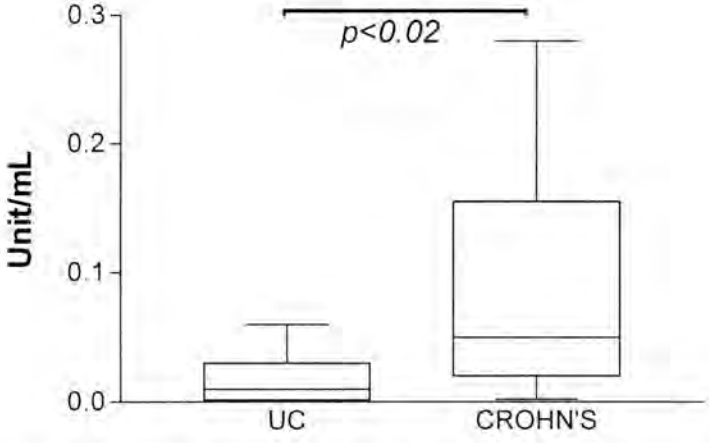

Figure: 6.7 Anti-ovalbumin IgG antibody in WGLF from patients with active IBD.

Serum antibodies:

The levels of IgG EndoCAb in serum are shown in figure 6.8. In the control group, the median (and range) concentration of IgG EndoCAb in serum was found to be 200 (103-318) MU/mL. In CD, the levels of this antibody were significantly increased (p<0.003), while in UC, the antibody levels were found to be significantly decreased (p<0.004) when compared with controls. The antibody levels in CD and UC were 279 (148-595) and 79 (18-224) MU/mL respectively.

The serum level of IgG EndoCAb did not correlate with IgG EndoCAb in WGLF.

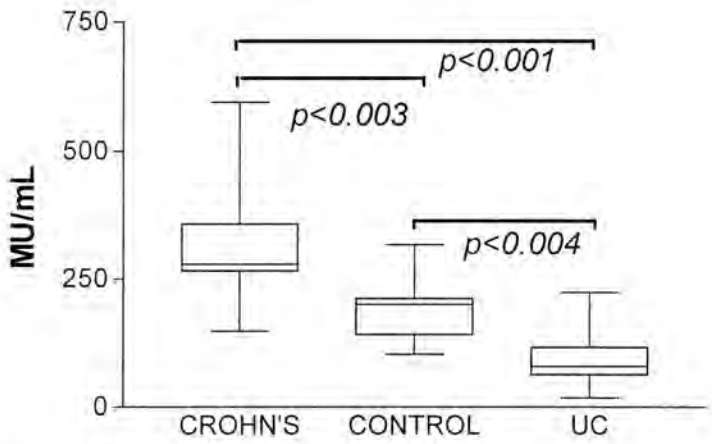

Figure: 6.8 IgG EndoCAb in serum from patients with active IBD and controls.

Cytokines

Figure: 6.9, summarises the results of IL-1β in WGLF. Out of 12 controls , 10 had no IL-1β detectable in WGLF. In the other 2 controls the IL-1β levels were 11 and 13 pg/mL. These two cases have already been discussed in chapter 4. The median (and range) value of IL-1β in WGLF in CD and UC were 496 (184-1200) and 120 (22-1200)

pg/mL. There was a significant difference in IL-1β concentration between these two patient groups ($p<0.03$).

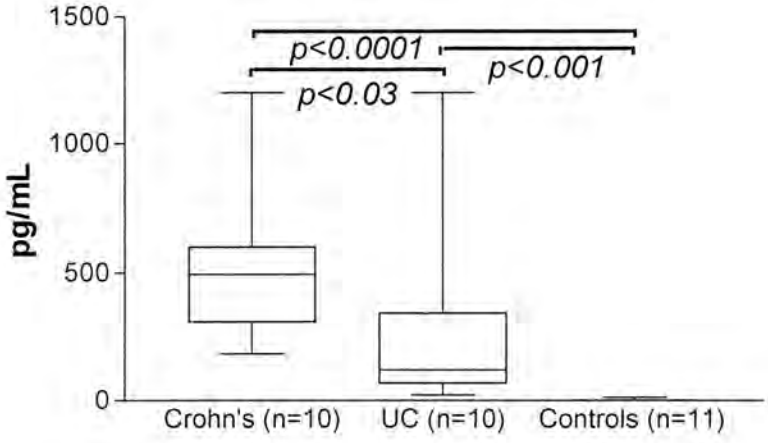

Figure: 6.9 IL-1β concentration in WGLF from patients with active IBD and controls

Cellular mediators

Figure: 6.10 illustrates the correlation between granulocyte elastase (GE), a marker of luminal neutrophilia and IgG in WGLF in patients with UC. There was no such relationship observed in CD (data not shown).

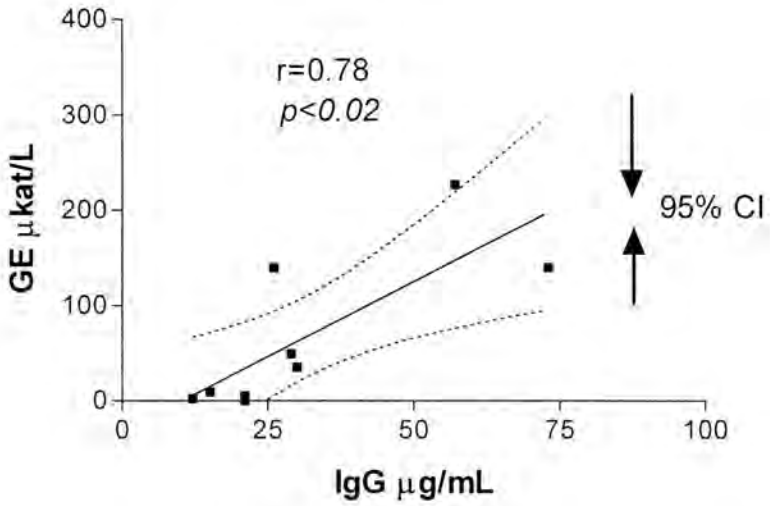

Figure 6.10 Correlation between IgG and GE in WGLF from patients with active UC

Serum IL-6 concentration was significantly higher in active CD when compared with controls (p<0.04) (Figure: 6.11). The level was also higher than UC but did not reach statistical significance at the 5% level (P<0.054). No difference was observed in serum IL-6 level in UC when compared with controls.

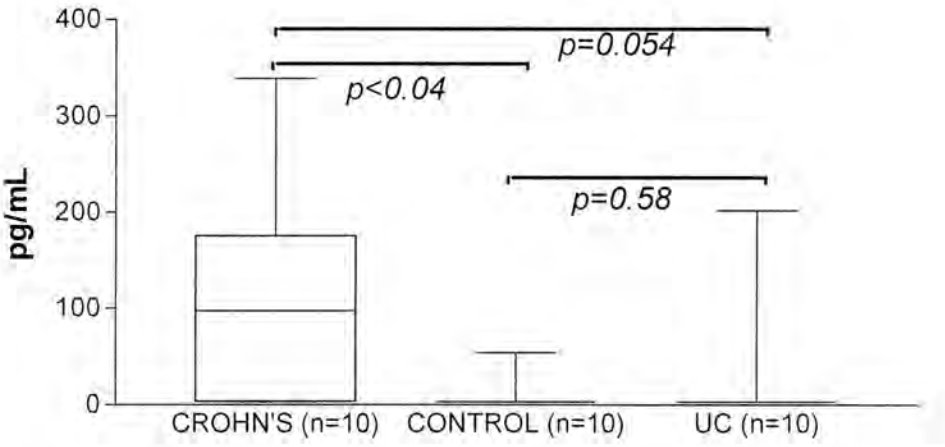

Figure:6.11 Serum IL-6 concentration in patients with active IBD and controls.

Discussion

The intestinal mucosa has two opposing functions: as a barrier to exclude numerous harmful antigens from micro-organisms and food, and to transfer antigens, a function essential for evoking specific responses (Sanderson and Walker, 1993). It is quite possible that primary mucosal immunodeficiency is present in the intestines of IBD patients, thus allowing agents to penetrate the intestinal mucosa, and subsequently initiate both a local inflammation and a heightened systemic immune response (MacDermott *et al*, 1981). The findings of heightened mucosal humoral responses against bacterial and food antigens in CD and UC from this study lend further support to the hypothesis.

Several earlier studies have indicated that local over production of IgG together with a relative mucosal IgA deficiency may be essential for the tissue lesion in Crohn' s disease (Baklien and Brandzaeg, 1976; MacDermott, 1988; Brandzaeg *et al*,1989). The data on total IgA in this study substantiate that there is decreased IgA concentration in WGLF from patients with active CD compared to controls or UC.

Clearly, this complex area needs further exploration. Whether primary mucosal immunodeficency is responsible for the impaired barrier function, a credible factor in the pathophysiology of CD, is not clear . Furthermore, the debate continues as to whether the dysfunction is a primary aetiological factor in CD or merely an epiphenomenon of the inflammatory process of the disease. There is evidence to support the concept that the functional derangement of macromolecular uptake is due to an intrinsic defect in the epithelial cells (Hollander *et al*,1986; Katz *et al*,1989 and May *et al*, 1993), and consequently gut permeability tests might predict CD before the

onset of serious symptoms. Conversely, other observations have suggested that the barrier disorder is secondary to inflammation (Katz *et al*, 1989; Howden *et al*,1991), in which case its severity could be a useful indicator of clinical activity in CD and a predictor of relapse (Howden *et al*, 1991; Wyatt *et al*,1993). However, some studies found normal permeability in active CD (Howden *et al*,1991; Ruttenberg *et al*,1978). In either case, this study has shown that there is abnormally high levels of both IgA (Figure: 6.2) and IgG (Figure: 6.4) antibodies against enteric bacterial antigens. The IgG antibody against a food antigen (Figure: 6.7) was also significantly higher in CD than UC . The heightened systemic immune response in CD is clearly supported by the findings of the high IgG EndoCAb (Figure:6.8) and IL-6 (Figure:6.10) levels in serum. It is quite probable that a leaky gut would enable enteric antigens to penetrate the mucosa to an increased extent, and the capacity for removal or degradation of this antigenic load by the macrophage or neutrophil defences might then be exceeded. The inability of macrophages to ingest the foreign material may result in a chronic granulomatous inflammation (IL-1β result is supportive, Figure: 6.9). It has been shown by culturing lamina propria mononuclear cells from patients with IBD that when the mononuclear cell population was depleted of macrophages, IL-1β activity was appreciably reduced (Smith & Jewell, 1996). When the secretory immune mechanisms are insufficient to exclude the antigen load from the gut lumen, a compensatory mucosal IgG response ensues. This may cause a local inflammatory reaction (Brandtzaeg,1987). This concept is supported by the following study findings: a) restoration of gut immunological barrier by promotion of IgA immune response in patients with CD by oral bacteriotherapy with *Lactobacillus* GG (Malin *et al*,1996). b) faecal stream diversion (which reduces bacterial antigenic load) may determine the recurrence of CD (Rutgeerts *et al*, 1991); c) remission of CD by parenteral nutrition (

Kushner *et al*, 1986)], or by elimination of dietary antigen when treating patients with elemental diet (Saverymuttu *et al*, 1985) or exclusion diets (Jones *et al*, 1985).

As has already been mentioned, CD and UC both may have very similar presentations and precipitants of clinical relapse but it is thought that they should be considered as completely separate diseases (Ferguson, 1996). Several distinguishing features between these diseases have been described elsewhere (Sawyerr & Ferguson, 1994; Fiocchi, 1998); such as, the different subclasses of IgG secreted in CD (IgG1, IgG2) and UC (IgG1 and IgG3). In this study, there was a significantly higher IgG antibody response against enteric bacterial antigens observed in both CD and UC compared to controls (CD>UC, Figure:6.4). This antibody (IgG EndoCAb) correlated well with the total IgG in WGLF (Figures: 6.5 and 6.6). Whether this antibody is the result of increased mucosal production or merely a serum leakage, is not yet clear. I will discuss more about this issue in chapter 7. However, it is of note that I have not assayed the subclasses of IgG.

The observation of higher total IgA in WGLF and lower IgG EndoCAb in serum from patients with UC compared to CD and controls (Figures: 6.1 and 6.8) needs further explanation. Previous studies showed that the production of IgA in the colonic mucosa of patients with UC were substantially raised (Bader-el-Din,1988; Danis,1984). There was a preferential increase in IgA1 subtype secretion. That was supported by the increased number of IgA1 cells in the colonic mucosa (Kett & Brandtzaeg,1987). There is further evidence of defective immunoglobulin production and hyporesponsiveness of peripheral blood lymphocytes in patients with UC (Danis & Heatley, 1983; Fiocchi, 1998). This evidence suggests that there is an unrestrained immune response in the intestinal mucosa of CD directed against the normal flora. There is also an IgG-

mediated immune response against normal colonic flora in the mucosa of patients with UC, but at a relatively lower defence than that in CD. This may suggest that IgG produced in UC is also directed against other antigens, such as epithelial cell proteins (Das,1977).

In UC, the inflammatory process is strictly limited to the superficial layer of colonic mucosa. It has already been shown from this laboratory that luminal neutrophilia (measured by GE) is a feature of active UC but not small bowel CD (Handy *et al*, 1996). This study confirms the previous findings (Figure:6.11).
Significantly increased IL-1β in WGLF and IL-6 concentrations in serum were found in IBD compared with controls. The increase is more pronounced in active CD compared to active UC (Figures: 6.9 & 6.10). This is in accord with the findings of other studies (Mahida *et al*, 1989; Smith & Jewell,1996). IL-6 is osteopenic and thought to be responsible for the reduced bone mineral density seen in CD (Pollak *et al*,1998). Therefore increased IL-6 levels may explain the finding of low bone mineral density in Crohn's disease, which is not seen in UC, at diagnosis (Ghosh *et al*, 1994).

The impact of treatment with immunosuppresive drugs (for example prednisolone) may affect antibody dynamics both systemically and mucosally. However, most of the patients in both groups were on treatment. However, it has been found in a recent study that treatment with immunosuppressive drugs did not have an impact on the serological results in patients with IBD (Ruemmele *et al*, 1998). Further study should be done to see whether IgG EndoCAb in serum could be used as a reliable serological screening test which would have potential in identifying or predicting relapse in patients with IBD, especially CD, as other tests (e.g. the dual sugar permeability test) are cumbersome, time-consuming and lack specificity.

Section Two

Ileal pouch-anal anastomosis (IPAA)

Introduction

Ileal pouch-anal anastomosis (IPAA) is the operation of first choice following proctocolectomy in patients with UC and familial adenomatous polyposis (Merrett, 1997). These patients virtually have very little colon. At operation, the anus is retained and a pouch is formed from the distal 40 cm of the terminal ileum. The pouch acts as a storage reservoir for the output of the small bowel; it is joined to the top of the anal canal and thus evacuation is possible by the normal route, eliminating the need for a permanent ileostomy. In keeping with the same hypothesis discussed in section one of this chapter, I was interested to see if the humoral antibody responses varied in patients with UC and those who underwent surgery.

Up to 30% of patients with UC will require removal of their colon and rectum (Thompson-Fawcett & Meadows,1998). Soon after IPAA, 90% of these patients reported an excellent quality of life; indeed, patients with pouches had the same quality of life as matched controls who had undergone cholecystectomy (Tompson-Fawcett & Meadows,1998). However, one of the long-term complications of IPAA is pouchitis which is the most common cause of pouch dysfunction. The incidence is 15-20% (Merrett, 1997) and the aetiology of pouchitis is unclear. Stasis and bacterial overgrowth have been implicated because stasis is the main functional difference and metronidazole, an anti-anaerobic bacterial agent is therapeutic in pouchitis. On the

other hand, it has been postulated that pouchitis represents a recurrence of UC, because pouchitis is seldom seen in patients who have a pouch for familial adenomatous polyposis (Thompson-Fawcett & Meadows,1998).

The bacteria associated with the colonic mucosa are predominantly anaerobic. The majority belong to the genus *Bacteroides* (Poxton *et al*, 1997). In particular, *B. fragilis* is one of the major components of the mucosal flora and is the most commonly isolated species from patients with gut-associated abdominal sepsis (Poxton *et al*, 1997).

To test the hypothesis, I decided to measure EndoCAb and anti-*B. fragilis* LPS IgA antibody responses in WGLF from patients with UC (without surgery) and those who had IPAA. The results were compared with those obtained from healthy controls from Edinburgh and Dhaka. In view of identification of the enterotoxigenic *B. fragilis* as a diarrhoeal pathogen in children in Bangladesh (Sack *et al*,1994), the Dhaka control group was included so that I could determine the variability of anti-*B fragilis* LPS antibody levels in healthy controls from two geographically distinct populations in Dhaka and Edinburgh.

Results

EndoCAb antibodies

Figure: 6.12 illustrates the levels of IgA EndoCAb detected in WGLF from patients with UC and IPAA. In UC patients, the concentration of IgA EndoCAb was significantly higher than that of patients with IPAA (both Pouch and Pouchitis group) ($p<0.003$ and $p<0.004$ respectively). There was also a significant difference in IgA EndoCAb levels within the IPAA patients ($p<0.008$).

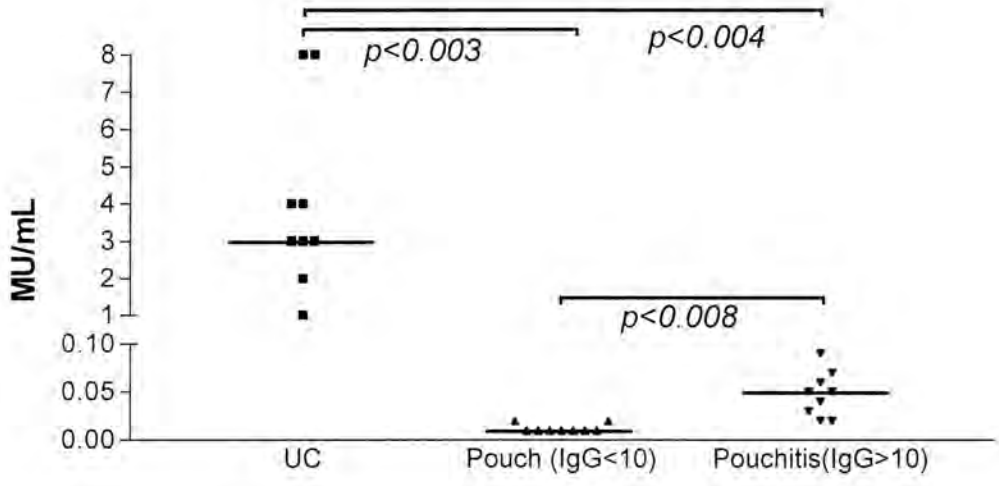

Figure: 6.12 IgA EndoCAb in WGLF from patients with UC and IPAA.

The serum IgA EndoCAb results are presented in figure 6.13. The results were similar to those observed with WGLF. There was a significant difference in serum IgA EndoCAb concentrations between patients with UC and IPAA ($p<0.001$). The serological control values were similar to the values of UC patients. There were, however, no differences observed within the IPAA patients (Figure 6.13).

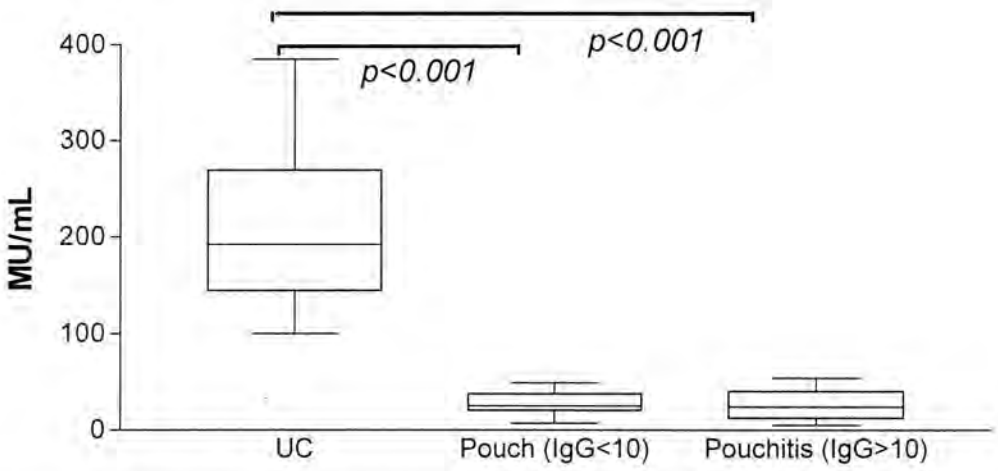

Figure: 6.13 IgA EndoCAb in serum from patients with UC and IPAA.

Anti-*Bacteroides fragilis* (*B.fragilis*) LPS IgA antibodies in WGLF.

Figure 6.14 shows the distribution of anti-*B.fragilis* IgA antibody in the healthy control groups from Edinburgh and Dhaka. Both the median and range values of anti-*B.fragilis* IgA antibody tended to be higher in the Dhaka controls than in the Edinburgh controls. However, the difference was not statistically significant (*P*=0.16).

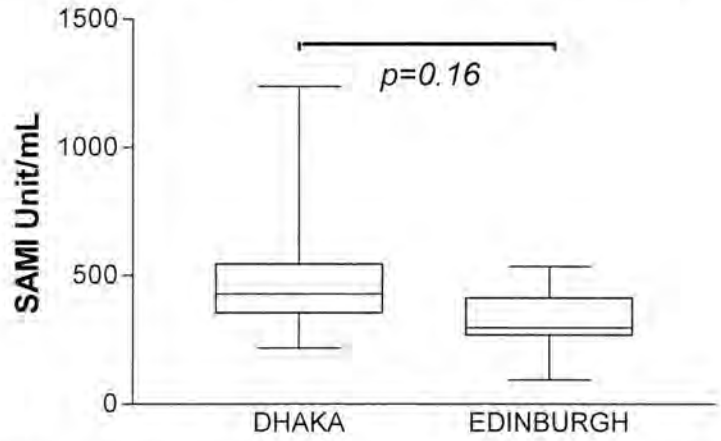

Figure:6.14 Anti-*B fragilis* LPS IgA antibody in WGLF from healthy volunteers.

The corresponding levels of *B. fragilis* antibody found in patients with UC and IPAA are shown in (Figure:6.15). There was a significant difference in anti-*B.fragilis* IgA concentration in patients with UC compared with pouch (P<0.004). Similarly, there was also a significant difference observed between patients with pouch and pouchitis (p<0.02). However, no significant difference was found between patients with UC and pouchitis (p=0.35). The median and range values of anti-*B.fragilis* IgA in patients with UC were higher than that of control groups from Edinburgh but this was not statistically significant (p=0.23). Likewise, no significant difference was observed between controls and patients with pouch (p=0.11).

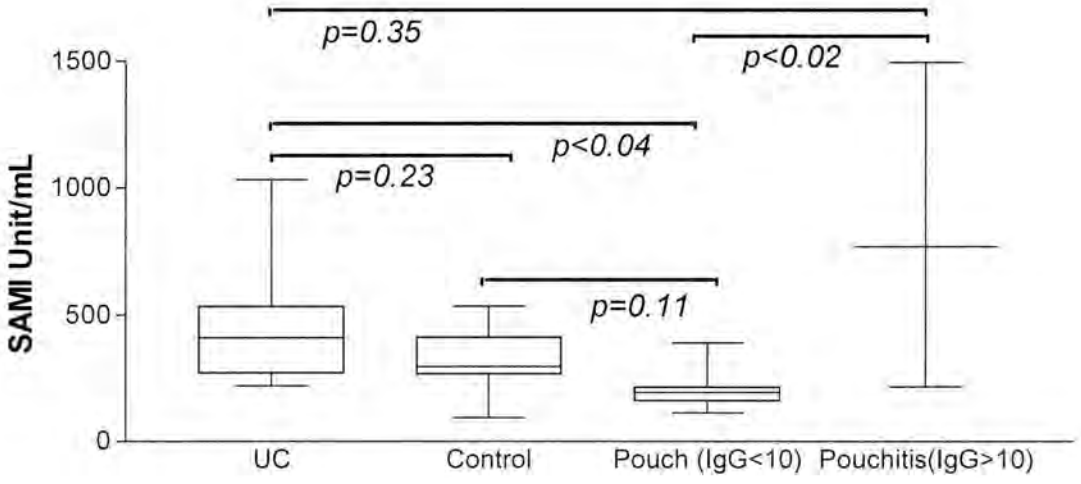

Figure: 6.15 Anti-B.fragilis LPS IgA antibody in WGLF from patients with UC, IPAA and controls.

Discussion

The higher IgA antibody responses against gut bacterial antigens in UC have already been discussed in the previous section of this chapter. It is known that the production of IgA, especially IgA2, increases along the intestinal tract with distance from the stomach (Mestecky *et al*,1989). The lower levels of IgA EndoCAb in patients with pouch was an expected finding, because patients with pouch have virtually no colon These factors may be responsible for the lower detection of IgA EndoCAb in a normally functioning pouch. However, in pouchitis, the higher IgA EndoCAb response supports the theory further that there is an abnormally increased mucosal immune response against non-pathogenic commensal intestinal bacteria in UC. It also lends support to the theory that pouchitis represents a recurrence of UC (Merrett,1997). However, these results would have been more meaningful with more patients. In addition, it would be informative to do other assays, such as total IgA, IgG EndoCAb in WGLF and IgG EndoCAb in serum. However, due to paucity of sample size as well as specimen I could not perform those assays.

The serological responses of IgA EndoCAb were similar both in UC and in control groups and are significantly higher than those values for both the pouch and pouchitis groups. This phenomenon has also been observed by others previously (Kilhamn *et al*, 1998). Kilhamn *et al* suggested that the weaker serum response seen in colectomized patients may be due to depressed immune cells that present to the systemic immune systems. These results lend further support to the idea of defective immunoglobulin production and hyporesponsiveness of peripheral blood lymphocytes in patients with UC (Danis & Heatley, 1983; Fiocchi, 1998).

It is interesting to note that there was no significant difference observed in anti-*B.fragilis* IgA antibody response between healthy controls from Dhaka and Edinburgh. In the Dhaka group, however, the antibody response was somewhat higher than that of Edinburgh groups. This may be due to higher incidence of diarrhoea caused by toxigenic *B.fragilis* (Sack *et al*,1994).

The anti-*B.fragilis* IgA antibody response was higher in UC patients when compared with Edinburgh controls. The value did not reach statistical significance, probably due to inadequate sample size. Significant differences were observed when antibody levels were compared with those from patients with pouch. This result is quite expected as pouch patients are colectomised and *B.fragilis* is predominantly a colonic bacterium. The noteworthy finding was that there was a significant difference in anti-*B.fragilis* IgA antibody responses between patients with pouch and pouchitis. In addition, there was no difference in anti-*B.fragilis* IgA antibody responses between UC and pouchitis. These results suggest the role of colonic bacterial flora in perpetuation of UC or pouchitis. Alternatively, this may also mean that breakdown of tolerance towards these organisms may be responsible for UC or pouchits. This concept is further supported by the fact that metronidazole (a chemotherapeutic agents that is very effective against Gram-negative anaerobic bacteria) is the one effective mainstay therapy in pouchitis (Thompson-Fawcett & Meadows, 1998).

In fact, pouch is a 'man-made' colonic reservoir. There are some studies suggesting that in a pouch, there may an overgrowth of colonic bacteria due to stasis. It has also been found that there is an increased ratio of anaerobes to aerobes with higher concentrations of *Bacteriodes* and *Bifidobacterium* species(Merrett, 1998).

The role of *bacteroides* bacteria in UC is not yet clearly defined. In a recent study, it has been shown that *Bacteroides thetaiotaomicron,* was isolated from biopsy specimens of all 3 patients with active UC but from only 4 of the other 18 samples from non-inflamed colonic mucosa (Poxton *et al*, 1997). Whether this is a true association is not yet known. However, the same research group had shown previously that the IgA antibody responses to at least two of the most common *Bacteroides* (*B.fragilis* and *B.vulgatus*) were directed largely toward a common epitope of the lipopolysaccharide (Poxton *et al*,1995) which may also be shared by other species of the genus. These workers also suggested that this may be a mechanism for protecting the mucosa from the potentially inflammatory molecules produced in the colon, thereby maintaining homeostasis. It is quite probable that there may be breakdown of this homeostasis in UC.

In conclusion, despite several limitations, some important pieces of information have emerged from this study. It is recognised that the role of *Bacteroides* in UC warrants further investigation. Apart from bacterial factors in the pathogenesis of pouchitis, other factors for example short chain fatty acid deficiency or mucosal ischaemia may have some role, and these also merit further study.

Chapter 7

Evidence of Local Production of IgG

Introduction

The accurate assessment of activity and extent of disease in UC and CD is crucial in the rational management of these conditions (Summers,1979). Objective parameters of disease activity are necessary for the assessment of the effects of drug therapy (Hodgson,1997) and facilitate the comparison of data collected in multi-centre trials on new therapeutic strategies in these conditions. Various scientific approaches to the measurement of disease activity have been proposed (Singleton,1987; Camilleri & Proano,1989), although none could be considered as a 'gold standard'. Some are heavily weighted towards subjective parameters which are variable. Laboratory tests such as ESR, platelet count, and the estimation of acute phase proteins, are useful, but may be normal in active IBD, particularly UC and small bowel CD (O'Mahony *et al*,1991). Studies from this unit have shown that total IgG in WGLF is an excellent measure of disease activity in IBD (O'Mahony *et al*, 1991; Choudari *et al*,1993). The authors suggested that the high IgG value in WGLF is derived from plasma leakage across the inflamed mucosa. The reasons for this conclusion were: firstly, there is a high concentration of albumin in lavage fluid from patients with active IBD, and a positive correlation between albumin and IgG levels was found. Secondly, there is no mechanism as yet established for transport of IgG between the systemic and mucosal compartments.

However, several studies have shown that the number of IgG-containing plasma cells in the gut is greatly increased in IBD (Baklien & Brandtzaeg, 1976; Rosekrans *et al*, 1980). Also, intestinal mononuclear cells from isolated IBD patients spontaneously secrete high amounts of IgG (MacDermott *et al*,1981; Verspaget *et al*, 1988). Furthermore, as already discussed in chapter 6, I have found high IgG EndoCAb in

WGLF from patients with IBD. It is relevant to know the source of the IgG antibody, as this has inflammatory potential. It is well known that IgG can assist in the opsonisation of antigen, and that it may be involved in triggering inflammation and enhancing immune responses against infectious agents.

Therefore, I decided to study whether IgG in WGLF was plasma-derived or locally produced. What proportion of the total IgG in WGLF is derived from plasma? In this study, I used the results obtained from the study of patients with IBD presented in chapter 6. I have tried to interpret these results to elucidate the source of IgG on the basis of arithmetic calculations.

Calculation of plasma loss

Calculation of plasma loss per day was done using the formula used by Ferguson *et al* (1996) for the calculation of blood loss. The original formula was:

$$\frac{\textbf{WGLF Hb concentration (}\mu\textbf{g/mL) X Volume of lavage/d**}}{\textbf{Blood Hb concentration (g/L)}} = \text{mL of blood loss/d}$$

= mL of [blood - Haematocrit] = plasma lost /d

(** calculation based upon 'intake and output' data during lavage. 'd'= day)

I have deducted the haematocrit value from the total volume of blood which gives the total plasma lost per day.

It has been shown by a sequential lavage study that once the WGL specimens have become clear, it is essentially a perfusate (Sallam, 1994). The perfusion rate was generally 20 mL/min. The volume of lavage required for each patient was extrapolated

to daily output. The specific antibody that can be lost per day through inflamed gut was determined. Likewise, it was also possible to estimate the daily production of antibody, assuming all antibodies are locally produced in the gut. Allowance was made for reagents required for the processing of WGLF.

The following formula was used to calculate the relative coefficient of excretion (RCE) of antibody:

$$\text{RCE} = \frac{\text{(antibody in WGLF)/ (antibody in serum)}}{\text{(albumin in WGLF)/(albumin in serum).}}$$

As transport of albumin in to the gut lumen is entirely passive (albumin is not produced in the gut) (Jonard *et al*, 1984), the RCE is useful for expressing the secretion rate independent of serum protein concentration and the net fluid transcolonic movement (Delecourt *et al*, 1995; Jonard *et al*, 1984). If a protein is passively transported from plasma to the colonic lumen, the RCE value for this protein is near 1 and inversely related to its molecular weight. An RCE value of greater than 1 indicates partial or total local gut synthesis and/ or facilitated transport (Delacroix *et al,* 1982).

I have used the results of two categories of specific antibody (EndoCAb and anti-cholera toxin antibody) responses in both the serum and WGLF from patients with active IBD as presented in chapter 6.

Results

It is essential to determine whether the estimated plasma loss is well correlated with the blood loss in patients with active IBD. Figure: 7.1 shows an excellent correlation (r=0.98) between estimated plasma loss and haemoglobin in WGLF from patients with active IBD.

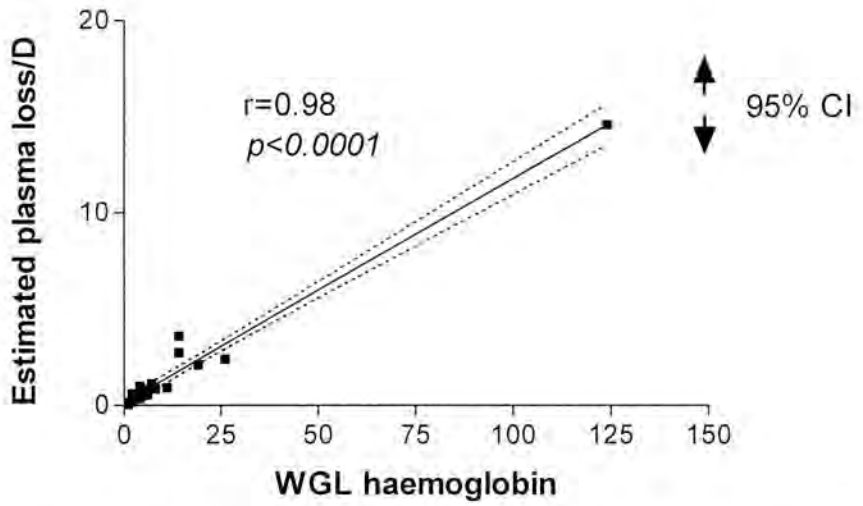

Figure: 7.1 Correlation between estimated plasma loss/day and haemoglobin in WGLF from patients with active IBD.

The estimated daily production and loss of IgG antibody against multiple gut bacterial antigens (EndoCAb) and cholera toxin in active UC are presented in figures 7.2 and 7.3. These results show that there was more specific antibody produced within the gut lumen than lost through the gut: 57% and 96% in cases of IgG EndoCAb and anti-CT IgG respectively. When compared with albumin, the RCE values for IgG EndoCAb and anti-CT IgG were 1.7 and 1.2 respectively (Figure: 7.4).

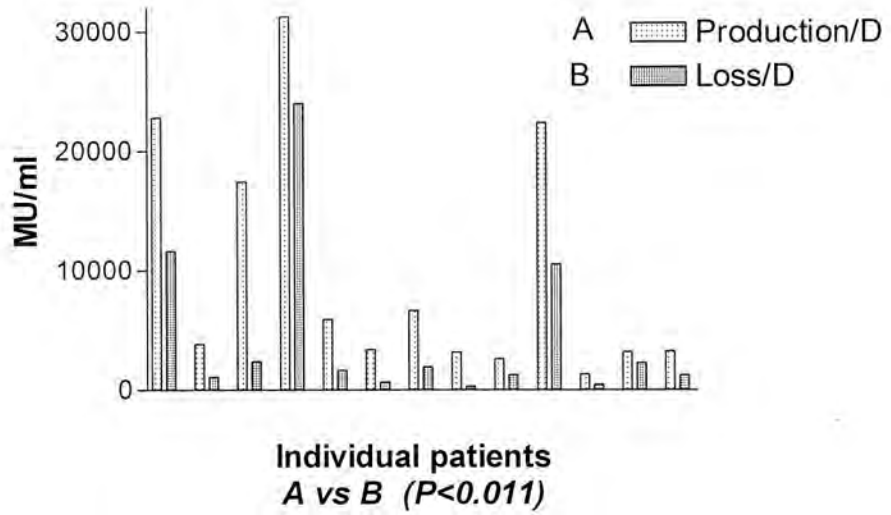

Figure: 7.2 Estimated IgG EndoCAb production and loss/day in patients with active UC.

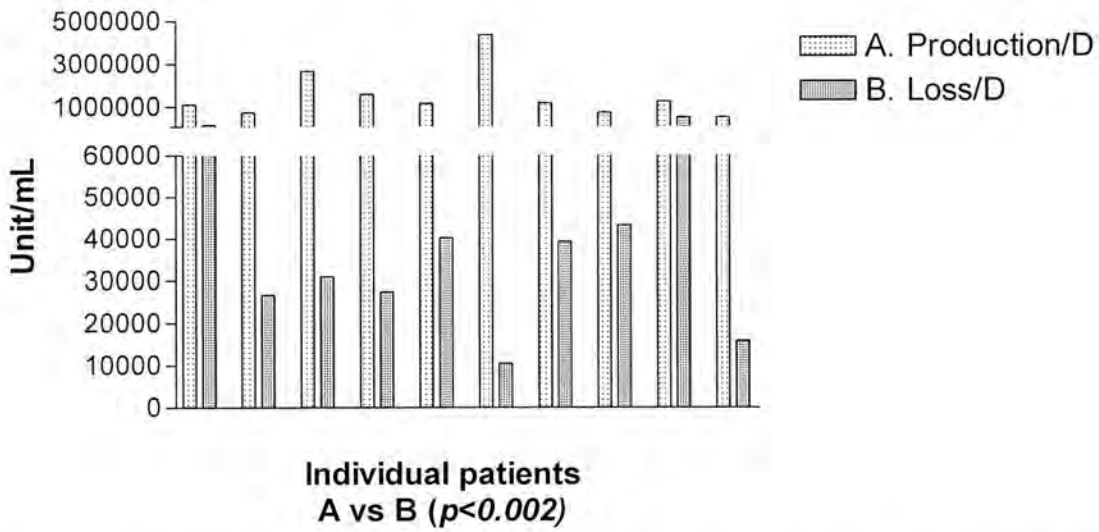

Figure:7.3 Estimated anti-CT IgG antibody production and loss/day in patients with active UC.

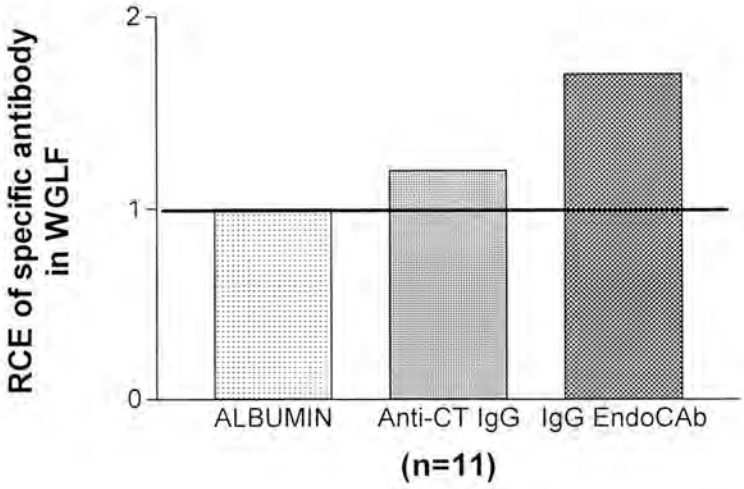

Figure: 7.4 Relative coefficient of excretion (RCE) of specific antibody in WGLF from patients with active UC.

Similarly, in active CD, the local production of specific antibody was also increased (Figures: 7.5 & 7.6). Both the IgG EndoCAb and anti-CT IgG were increased by 99%. The RCE values for IgG EndoCAb and anti-CT IgG were higher in active CD than active UC. These values were 1.8 and 2 respectively (Figure:7.7).

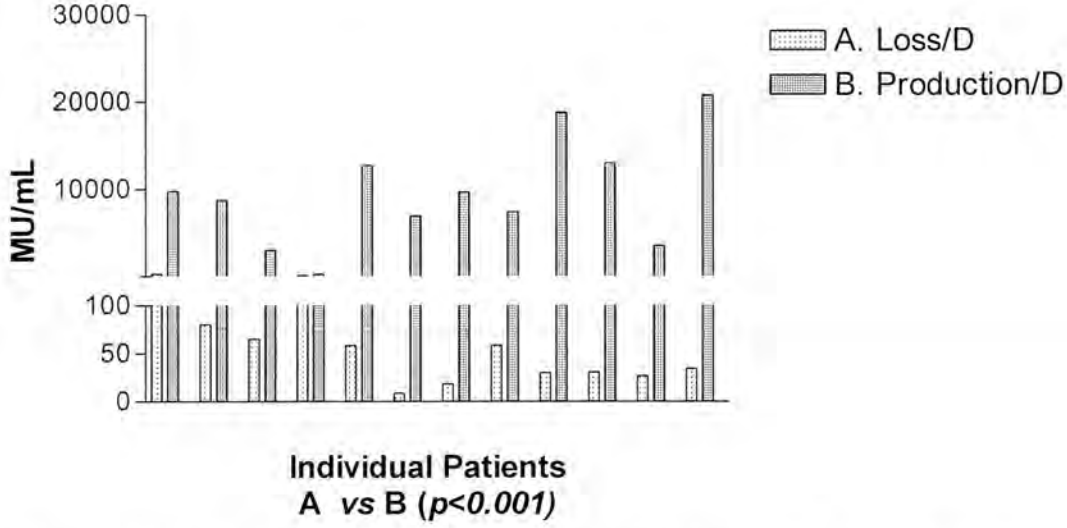

Figure: 7.5 Estimated IgG EndoCAb production and loss/day in patients with active Crohn's disease.

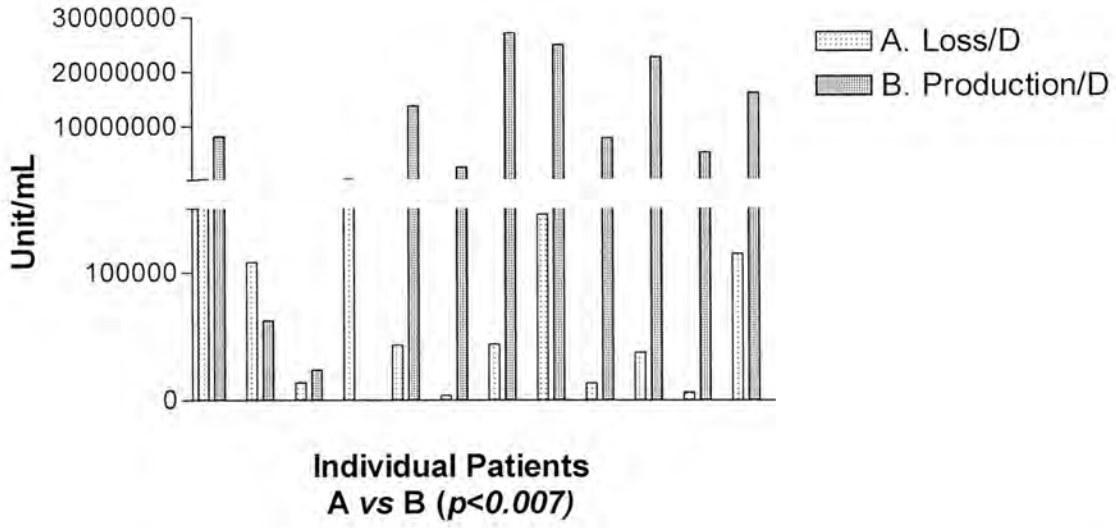

Figure: 7.6 Estimated anti-CT IgG antibody production and loss/day in patients with active Crohn's disease.

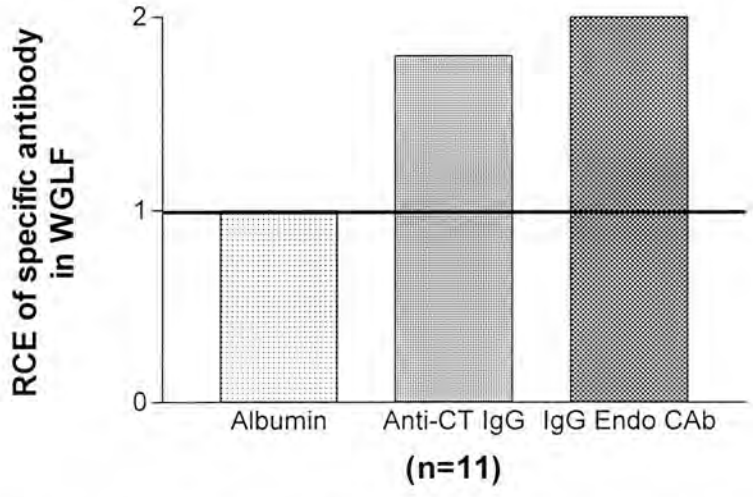

Figure: 7.7 relative coefficient of excretion (RCE) of specific antibody in WGLF from patients with Crohn's disease.

Discussion

This study demonstrates an increased local production of IgG antibody in the gut. It has been shown histologically by several investigators (Soltoft,1969; Brandtzaeg *et al*,1974; Karen *et al*,1984; Rosekrans *et al*,1980) that the number of IgG positive lymphocytes are increased in the inflamed mucosa (Scott *et al*,1983). IgG-producing cells constitute 3- 5% of the lymphocytes in the intestinal mucosa. In IBD, however, there is a dramatic increase in intestinal Ig- producing cell populations, particularly, IgG. It has been shown that the number of IgG-producing lymphocytes in IBD can be increased by up to 200 -fold. This increase appears to be dependent on the degree of inflammation (Brandtzaeg *et al*, 1985). In this study, the antigen-specific IgG antibody levels were found to be increased by 56-99% over the estimated loss through gut lumen (in patients with moderate to severe active IBD). The antibody production was higher in CD than UC (Figures: 7.6 & 7.7) as has been previously described and discussed in chapter 6. Macpherson *et al (*1996), found an increased IgG response against normal colonic flora in endoscopically obtained mucosal washings from patients with active IBD. The response was smaller in active UC than active CD. A major disadvantage of the endoscopic mucosal wash technique, compared with WGLF, is that the level of mucosal immunoglobulins only represents loss from an isolated defined area which is not necessarily representative of the whole gut. However, those investigators elegantly demonstrated a higher binding of mucosal IgG to commensal bacterial protein in patients with CD and UC than that found in serum. Furthermore, mucosal immunoglobin did not show significant binding to non-intestinal commensal bacterial proteins, whereas there was strong binding by serum IgG. Again, as has been discussed in chapter 4 of this thesis, despite higher serum IgG and higher albumin loss through gut there was no evidence of higher IgG amounts in

WGLF from the Dhaka control groups. These data all lend support to the postulated local production of IgG in patients with IBD.

The higher antibody response in CD merits further explanation. It has been well established that in CD there are activated T cells within the lamina propria (Breese *et al*, 1993; Mullian *et al*, 1992). The increased production of IgG specific for commensal bacterial antigens in active CD may reflect a Th1(proinflammatory response) from these activated cells (Liblau *et al*, 1995). It is thus conceivable that bacterial protein antigens are recognised by the T cell receptors which might result in such a profound immune activation leading to damage to epithelium. This may also explain the greater response seen in active CD compared with active UC.

In the present study, the estimated plasma loss correlates well with blood loss through the gut (Figure: 7.1). The results also show that there was greater plasma loss per day in active CD compared with active UC. Although the estimated plasma loss in this study is based on the formula for the calculation of daily blood loss through the gut, these results are in accordance with those of other studies (Macpherson *et al*, 1996; Saitoh *et al*, 1995).

Some important questions arise: firstly, what is the cause of increased mucosal production of IgG, or is there an increased population of IgG-bearing lymphocytes in IBD? Secondly, what are the implications of this increased local production of antibody? It has been shown previously that the secretory Ig responses (e.g. secretory IgA) apparently fail to cope with pathogenic agents in the diseased mucosa from patients with CD (Baklein & Brandtzaeg, 1976). Due to the failure of this 'first line of defence', an additional 'second line defence' seems to be established by local IgG

production (Brandtzaeg, 1987). This phenomenon has already been observed; it is recorded and discussed in chapter 6. It has been proposed that constant exposure of Peyer's Patches to environmental antigen stimulates B cells to proliferate and switch immunoglobulin isotypes in a vectorial manner (IgM → IgG →IgA) according to the order of genes on the chromosomes (Cebra *et al,*1982; Fuhrman & Cebra, 1981). Basically, T cells control the quality as well as the quantity of the antibody response (Elson,1985). It may be that continual antigen-driven division of B cells in the absence of signals from helper T cells for the terminal differentiation may result in increased IgG production instead of IgA in the gut. This 'compassionate' shift from IgA to IgG might dominate the local humoral immunity in IBD. Although this may be beneficial as a 'second line of defence', it may however, give rise to a deleterious reaction which can induce and maintain chronic inflammation (Brandtzaeg, 1995). It has been shown that increased IgG production is an early event in the pathogenesis of inflammation in CD and UC, and that the IgG may contribute to ongoing inflammation by its proinflammatory potency (Ruthlein *et al,*1991). It is well recognised that IgG, in particular IgG1, is a potent complement activator. In fact, both IgG1 and activated complement have been demonstrated in the epithelium in UC (Halstensen *et al*, 1990). Thus it seems likely that IgG and complement may contribute to persistence of chronic inflammation in IBD. As with IgG, it is unknown, whether complement components are derived from plasma leakage or locally produced by the activated macrophage in the gut. My results help to elucidate mechanisms that may be involved and this area merits further study.

Recently, it has been reported from an in vitro study that IgG can be transported across model human intestinal epithelial cells by the process of transcytosis (Christ *et al,*1997). By using the human cell line T84, the authors have demonstrated that there

is a pathway involving the Fc receptor FcRn within human intestinal epithelia that mediates the bi-directional transport of IgG across intestinal epithelia. The reported work is fascinating but needs to be confirmed.

Chapter 8

Intestinal Antibody Responses to Food Protein are Reduced in Subjects Who Have High Levels of Gut Bacterial Antibodies

Introduction

The environmental antigens from food and the indigenous microbial flora are in constant contact with mucosal surfaces and provide a continuous stimulus for the entire immune system. Although a common result of such stimulation is the induction of mucosal and systemic immunity, an alternative outcome is a state of unresponsiveness or tolerance (Husby *et al*, 1994). In view of the high antigenic load from the contaminated environment in healthy volunteers from Dhaka, the higher level of IgA antibody in the gut of the Dhaka group compared to the Edinburgh group (chapter 4) is of interest. Additionally, the higher IgA response may be related to non-specific immune activation resulting from the postulated high bacterial antigenic load.

Considering the above facts, I decided to test the hypothesis that, in developing countries, the drive for production of a high humoral response to bacteria would have the additional effect that antibodies to other gut antigens, such as foods, might be absent or of low titre.

To address this question, I decided to measure antibody responses against a food protein antigen in WGLF from healthy subjects both from Edinburgh and Dhaka. As egg is one of the regular food items in both countries, I chose ovalbumin as the test food antigen.

Results

Antibodies in WGLF

Figure 8.1 depicts the anti-ovalbumin IgA concentration in WGLF from the healthy subjects from Dhaka and Edinburgh. The median and (range) concentration of anti-ovalbumin IgA concentrations in WGLF in the Dhaka and Edinburgh groups were 0.33 (0.05-1.28) Unit/mL and 7.5 (2-37) Unit/mL respectively. There was a significant difference in anti-ovalbumin IgA concentrations between the Dhaka and Edinburgh groups ($p<0.001$).

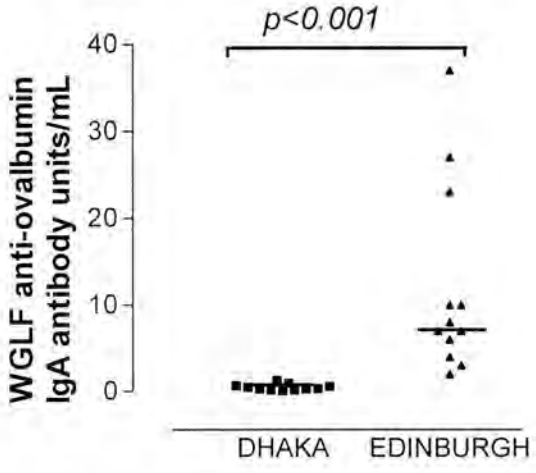

Figure: 8.1 Anti-ovalbumin IgA antibody in WGLF from healthy controls.

Similarly, there were significantly higher levels of anti-ovalbumin IgM antibody found in the Edinburgh groups ($p<0.003$) (figure 8.2). The median and (range) concentration of anti-ovalbumin IgM antibody in WGLF from healthy subjects from Dhaka and Edinburgh were 0.34 (0-4.2) Unit/mL and 3 (0-14) respectively.

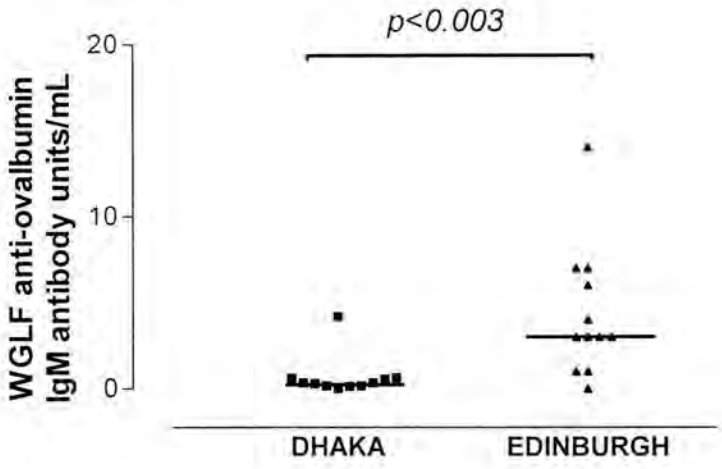

Figure 8.2 Anti-ovalbumin IgM antibody in WGLF from healthy subjects.

No anti-ovalbumin IgG antibody was detected in WGLF from either of the groups.

Serum antibodies

Figure 8.3 illustrates the anti-ovalbumin IgM antibody levels in serum from healthy subjects in Dhaka and Edinburgh. The median and (range) concentrations of anti-ovalbumin IgM antibody in controls from Dhaka and Edinburgh were 454 (83-620) Unit/mL and 33.5 (11-100) Unit/mL respectively. There was a significant difference in anti-ovalbumin IgM concentration between the two groups ($p<0.002$).

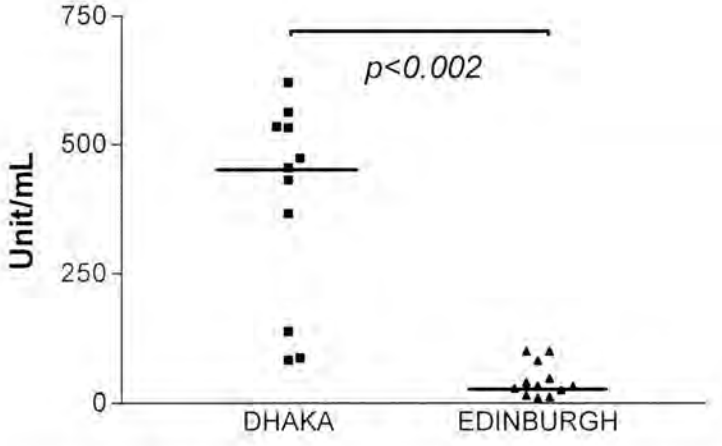

Figure: 8.3 Anti-ovalbumin IgM antibody in serum from healthy subjects.

The median and (range) values of anti-ovalbumin IgA in serum were similar in both of the Dhaka and Edinburgh groups. These values were 13(7-42) Unit/mL and 11(5-22) Unit/mL respectively (figure:8.4).

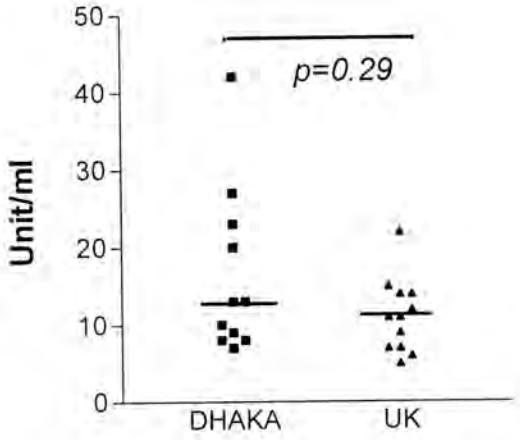

Figure 8.4: Anti-ovalbumin IgA antibody in serum from healthy subjects.

Likewise, there was no significant difference in anti-ovalbumin IgG antibody in serum in both the groups (figure:8.5). The median and (range) values of serum anti-ovalbumin IgG antibody in the Dhaka and Edinburgh groups were 11(4-19) Unit/mL and 17(6-87) Unit/mL respectively.

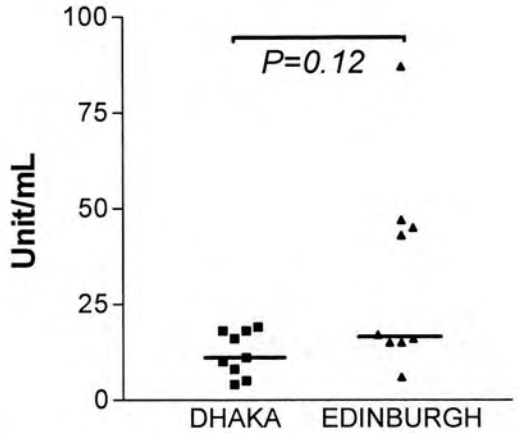

Figure: 8.5 Anti-ovalbumin IgG antibody in serum from healthy subjects.

Discussion

Oral exposure to antigen has several potential outcomes, which include: a) induction of systemic immunological hyporesponsiveness (tolerance), b) systemic priming, and/or c) the induction of local secretory IgA responses in the absence of measurable systemic immune responses . Mucosal exposure to living and multiplying pathogens leads to local and systemic priming, whereas the most frequent outcome of an oral encounter with soluble antigen is systemic tolerance (Strobel & Mowat, 1998).

The results of the present study were interesting. The increased IgA and IgM antibody responses against ovalbumin in WGLF from Edinburgh control groups compared with Dhaka groups merit consideration. Lymphocytes in Peyer's patches (PP), the lymphoid follicles in the intestinal wall, are exposed to a wide variety of dietary and bacterial antigens from the gut lumen (MacDonald, 1983). Studies have shown that exposure of PP B cells to specific environmental antigens results in these B cells becoming committed to giving rise to progeny capable of making secretory IgA (Gearhart & Cebra, 1979; 1981; Fuhrman & Cebra, 1981). On the other hand, the influence of environmental antigens on T cells in PP is not well described. T cells capable of suppressing anti-lipopolysaccharide (LPS) B cell responses can be found in the PP of normal mice but not in germ-free mice. Thus suggests that they are generated by the exposure of PP T cells to LPS from the normal intestinal flora (McGhee *et al,* 1980).

After the oral administration of a single large dose (40mg) of ovalbumin to mice, it has been reported that the PP not only contains T cells capable of helping anti-ovalbumin IgA responses, but also contain T cells which can suppress anti-ovalbumin IgG

responses (Richman *et al*,1981). Conversely, it has also been shown that chronic low levels of antigenic stimulation, which is probably the way in which most antigens are presented at the mucosal surfaces, down-regulate the secretory IgA response in the PP. This effect via the Ts (Suppressor T cell), may be a means of preventing excessive mucosal immune responses to antigens of little or no pathogenic significance (MacDonald,1983).

It is well recognised that the outcome of oral exposure to antigen depends on the nature of the antigen, and the dosage and the frequency of antigen administration. In addition, host factors such as genetic background, host immaturity and the role of the gut flora are important factors modulating antigen responses (Strobel & Mowat, 1998). It was not possible to ascertain the dose and frequency of intake of ovalbumin from chicken egg between the two heterogeneous population groups tested. The higher anti-ovalbumin IgA and IgM responses in WGLF in Edinburgh subjects or lower responses in the Dhaka groups might have been due to the variation in dosage and frequency of antigen. It is also quite probable that the lower antibody responses against ovalbumin in Dhaka control groups may be due to 'antigen-driven bystander suppression'. This is the mechanism by which CD 8^+ T cells, generated after induction of oral tolerance, are triggered in an antigen-specific fashion and release cytokines (e.g. TGF-β) that mediate suppression (Miller *et al*,1991). It has been shown in murine models that LPS combined with oral ovalbumin suppressed anti-ovalbumin antibody responses (Kim & Ohsawa, 1995). This is similar to the finding that LPS given orally to rats in conjunction with myelin basic protein (MBP) promotes their hyposensitization to MBP (Khoury *et al*,1990). The mechanism for this phenomenon has been suggested as competition at the level of antigen-presenting cells (APC) binding between multideterminant antigens and immunogenic fragments of food proteins (Herrmann *et*

al, 1990). It is, therefore, likely that a higher bacterial antigenic load in Dhaka subjects may be responsible for their lower antibody responses against ovalbumin.

Anti-ovalbumin IgG antibody was not detected in WGLF from either of the test groups. This result is in accordance with others which suggest that locally produced food-specific IgG is generally absent in normal individuals (Strobel & Mowat, 1998).

Regarding the data of serum antibodies against ovalbumin, the IgM antibody response in Dhaka subjects indicates that there was systemic priming in the Dhaka group. In a recent review by Strobel & Mowat, they suggested that, under certain conditions depending on the nature of the antigen and poorly understood host factors, systemic priming after oral antigen may also result (Strobel & Mowat,1998).

Nevertheless, the distribution of anti-ovalbumin IgA and IgG antibodies in serum were similar in both of the test groups. Since antigen-specific subcutaneous immunisation was not done in either of the groups, it is difficult to comment on this aspect of the results. With hindsight, if both groups had been subcutaneously re-challenged with ovalbumin, and if subsequent follow up serum and proliferation studies had been done and if the subclass of IgG antibodies against ovalbumin had been measured, we would be to speculate. These results would have provided the different responses of T helper subpopulations (IgG2 subclass is Th1 dependent, IgG1 is Th2 dependent) . Studies suggested that sensitivity to tolerance induction varies among T cell subsets. $CD4^+$ T cell can be divided into two major subgroups based on their production of cytokines. $CD4^+$ Th1 cells produce IL-2 and IFN-γ and mediate cellular immunity such as delayed hypersensitivity. $CD4^+$ Th2 cells produce IL-4, IL-5, IL-6, IL-10 and provide help for B cell antibody responses (Mosmann & Coffman, 1989, Husby *et al*,1994). It

has been shown in mice that parenteral injection of soluble protein antigen tolerized Th1 but not Th2 cells (Burstein *et al,*1989); the hyporesponsiveness in Th1 cells was mediated by IL-4 production by Th2 cells (Burstein & Abbas,1993). There seems to be a gradient of sensitivity to tolerance induction, with Th1 cells >Th2 cells> B cells (Husby *et al*, 1994).

It is certain that humans ingest food antigens daily in quantities that should result in tolerance, and a small fraction is known to be absorbed into the circulation (Husby et al, 1985). Despite the apparent occurrence of tolerance to food antigens, secretory and serum antibodies to them are readily detectable in humans (Gunther *et al*,1960, Scott *et al,* 1985). The gradient of sensitivity of T cell subsets and B cells may explain this apparent paradox. It is worth mentioning that an increasing body of evidence supports the concept that 'certain systemic infections in early life may reverse the Th response type by upregulating the Th1 and depressing the Th2 type response'(Hertzen,1998). The higher incidence of infectious disease in Bangladesh may be seen as relevant to the above statement

It is generally agreed that most studies related to antigens and 'tolerance' are in animal models. Human studies are few in number. Despite certain limitations, this present study has provided some useful information that may underpin future research. There are possibilities to exploit orally induced tolerance in the treatment of human disease such as allergy or autoimmunity. In addition, these results have other implications, especially related to the strategies for oral vaccines against infectious diseases.

Chapter 9

Cell Migration: Eosinophils and the Gut

Introduction

It was known to the pathologists of the last century that leukocytes migrate from the blood across the walls of microvessels and accumulate in inflamed tissue. The purpose of this migration and the process (called 'diapedesis') was unknown until Elias Metchnikoff showed that leukocytes engulf and kill bacteria and recognised diapedesis as a fundamental mechanism of host defence (Metchnikoff,1901). After the discovery of interleukin 8 (IL-8) in the late 1980's, chemokines were seen as the stimuli that largely control leukocyte migration (Baggiolini, 1998).

As already discussed in the literature review section of chapter 1, the granulocytes, unlike lymphocytes cannot re-circulate. Eosinophils are granulocytes that originate in the bone marrow and migrate to the tissue. It is a commonly held belief that they have specialised roles in the host defence against non-phagocytosable multicellular parasites (Weller,1991). Their anti-parasitic host defence action is mediated by release of cationic granule proteins which are toxic to parasites and may sometimes be detrimental to the host's own cells.

The histology of the intestinal mucosa in IBD is characterized by epithelial cell damage and tissue infiltration by lymphocytes, monocytes and neutrophils (Lowes & Jewell, 1990). The role of some of these cells, such as eosinophils and mast cells, is less clear, although there is increasing evidence that both cell populations are functional in the pathophysiology of IBD (Sarin *et al*, 1987; Bischoff *et al*,1996). Activated eosinophils release specific cationic proteins, such as major basic protein and eosinophilic cationic protein (ECP), which are cytotoxic to a variety of target cells,

including intestinal epithelial cells (Gleich *et al*,1979; Motojima *et al,* 1989). ECP levels in serum and other body fluids, such as sputum, nasal secretions and bronchoalveolar lavage fluid, are elevated in some allergic and inflammatory conditions, and may provide information regarding involvement of eosinophils in the pathological process of inflammation (Martin *et al,* 1996).

The mechanism of intestinal recruitment of eosinophils is not clearly known. However, three main cytokines namely, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5 are known to be responsible for recruitment and activation of eosinophils (Dvorak,1980). Immunohistochemical studies showed that IL-3, GM-CSF and IL-5 could be found in biopsy tissues from patients with eosinophilic gastro-enteritis (Desreumaux *et al*, 1996). In addition, it has been demonstrated that activated eosinophils possess the capacity to synthesise these cytokines and can regulate their own proliferation and differentiation perhaps by autocrine and /or paracrine activities (Desreumaux *et al*, 1996; Kita *et al,* 1991; Moqbel *et al*, 1991; Deseumaux *et al*, 1992). IL-5 is considered an eosinophil-specific haemopoetic growth factor in humans (Bagley *et al*, 1997). Studies with *in situ* hybridisation techniques on surgical specimens from paediatric patients with active CD, IL-5 mRNA was detected within the infiltrating cells in the mucosa. Most of the labelled cells were eosinophils. The number of IL-5 -expressing cells correlated with the histological grade of inflammation (Hankard *et al,*1997).

Recently a new chemokine was identified and isolated in the bronchoalveolar lavage (BAL) of guinea pigs after aerosol challenge with ovalbumin (Jose *et al*,1994). Intradermal injection of this BAL was followed by the accumulation of guinea pig eosinophils into the dermis (Jose *et al*, 1994). Purification of BAL fluid revealed a new

protein, termed Eotaxin. It has been demonstrated that the Guinea pig Eotaxin was able to induce [Ca^{2+}] transients and chemotaxis in guinea pig and human eosinophils (Jose *et al*, 1994; Rothenberg *et al*, 1995; Collins *et al*, 1995). Recently cloned human Eotaxin has been shown to be a 8.4 kDa, 74 amino acid residue polypeptide. Cells known to produce eotaxin include eosinophils themselves, macrophages, lymphocytes, pseudostratified ciliated columnar epithelium, endothelium, and thymic epithelium (Gonzalo *et al*,1996) . Human Eotaxin was found exclusively to induce chemotaxis of human eosinophils, with no effect on lymphocytes, monocytes and neutrophils (Ponath *et al*, 1996). It has also been demonstrated *in vitro* that eotaxin is a potent activator of eosinophil 'effector' function by means of activating the respiratory burst (Elsner *et al*, 1996).

An interesting clinical case (described below) prompted me to ask the following questions:

a) How do eosinophils accumulate selectively in certain diseases and contribute to mucosal damage?

b) How do they accumulate in high numbers despite being a minority cell type in the circulation?

Index Case:

D.R. (Date of birth: 24.10.81) was referred to the GI unit on March 1995 with persistent bloody mucoid diarrhoea. He first became unwell in April 1994 with sore throat, malaise and a fine rash over his trunk. His general practitioner had made a clinical diagnosis of glandular fever, but subsequent tests (monospot and antibody titre against EBV) confirmed past infection only. He also complained of abdominal pain which was mainly epigastric, and diarrhoea. Since that time he had suffered from

recurrent episodes of epigastric pain and diarrhoea. He lost weight and was crossing centiles for weight. Since October 1994, he continued to have persistent diarrhoea, up to 5-6 motions per day with urgency. The diarrhoea was explosive in nature and very watery, often containing bright red blood. His initial investigations showed a total white cell count of 12.4/ mm ^{3} with an eosinophilia of 25%. Platelets were 530 and ESR was 50. IgG level was slightly raised at 15.6 g and IgA and IgM levels were normal. Among liver enzymes, γ-GT was slightly raised at 66 units (normal, 8-49) and other investigations gave normal results. No enteric pathogens including ova and parasites were isolated from stool. Very low levels of specific IgE against egg and wheat, and no IgE antibody against milk, were found in his serum.

D. R.'s mother suffers from irritable bowel syndrome. D.R.'s elder brother suffered from asthma when he was younger but there was no other family history of atopy. There were two cats and a guinea pig at home and his grandmother has a dog. D.R. was treated with Mebendazole for possible worm infestation.

Investigations done at the Royal Hospital for Sick Children, Edinburgh, showed persistent eosinophilia. A barium enema showed a very fine granular appearance in the mid-transverse colon but no ulceration was identified. His colonoscopy and subsequent biopsy results showed severe active colitis with a prominent eosinophil component (Figure: 9.1, Eosinophils are pink in colour with Carbol Chromotrope staining). Ulcerative colitis was thought to be the likeliest diagnosis in view of the disruption of crypt architecture, goblet cell depletion and crypt abscess formation, which are not usual features of eosinophilic colitis.
The subsequent investigations conducted in the GI unit showed: WGLF IgG 20 μg/mL, IgA 123 μg/mL, and WGLF cytology analysis showed an abundance of eosinophils.

His upper GI endoscopy and biopsies were normal. He was treated with Prednisonlone. In view of his persistent eosinophilia, a bone marrow examination was performed and the report suggested reactive peripheral eosinophilia, but with no evidence of eosinophilic leukaemia or a primary bone marrow problem. There was no evidence of eosinophil infiltration in any other organs. A diagnosis of 'Enteropathic hypereosinophilic syndrome' was made. He continued to receive a low dose of Prednisolone to reduce his blood eosinophil counts which returned to within the normal range after three months. He remained well when last seen in November 1998.

In the following section, I record IL-8, IL-1β, granulocyte elastase (GE) and IgA EndoCAb determinations done relevant to my interests in eosinophil migration to the gut mucosa. These results were discussed in chapters 4 and 6.

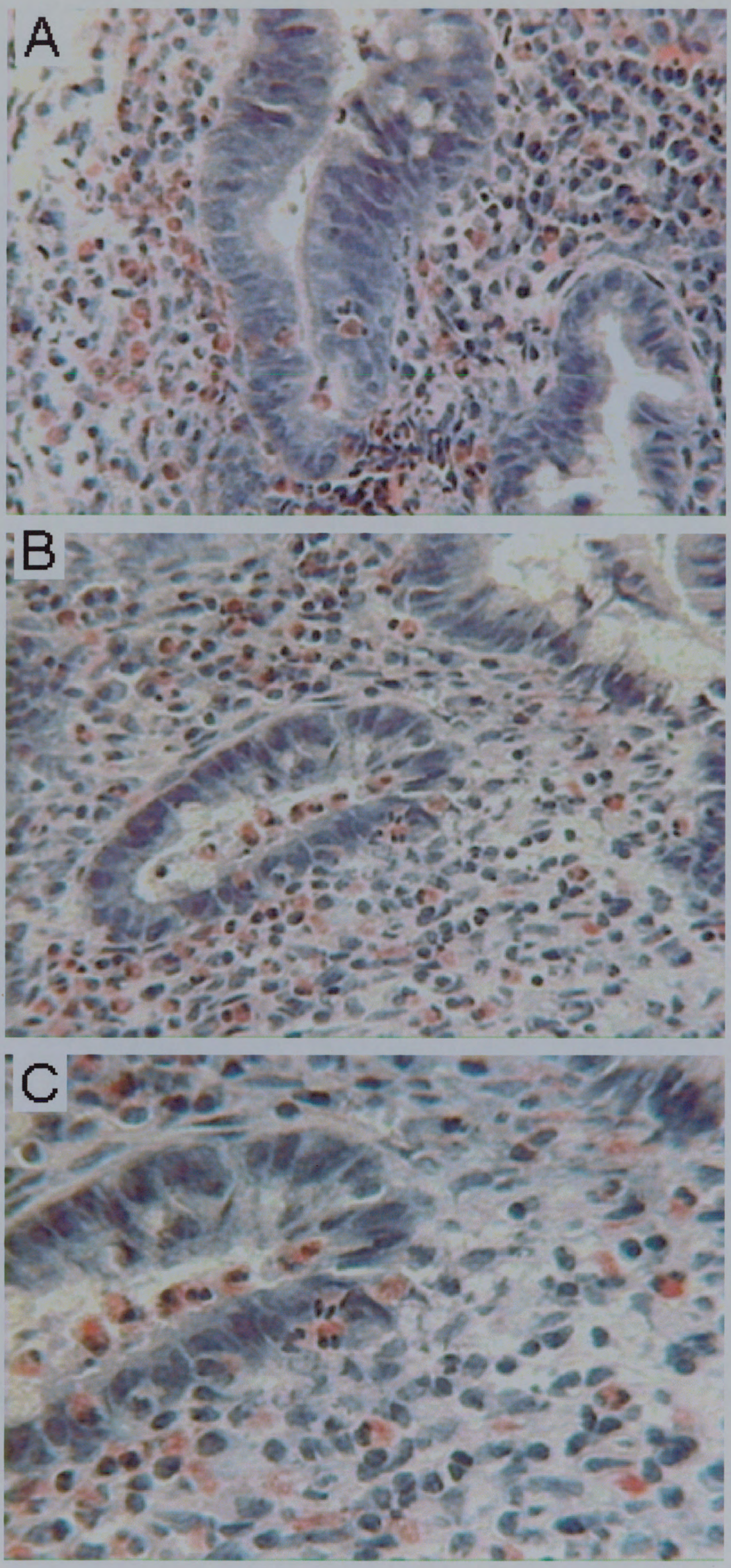

Figure: 9.1. Colonic biopsies from the index case, carbol chromotrope staining.
A&B – original magnification: x 25; C - original magnification: x 40.

Results:

ECP in WGLF

Figure 9.2 illustrates the ECP concentration in WGLF from the index case. During the active stage of his disease, the ECP concentration was found to be as high as 1193 μg/L, while in remission this value came down to 50.3 μg/L.

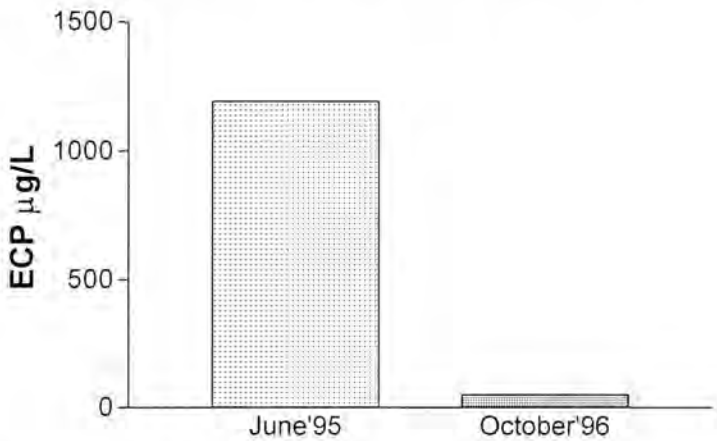

Figure: 9.2 ECP concentration in WGLF from the index case during active and remission stage of the disease.

Figure 9.3 depicts the concentration of ECP in WGLF from IBD patients and healthy controls. The median and (range) values for ECP in WGLF from Dhaka and Edinburgh controls were 70 (12-98) μg/L and 12 (6-29) μg/L respectively. There was a significant difference ($p<0.002$) in ECP concentration in WGLF between Dhaka and Edinburgh control groups. In IBD patients, the median and (range) concentrations of ECP in WGLF from UC and CD were 69 (42-153) μg/L and 74(29-443) μg/L respectively. These values were significantly higher than the values from the Edinburgh controls ($p<0.006$ for UC and $p<0.0001$ for CD). No significant differences in ECP concentration were observed between patients with UC and CD.

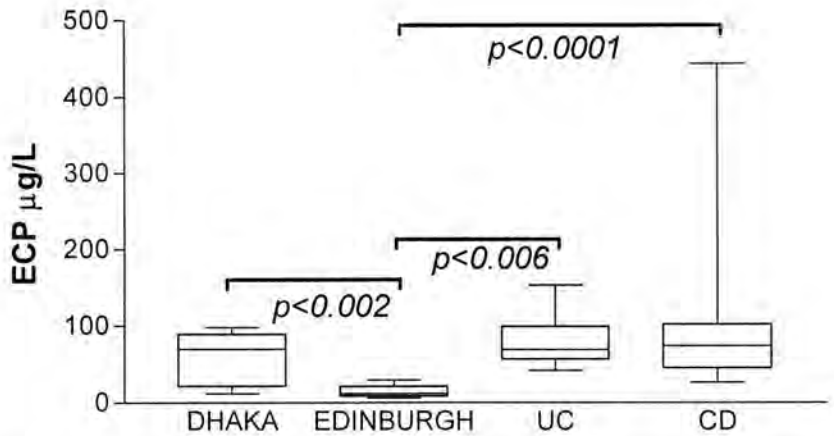

Figure: 9.3 ECP concentration in WGLF from patients with active IBD and healthy controls.

A significant positive correlation (r=0.8, $p<0.03$ for UC and r=0.75, $p<0.001$ for CD) was noted between total IgG (an established objective marker of gut inflammation) and ECP in WGLF from patients with active IBD (figures: 9.4 and 9.5).

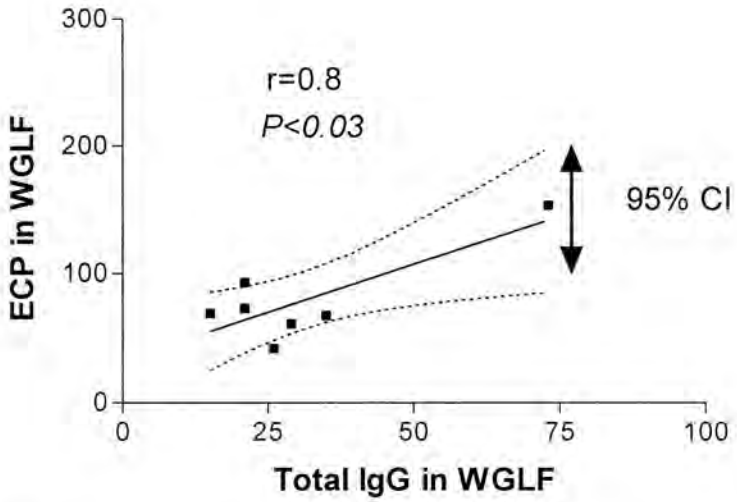

Figure: 9.4 Correlation between total IgG and ECP in WGLF from patients with active UC.

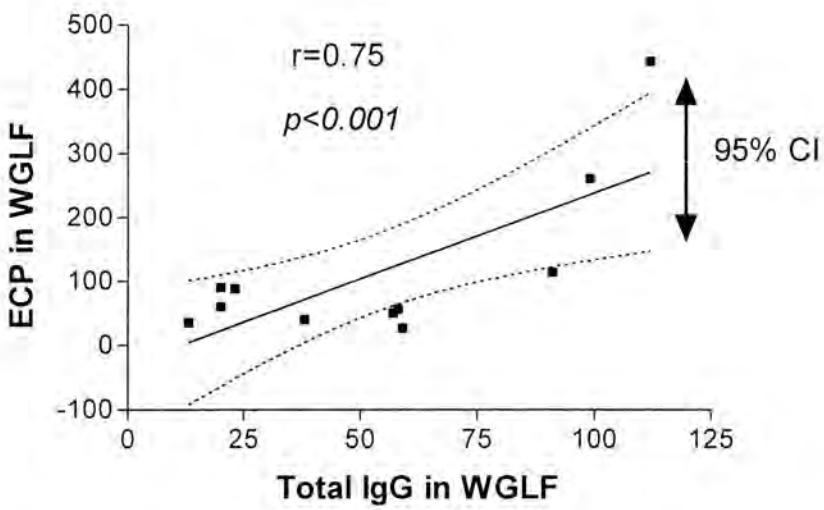

Figure: 9.5 Correlation between total IgG and ECP in WGLF from patients with active CD.

In active CD, a significant positive correlation was found between ECP, total IgA and IgA EndoCAb (Figures: 9.6 and 9.7). No such correlation was found in patients with active UC.

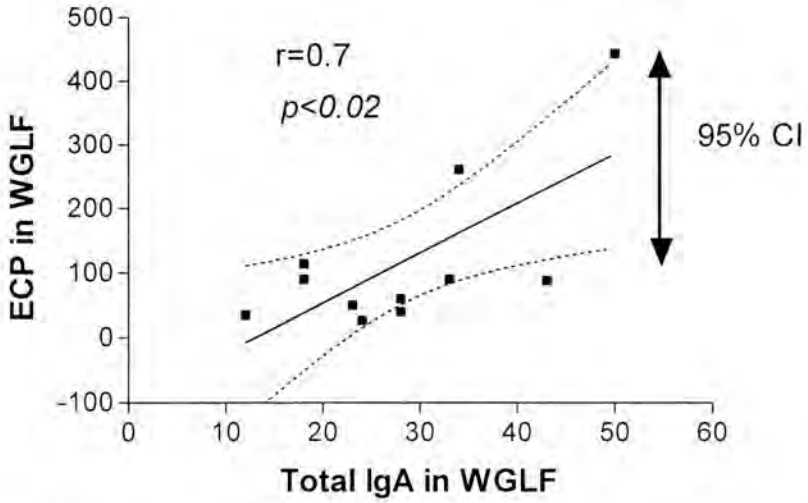

Figure: 9.6 Correlation between ECP and total IgA in WGLF from patients with Crohn's disease.

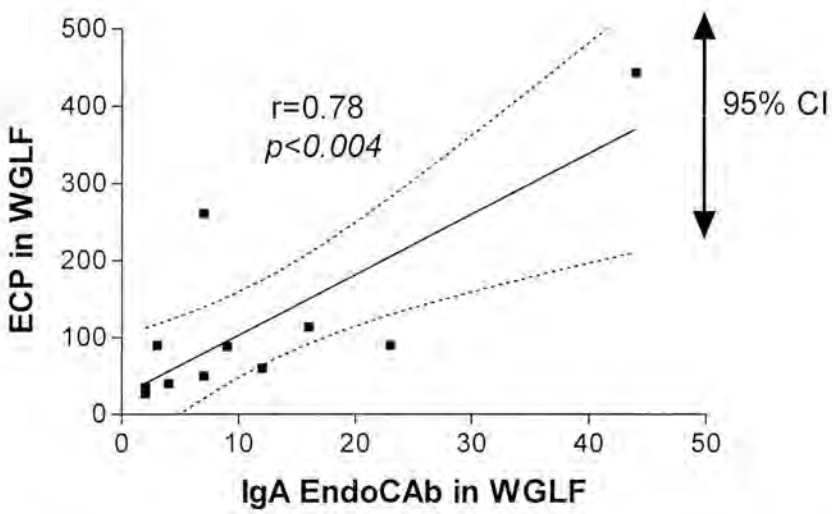

Figure: 9.7 Correlation between ECP and IgA EndoCAb in WGLF from patients with active Crohn's disease.

IL- 5 in WGLF

There was no IL-5 detected in WGLF either from patients or from healthy controls by the standard ELISA techniques.

The compiled results of GE, IL-8 and IL-1β are presented in figures 9.8 a,b, and c. The reason for presenting these compiled results is to show that despite the virtual absence of GE, IL-1β and IL-8 in WGLF, there were high ECP and eotaxin levels in the Dhaka control group. In addition, there was no correlation noted between GE, IL-1β, IL-8 and ECP or eotaxin in WGLF from patients with active IBD.

Figure: 9.8a
Granulocyte elastase (GE) in WGLF from patients with active IBD and healthy controls

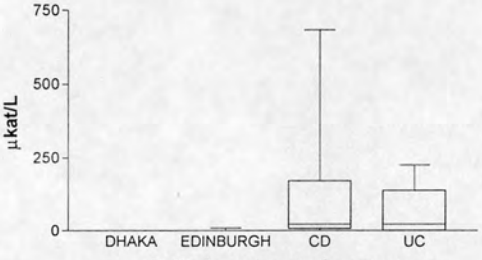

Figure:9.8b
IL-8 in WGLF from patients with active IBD and healthy controls

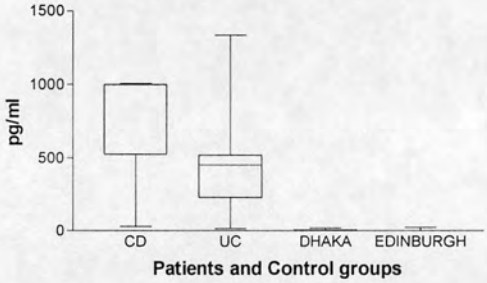

Figure:9.8c
IL-1β in WGLF from patients with active IBD and controls

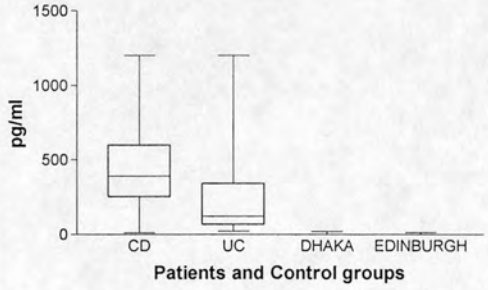

Figure: 9.8 a,b,c. Compiled results of GE, IL-8 and IL-1β of patients with IBD and healthy controls.

Eotaxin in WGLF

The results of the eosinophil specific chemokine eotaxin levels in WGLF are presented in Figure 9.9. The median and (range) concentrations of eotaxin in WGLF from the healthy control groups from Dhaka and Edinburgh are 20 (15-28) pg/mL and 14 (12-17) pg/mL respectively. There was a significant difference in eotaxin concentrations in WGLF from the control groups of Dhaka and Edinburgh (p<0.004). In active IBD patients, the median and (range) concentrations of Eotaxin in WGLF from UC and CD were similar. These values were 17(15-24) pg/mL and 18(17-19) pg/mL respectively and were significantly higher than the values of Edinburgh controls (p<0.002 for UC, and p<0.007 for CD).

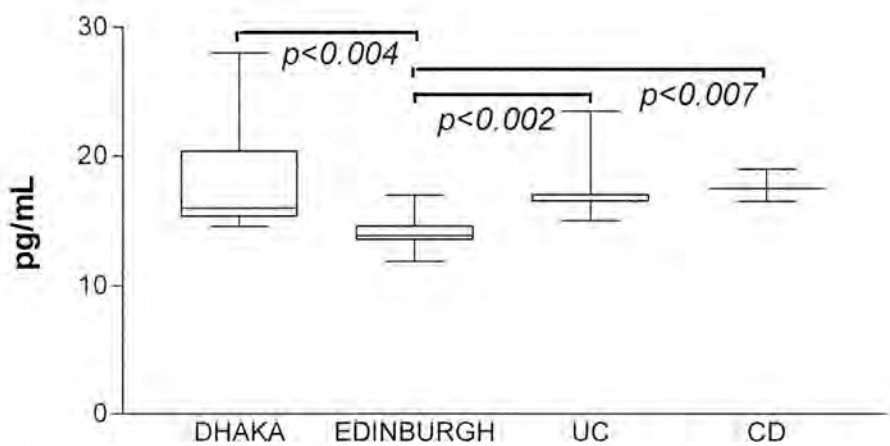

Figure: 9.9 Eotaxin concentration in WGLF from patients with active IBD and controls.

A significantly positive correlation (r=0.8, p<0.03) was found between levels of eotaxin and ECP in WGLF from patients with active UC (figure: 9.10). There was no such correlation found in patients with active CD nor in healthy control groups from Dhaka and Edinburgh.

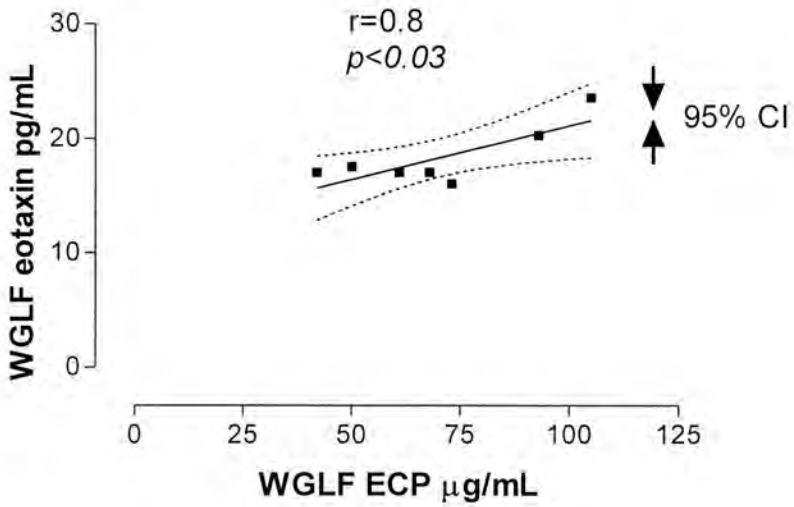

Figure: 9.10 Correlation between ECP and eotaxin in WGLF from patients with active UC.

Discussion

Gastrointestinal mucosa, unlike healthy skin and respiratory mucosa, normally contains eosinophils. The constitutive presence of eosinophils in the gut is probably very important in host defence, but also in the pathogenesis of diseases such as eosinophilic gastroenteritis and coeliac disease (Talley *et al*, 1992). In fact, normally, the level of eosinophils in the body is tightly regulated and accounts for only a small minority of peripheral-blood leukocytes. A marked accumulation of eosinophils has been found in several important disorders, including allergic disorders, parasitic infections and cancer (Weller, 1991). The aim of the study was to identify signals of eosinophils migration and their role in the genesis of mucosal damage in IBD.

Previous studies of the bowel mucosa in IBD have focused on the involvement of neutrophils but have given little consideration to the eosinophil population. Rectal biopsy specimens from patients with UC (Lumb & Protheroe, 1955; Truelove & Richards, 1956) and surgical CD specimens (Dvorak, 1980) have been reported to have an increased amount of eosinophil granule proteins. Increased levels of ECP have also been shown in faeces from patients with IBD (Berstad *et al*, 1993; Bischoff *et al*, 1997). However, it has recently been suggested that measurement of secretory proteins in faecal extracts may be highly misleading, since they are not representative of the total amount of proteins secreted into the gut lumen (Ferguson *et al*, 1995). In addition, degranulation of eosinophils may occur by the trauma during colonoscopic biopsy as well as processing of the specimen (Talley *et al*, 1992). Therefore, measurement of eosinophil proteins in WGLF was considered a better approach for investigating the role of eosinophils in IBD.

In this study, the ECP concentration in WGLF from patients with active IBD was found to be significantly higher than that from control groups (figure:9.3). It was also observed from the index case that the ECP level decreased when the patient was in clinical remission (figure 9.2). However, it is pertinent to question whether the ECP detected in WGLF from patients with active IBD originated from the gut or whether it could have resulted from plasma leakage through the inflamed bowel. An elegant study was done by Croft (1996), who measured ECP concentrations in WGLF and serum collected at the time of lavage. During the WGL procedure, lavage fluid is taken at a rate of 20mL/minute (1200 mL/hour) in adults. Considering the ECP values found in WGLF from patients with IBD, for serum to be the source of the ECP, in excess of 1.2 litres per hour would have to have been secreted into the GI tract. This is not possible since such a high volume of fluid loss would have resulted in patients becoming rapidly hypotensive (Croft, 1996). In addition, WGLF is a perfusate and is considered more valid than faecal examination for the estimation of the total amount of mediators released into the gut lumen (Ferguson *et al*, 1995).

It is recognised that the IgG levels in WGLF are an excellent objective marker of gut inflammation and can be correlated with clinical disease activity score in active IBD (Choudari *et al*, 1993). Data have also been presented in chapter 7 of this thesis to support the view that there is local production of IgG in the gut. The significantly positive correlation between ECP and IgG in WGLF from patients with active IBD (Figures: 9.4 & 9.5) suggests that the level of ECP is related to the degree of mucosal inflammation. Furthermore, it has been shown that activated eosinophils are capable of secreting pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α (Weller, 1992; Costa *et al*, 1993). These findings may suggest that IBD is accompanied by eosinophil activation and degranulation in the gut. However, whether activation and degranulation

of eosinophils in IBD is a spontaneous phenomenon or induced by other factors in the gut is not yet clear. Studies of immunoglobulin-mediated eosinophil degranulation indicated that IgE (Capron *et al*, 1981;Capron & Dessaint, 1990) and especially secretory IgA and IgG are potent stimuli of eosinophil degranulation (Abu-Ghazaleh *et al*, 1989). It is possible that increased local production of IgG in the gut induces eosinophil degranulation in the lamina propria of inflamed mucosa in patients with active IBD.

It is reasonable to raise several questions regarding mechanisms behind the activation and local accumulation of eosinophils in the gut. What is the initial stimulus for gut inflammation that results in expansion and activation of the eosinophil population in the lamina propria and which cell responds initially to the stimulus? Do eosinophils follow lymphocytic signals or signals from other granulocytes, such as neutrophils? Are there any local mechanisms involved in the recruitment of eosinophils? In addition, what role do they play in the pathogenesis of disease; are they effector, director or collaborator?

It has been shown that the eosinophil receptor for IgA binds secretory IgA more potently than other forms of IgA and hence induces degranulation (Abu-Ghazaleh *et al*, 1989). As eosinophils usually localise to mucosal surfaces in the gut, their IgA receptors can engage secretory IgA at this site and help in degranulation. The index case had a very high level of IgA and ECP in WGLF. In addition, ECP concentrations in WGLF from the Dhaka control group were significantly higher than for the Edinburgh control group (figure: 9.3). As already discussed in chapter 4 the total IgA in WGLF from Dhaka healthy controls was also significantly higher than in the Edinburgh

control group. These results clearly support the hypothesis that IgA may have some role in induction and degranulation of eosinophils.

Beeken *et al,* (1987) showed that intestinal eosinophils are capable of phagocytosis and play a role in the defence against bacterial invasion in the gut. In view of the higher microbial antigenic load in the gut of the Dhaka population (Albert *et al*, 1991), it does seem that microbial antigens might be an additional aetiological factor for the high ECP values in this population. In active CD, a positive correlation between ECP and total IgA (figure: 9.6) as well as IgA EndoCAb (figure:9.7) lends further support to these statements. The roles of non-pathogenic intestinal bacteria and IgA in CD have already been discussed in chapter 6. However, there was no such correlation noted in patients with active UC. Possible immunological differences in disease pathogenesis exist between CD and UC (as discussed in chapter 6, CD is Th1 and UC is Th2 mediated). In addition, sites of inflammation (small bowel or colon: apart from anatomical structure, bacterial populations are also different in these two sites) are likely to determine this discrepancy.

In most well studied examples of gut inflammation such as IBD, activated T cells and macrophages are considered to be the primary co-ordinators and it is generally believed that eosinophils are recruited into the inflammatory lesion secondary to the activation of primary immunoregulatory cells (Levy & Kita, 1996). The summarised results of IL-1β, IL-8 and GE assays (figure: 9.8 a, b, c) showed that despite the virtual absence of IL-1β, IL-8 and GE there were high ECP levels in the WGLF of the Dhaka control groups. These results clearly suggest that eosinophils are not just fellow travellers with granulocytes, neutrophils, or macrophages. Whether eosinophils follow lymphocytes in the event of inflammation is not known. On the other hand, it has been

found that tissue lymphocytes are increased in eosinophilic gastroenteritis (EG) (Levy & Kita, 1996). This area needs further study.

In an excellent study conducted in patients with EG it is suggested that local mechanisms must be involved in recruitment and activation of eosinophils in the gut (Desreumaux *et al,* 1996). EG is a rare disorder defined by three criteria: the presence of gastrointestinal symptoms, an eosinophilic infiltration of one or more areas of the gastrointestinal tract from oesophagus to colon, and no evidence of parasitic or extraintestinal disease (Cello, 1979). Unlike the idiopathic hypereosinophilic syndrome, eosinophil infiltration in EG is limited to the gut without extension to other organs and frequently occurs independently of blood eosinophilia (Spry, 1988). This suggests that local mechanisms must be involved in recruitment and activation of eosinophils in the gut . These mechanisms could involve key regulatory cytokines that control the production and function of eosinophils. In this study, IL-5 was not detected in WGLF from either patients or control groups. I have not undertaken 'spiking' experiments with IL-5 in WGLF. However, in-house experiments with other cytokines showed that IL-6 and TNF-α were also not detected in WGLF (Hazel Drummond, personal communication). 'Spiking' experiments with these cytokines suggest that IL-6 and TNF-α are very labile. In addition, perhaps other unknown factors present in WGLF are detrimental to the detection of these cytokines. IL-5 is a homodimeric glycoprotein of 25 to 40 Kd, held together by two interchain disulphide bonds (Bagley *et al,* 1997). It is probable that the reason for undetectable IL-5 in WGLF might be similar to that for the apparent absence of IL-6 and TNF-α. This would need to be confirmed.

For the first time, it has been shown in this study that eotaxin is secreted into the gut lumen in both health and disease. Higher eotaxin concentrations in WGLF from the

Dhaka control group (figure: 9.9) may be the reason for higher ECP concentration in that group compared to the Edinburgh controls. A significantly positive correlation was found between eotaxin and ECP in WGLF from patients with active UC (figure: 9.10). There was no such correlation noted in patients with CD and in either healthy control groups from Dhaka or Edinburgh. These interesting results merit further clarification. Eosinophils are predominantly tissue-dwelling cells; there are normally several hundred times as many eosinophils in tissues as in blood (Weller,1991). Their actual life span is unknown, but they live longer than neutrophils and may survive for weeks within tissues (Spry,1988). However, in disease states, where the process of activation and degranulation of eosinophils leading to inflammation and tissue damage is rather rapid, this cell turnover may be responsible for the positive correlation between 'eotaxin' and ECP in patients with active UC. Due to the paucity of sample size (in five CD cases only), it is very difficult to comment on the lack of correlation between eotaxin and ECP in CD patients. In addition, as already discussed, other non-specific factors may be involved in activation and degranulation of eosinophils in patients with CD.

In summary, this study demonstrates that eosinophils may contribute to mucosal damage in active IBD. ECP in WGLF may represent another marker of inflammation in IBD. Eosinophils are not fellow travellers, they actively migrate to tissues. Eotaxin plays a key role in attracting eosinophils to the site of inflammation leading to tissue damage by releasing ECP and perhaps some other reactive oxygen species. The identification of molecules specifically involved in eosinophil- induced disease pathogenesis offers hope for the development of new therapeutic agents that specifically target eosinophil pathways.

Discussion

Chapter 10

General Discussion, Overview, and Future Directions

Infectious disease continues to be a major cause of mortality and morbidity throughout the world and especially in developing countries. Despite the existence of multiple mechanisms which control microbial populations at mucosal surfaces, the mucous membranes remain the most important portal of entry for microbial infections. The ability, therefore, to enhance mucosal resistance to infection has enormous clinical importance. My long-term research objectives are to investigate the gut immune system in the context of health and disease of people in the developing world, especially in the fields of diarrhoeal disease and undernutrition. In this treatise, the major aim was to develop antibody tests which could be used as tools to examine the gut humoral immune responses to bacteria of the gut flora. Combined with studies of other facets of gut immunity for which study methods are already available, these tools should facilitate further investigations into active immunity and tolerance in the mucosal and systemic compartments.

The technical developments related to measuring antibody in WGLF have been presented in section 2 of chapter 2. The WGL technique has been established recently as a clinical tool for studying various humoral immune functions and inflammatory parameters in the gut . In chapter 3, evidence has been presented to support the view that antibody tests with WGLF are essentially reproducible. The techniques that I have described offer much hope in the further exploration of gut immunopathogenicity and the pathophysiology of intestinal disease.

The intestine is considered the largest lymphoepithelial organ in the body with a surface that is exposed to the heaviest burden of environmental antigens. In view of the higher microbial antigenic load from the contaminated environment in Dhaka population, I hypothesized that there would be evidence of gut damage and inflammation in healthy people of Dhaka. In addition, there may be higher levels of IgA antibody in the intestine due to humoral responses to bacteria. The results presented in chapter 4 suggest that there was no evidence of gut damage or inflammation in the healthy Dhaka population. However, the results support the second part of the hypothesis, demonstrating a higher IgA antibody production in the gut of the healthy people of Dhaka. The reason for the increased total IgA in WGLF from healthy controls from Dhaka may be due to non-specific immune activation. Furthermore, to prevent gut inflammation or bacterial translocation, it is probable that there may be an increased drive in the production of S-IgA.

As discussed in the literature review, IgA1 and IgA2 subclasses are present in the gut and these have subtle structural and functional differences, including sensitivity to bacterial proteases. Recently, it has been shown in a murine model that two functionally distinct types of S-IgA antibodies exist. These antibodies are produced by two separate B cell populations, B-1 and B-2 (Mestecky *et al*,1999). The B-1 lymphocytes (formerly Ly1+ or CD5+), make an important contribution to the lamina propria IgA plasma cells. These produce less specific, perhaps polyreactive 'natural' antibodies. These antibodies actually play a role in the maintenance of the normal intestinal bacterial flora (Mestecky *et al*, 1999). However, pathogenic micro-organisms stimulate the immune systems after invasion through Peyer's patch **M** cells (Owen & Davis,1997). These stimulate locally present conventional B-2 cells which are then disseminated to the lamina propria of the gut, and mature into IgA plasma cells.

These plasma cells then produce specific IgA antibodies with high affinity for the pathogen which they exclude (Mestecky *et al*,1999). These murine studies should not be extrapolated to Man without caution, for the existence of such functional dichotomy of S-IgA antibodies in human is unknown. Certainly this is a fertile area for future studies. An immunology honours student placed in the GI lab is currently setting up studies to investigate this area. Knowledge from such studies will hopefully be helpful for developing oral vaccines for enteric infections, especially for developing countries.

The immune system of the gut is separate and distinct from the systemic immune system. It differs in many respects: in the cells and immunoglobulin isotypes involved, in the various effector functions, and in its immunoregulation (Ferguson *et al,* 1994). There are complex interrelationships between the normal gut flora, intestinal infection, invasion of the tissues by gut bacteria or their products, sepsis syndrome, shock, and multi-organ failure. Gram-negative sepsis and endotoxic shock are complicated pathophysiological entities which have evaded effective medical treatment for centuries. This sepsis syndrome is a major cause of mortality and morbidity in the immunocompromised, especially in the neonate, elderly and in post-operative patients. Estimated mortality in patients with severe sepsis and related haemodynamic compromise ranges from 40-90% depending upon various settings and the time of intervention. It is one of the leading cause of death in children hospitalised for diarrhoeal diseases in developing countries (Strulence *et al*, 1985).

In the field of sepsis, the source of endotoxin (LPS) is debated (gut versus bacteraemia). It has been shown that low serum anti-EndoCAb antibody is an independent predictor of adverse outcome following cardiac surgery (Bennett - Guerrero *et al*, 1997). Antibodies to LPS are often protective (Poxton, 1995).

Nevertheless, systemic immunisation is essentially ineffective for induction of mucosal immune responses. Since the majority of infectious micro-organisms are encountered through mucosal surface areas, it is logical to consider the induction of protective antibodies and T cell responses in mucosal tissues. Various core types of LPS of *E. coli* (a prototype of Gram-negative bacteria) were well studied in the Department of Medical Microbiology, University of Edinburgh. The results are presented in chapter 5. The study demonstrated for the first time that there are mucosal IgA antibody responses present against the specific LPS core types of *E.coli* in the gut. There are some important potential implications of this study. However, little is known of how LPS epitopes, especially in the core /lipid A regions, are presented to the inductive arm of mucosal immune systems, and notably the roles of T cells and immunological memory are unclear. It is necessary to investigate the relationship between LPS core types and O types and pathogenicity. In addition, it is worthwhile to study the protective capabilities of specific monoclonal IgA antibodies against LPS core types, especially whether antibodies recognising a single epitope on the target organism can provide protection against colonisation or invasion of the gut mucosa. Immunoblot study (figure 5.8 in chapter 5) clearly demonstrated that mucosally presented vaccine against infectious diarrhoea is an avatar. It seems quite probable now, a vital question, which could be answered using this study technique, is whether patients with sepsis syndrome and multiorgan failure have adequate intestinal mucosal antibody to LPS or whether there is transient or prolonged mucosal antibody failure in this situation. However, my study underpinned several other studies which are currently being undertaken in the Department of Microbiology under the supervision of Professor Ian Poxton. If he precise mechanisms causing septic shock are determined, the prospect of the development of effective therapy becomes more likely. It might not even be necessary to use human antibodies for this purpose. Passive administration of

heterogenous antibody via gut could theoretically be an attractive and economic option.

Immune phenomena are believed to play a key role in the pathogenesis of tissue damage in IBD. Results of studies on intestinal humoral immunity in this chronic condition are conflicting. Genetic factors predispose to IBD but a contribution of the gut flora cannot be ignored. The results presented in chapter 6 suggest that aberrant immune responses to non-pathogenic luminal bacteria or bacterial products may be responsible for initiation and perpetuation of chronic intestinal inflammation in IBD. In addition, it is possible that primary mucosal immunodeficiency is present in IBD patients, thus allowing agents to penetrate the intestinal mucosa and subsequently initiate both a local inflammation and heightened systemic immune response against bacterial and food antigens.

In CD, whether a primary mucosal immunodeficency is responsible for the impaired barrier function, a plausible factor in the postulated pathophysiology, is not clear. Furthermore, whether the dysfunction is a primary aetiological factor in CD or is merely an 'epiphenomenon' of the inflammatory process of the disease, merits further study. The results of serum IgG EndoCAb estimation in CD are fascinating and it suggest that this could be used as a reliable serological screening test. Further studies are needed to identify the sensitivity and specificity of this test in identifying and predicting relapse in patients with IBD, especially CD, as other tests (i.e. the dual sugar permeability test) are cumbersome, time-consuming and lack specificity.

Although some immunological features seem to be shared by CD and UC, there are important distinguishing features, possibly reflecting different loops of immune-

mediated intestinal inflammation. Evidence indicates that macrophage and T cell derived cytokines play a key role in the amplification and perpetuation of the inflammatory response in both disorders (Sator,1994). A number of quantitative changes in the secretion (i.e. IgG1 & IgG2 in CD, IgG1 & IgG3 in UC) and /or activities of both proinflammatory and regulatory cytokines have been reported in CD and UC. Considering the variation in the magnitude of these changes, there seem to be different cytokine profiles into which the inflammatory process may fall during the course of the disease. Studies from murine models indicate that two T lymphocyte subsets may be defined depending on their cytokine secretion profiles: Th1 lymphocytes producing interleukin (IL)- 2 and interferon γ (IFN-γ), and Th2-lymphocytes producing IL-4, IL-5, and IL-10. Combined data from both human and experimental studies suggest that in CD the local immune response tends to be predominantly Th1, whereas in UC Th2 mediated phenomena tend to predominate. Recently, it has been shown that IL-12 plays an important role in the generation of Th1 type cell clones and was found in increased concentration in the intestinal mucosal sample of patients with CD (rarely detected in normal intestinal mucosa and in unstimulated lamina propria mononuclear cells from patients with UC) (Pallone & Monteleone, 1998). The IL-12 induces synthesis of IFN-γ by lamina propria T lymphocytes and this process is further enhanced by other cytokines produced in the human intestine, such as IL-7 and IL-15 (Pallone & Monteleone, 1998). Driven by IL-12, IL-7 and IL-15 may thus promote Th1 cell expansion and contribute to the breakdown of tolerance towards the resident luminal antigens (Duchmann *et al,* 1995;1996).

However, several questions are still unanswered. What induces IL-12 production in CD? Is there any disease-specific stimulus for this or does it reflect macrophage

activation by T cell derived cytokines or luminal bacteria or both? Nevertheless, it seems clear that IL-12 may be involved in mediating the immune response in CD, and the inhibition or blocking of its biological effects may be a promising way to control the inflammatory process in CD.

The results on studies of bacteroides organisms UC and pouchitis are compelling. Whether any particular species of *Bacteroides* is associated with UC [*B. thetaiotaomicron* (Poxton *et al,* 1997), *B. vulgatus* (Onderdonk *et al*, 1981)], or whether the breakdown of tolerance towards predominant anaerobic resident colonic bacteria is responsible for UC, is currently unknown. Avenues for further studies are thus clear.

In the field of IBD, the source of gut antibodies is debated. One of my research aims was to establish, by studying patients with active IBD, whether the gut antibodies are serum- derived or locally produced. I have already discussed the limitations of the various methods of studying gut antibodies. For example, measurement of secretory proteins in faecal extract may be highly misleading, since they are not representative of the total amount of proteins secreted into the gut (Ferguson *et al,* 1995). Endoscopic biopsy are susceptible to trauma and only represent isolated areas which are not necessarily representative of the whole gut. In addition, cell culture in an artificial medium is necessary. Most of the *in vitro* studies using cultured cells are rely on stimulation by an antigen which may be inappropriate or dissimilar to luminal antigens. The WGL technique is an excellent non-invasive tool for the study of gut antibodies without trauma. To study the source of gut antibodies, an attractive arithmetic model has been contrived in chapter 7. The study demonstrates an increased local production of IgG antibody in the gut. It has been shown for the first time that depending upon the nature, quality and number of antigens (single or

multiple), local production of antigen-specific IgG antibody in the gut mucosa was increased by 56-99% in active IBD. As mentioned earlier, it is quite probable that due to the failure of the 'first line defence' (S-IgA considered as a first line defence in the gut mucosa), an additional second line defence seems to be established by local IgG production. It does seem that in IBD, continual antigen-driven division of B cells in the absence of signals from helper T cells for the terminal differentiation may result in increased IgG production instead of IgA in the gut. Although this may be beneficial as a second line defence, it may give rise to a deleterious reaction as IgG antibody has 'phlogistic' potential. Using my arithmetic model, others in this lab have recently shown that there is increased local production of complement in the gut of patients with active IBD (Kenneth Humphreyes, personal communicaton). It is now conceivable that locally produced IgG (a potent complement activator) and complement are contributing to the persistence of chronic inflammation in IBD. What initiates complement attack, and the identification of the antigens or autoantigens will be a 'Holy grail' in IBD research.

Initially I hypothesized that in developing countries, the drive of production of a high humoral response to bacteria would have an additional effect, and that antibodies to other luminal antigens, such as food, would be absent or of low titre. The hypothesis has been tested and the results presented in chapter 8. The induction of immunological unresponsiveness by feeding soluble antigens, termed 'oral tolerance' is a well-known phenomenon. However, most of the data generated related to this topic have come from studies on murine models. The literature on oral tolerance in the human remains scanty, partly because of the difficulties in identifying safe, immunogenic neoantigens in humans to be used in research on the induction of tolerance. However, my study on systemic and humoral immunity to antigens regularly encountered, such as gut bacteria or food antigens, supports the concept of a degree

of down-regulation of systemic immunity to gut luminal antigens. A study conducted by a colleague in this lab, feeding neo-antigen keyhole limpet haemocyanin (KLH) to human volunteers, demonstrated that oral tolerance does occur in humans. The mechanisms leading to oral tolerance include clonal deletion, anergy and active suppression. The determining factor in this process is the dose of fed antigen. It is recognised that low dose favours active suppression, whereas high dose favours clonal deletion and anergy (Strobel & Mowat,1998, Husby *et al*, 1994). Nevertheless, oral tolerance is an important physiological phenomenon and is likely to be relevant to the understanding of pathogenesis, and the development of new forms of therapy in several inflammatory gut diseases.

Recently the 'hygiene hypothesis' has gained wide acceptance. The key element of the hypothesis is the Th1/Th2 paradigm. This hypothesis suggests that infection in early life by micro-organisms that elicit a vigorous cell-mediated immunity (Th1 type response, e.g. *Mycobacterium tuberculosis*) may prevent the development of atopy or atopic diseases (e.g. asthma, Th2 type response). It is possible that the higher incidence of infectious disease (Th1 response) in Bangladesh may be a cause for low antibody against food antigen (Th2 response) in the gut. In addition, lately, it has been shown that the B subunit of cholera toxin is an immunomodulator. Coupling of antigen to the B subunit prior to oral delivery can dramatically decrease the dose required to stimulate tolerance (Williams *et al*,1999). This may be a reason for low food antibody in the gut in a population where cholera is endemic. These results have certain implications in the strategies for oral vaccines against infectious disease.

Eosinophils have been implicated in a number of gastrointestinal diseases including parasitic infestations, allergies and coeliac disease. There is some scepticism

regarding the participation of eosinophils in mucosal inflammation in IBD, perhaps related to the fact that eosinophils are terminally differentiated, minority, end stage leukocytes. In addition, the discharge of potentially toxic, proinflammatory granule proteins may make the cells less readily identified by routine histology (Levy and Kita, 1996). Thus, eosinophils which have not been identified may already have participated in the inflammatory processes. However, the results presented in chapter 9 suggest that eosinophils contribute to the mucosal damage in active IBD. Their granular protein ECP can be used as a marker of inflammation in IBD. Evidence has been presented to support that eosinophils are not fellow travellers, but migrate to the tissue by independent mechanisms. In fact, recently, mechanisms involved in eosinophil migration and related chemoattraction have been described (Williams, 1998). In addition, eotaxin receptor CCR3 which is highly expressed on eosinophils has been identified. Thus, eotaxin and its receptors emerge as prime targets for potential therapeutic intervention. Blockade of the receptor with an antagonist may render the circulating eosinophils insensitive to the attractant action of locally produced chemokines and may be effective in preventing the pathogenesis associated with eosinophil accumulation.

Appendix: Reagents and Instrumentation

WGLF Processing Methods and Reagents

1. Collect two Universal (2x30 mL)containers of clear lavage fluid

2. If the collected samples do not appear very clear or do not filter relatively quickly then centrifuge the samples at 1500g for 5 minutes.

3. Dispense 3x1mL unfiltered and untreated lavage fluid into sample tubes. Add 20 μl sodium azide (1%). Label with Laboratory no. and UF+UP (Unfiltered and Unprocessed).

4. Filter approximately 10 mL into a Universal or a flask. use Whatman GF/A 12.5 filter paper.

5. Transfer 5 mL into a Universal A. Transfer 5 mL unfiltered lavage into a Universal B.

6. Treat both the Universal aliquots as follows:

Add 0.5mL SBTI (Soya bean trysin inhibitor), and mix.

Add 0.28 mL EDTA (BDH Cat No: 10093), and mix

Add 0.12mL PMSF (Phenylmethylsulphonylfluoride, Sigma Cat No P-7626)

Add 0.06 mL Na Azide and mix.

Leave for 2 minutes

Add 0.3 mL NBCS (Newborn calf serum, sigma Cat No N 4762 heat-inactivated)

7. Dispense 10x250μl and 1x1 mL from A into microcentrifuge tubes and lab freezer tubes respectively, labelled with approprite lab no.

8. Dispense 2x1mL from B into freezer tube labelled with lab no, and UF +UP.

9. Dispense 2x1mL untreated filtered lavage into freezer tube, add 20 μl Sodium azide, label with lab no and F+UP (Filtered and Processed).

10. Strore 1 of each F/P, F/UP, UF/P and UF/UP at -70° C.

In-House ELISA reagents

Plates

- Immulon 1 129 A (Dynatech) were used for total immunoglobulin assay.
- Immulon 2 129 B (Dynatech) were used for antibody ELISA's.

Coating antibodies

- Goat antihuman IgG: Fc specific, Sigma Chemical Co. (Cat No 2136)
- Goat antihuman IgM: μ chain specific, Sigma Chemical Co, (Cat No I-2386)
- Goat antihuman IgA: α chain specific, DAKO Chemical Co (Cat No A-0262)
- Ovalbumin: (Sigma Cat No A-5503).
- Monosialoganglioside (GM_1): (Sigma) 1mg/mL in 2:1 chloroform:methanol
- Cholera Toxin: (List Biologicals)

Conjugated antibodies-alkaline phosphatase conjugate

All made up in the appropriate diluent

- Goat antihuman IgA, Sigma Chemical Co (Cat No A-3063)
- Goat antihuman IgM, Sigma Chemical Co (Cat No I-2386)
- Goat antihuman IgG, sigma Chemical Co (Cat No A-3187)

Other Reagents

- Sterile water

1 litre sterile water containers (Baxter Healthcare Code F 7124)

- Coating buffer

Carbonate-bicarbonate coating buffer, 0.05 M, pH 9.6 at 25°C. Dissolve the contents of 10 capsules (Sigma Chemical Co. Cat No C-304) in 1 litre water.

- Sodium chloride

0.9% sodium chloride in 1 litre sterile containers (Baxter Healthcare Code F7124)

- Wash solution

0.9% saline + 0.05% Tween 20 (Polyoxyethylene-Sorbitan Monolaurate, Sigma Chemical, Cat No P-1379)

- Adult Bovine serum

Adult bovine serum (filtered through 22 μm filter) (SAPU, Law Hospital, Carluke, Lanarkshire, ML8 5ES (Product No S026-220)

- Diluent

0.9% saline +0.05% Tween 20+ 1% adult bovine serum.

- Diluent for CT assay

0.9% saline +0.05% Tween 20+ 1% bovine serum albumin

- Diluent for EndoCAb and *E.coli* LPS cores

I litre freshly-distilled water + 1 vial powdered Dulbecco Phosphate Buffered saline + 1 mL Tween 20+ 5 mL 10% Na azide +40g PEG 800 (final conc 4%) + 10g serum albumin (endotoxin free BSA, Sigma Cat No A-1933).

- DEA (diethanolamine) substrate

To make 5 litres of DEA substrate:

1. Add 500 mL diethanolamine-concentrated liquid (BDH Laboratory Supplies, Analar Reagent Product No 10393 4J)

0.51g magnesium chloride (Mg $Cl_2 6H_2O$)

1.0g sodium azide (NaN_3)

4 litre sterile water

2. Adjust the pH to 9.8 with 6M hydrochloric acid (HCl)

- P-nitrophenyl phosphate

5 mg phosphatase substrate tablets-disodium p-nitrophenyl phosphate hexahydrate (Sigma Chemical Co. Product No 104-105)

- Alkaline phosphatase substrate

Fresh substrate is constituted approximately 30 minutes before required. Dissolve 1 tablet phosphatase substrate per 5 mL DEA buffer; mix thoroughly.

Haemoglobin Reagents

All reagents are analar grade and obtained from Merk (BDH) unless otherwise stated

- Oxalic acid Reagent: 4.0 g oxalic acid (Analar) is made up to a 10mL volume with distilled water, and dissolved in a water bath at 100°C. 0.10 g uric acid and 0.11g mannitol are then added, and the reagent left at 100 °C for 5 minutes. Any undissolved reagent is left to settle out. The supernatant is pipetted off while hot in the fume cupboard and this is the test reagent. The reagent is prepared fresh before use.
- Ethyl acetate/acetic acid 10/1 v/v: Add 50 mL glacial acetic acid to 500 mL ethylacetate in a fume cupboard.
- 3.3M Potassium Acetate (294g/L): Dissolve 29.4g potassium acetate(BDH GPR) in 100 mL distilled water. Store at room temparature.

- 4.3M Potassium Acetate in 1M Potassium Hydroxide (56g/L): Dissolve 147g potassium acetate in 300 mL distilled water, add 28g potassium hydroxide (fume cupboard), dissolve with stirring and make up to 500 mL with distilled water.

- N-butanol (Rathburn Chemicals Ltd, Walkerburn)

- 6.2M H3PO4/Acetic acid 9/1 v/v): Dissolve 68 mL orthophosphoric acid in 300 mL water and add 58 mL glacial acetic acid.
- PEG 4000 reagent: Dissolve 60g PEG 3350, 9g NaCl, and 0.2 g sodium azide in 1 litre of distilled water. Use to make Drabkins reagent.
- Drabkin's Reagent: (Sigma Cat No. 525-2) 6 vials.

Each vial contains 1 g sodium bicarbonate, 0.2g potassium ferricyanide, and 0.05g sodium cyanide. Take 1 vial and reconstitute to 1 litre with PEG 4000 reagent.

[Caution: this is a dangerous solution and must be appropriately labelled, handled and stored.]

LPS extraction reagents :

- **PCP reagent**, 90% w/v aqueous phenol, chloroform and petroleum spirit in the ratio of 2:5:8 by volume. (Use pyrogen-free glassware throughout)

- **w/v aqueous phenol** (add 1 part of pyrogen-free water to 8 parts of 90% w/v aqueous phenol solution. **Diethyl ether/acetone** in the ratio of 1:5 by volume.

PAGE reagents:

1. **40% w/v aqueous acrylamide solution**

100g acrylamide (BDH Electran), 2.7g methylenebisacrylamide (BDH Electran), Pyrogen-free water to 250 mL

2. **15 g/L w/v ammonium persulphate solution.**

300 mg ammonium persulphate (BDH Analar) in 20 mL of pyrogen-free water.

3. **0.05% w/v aqueous bromophenol blue solution**. 10 mg of bromophenol blue (BDH water soluble) in 20 mL of pyrogen-free water.

4. **Water saturated butan -2-ol**. Place 60mL butan-2-ol (BDH Analar) into a 100 mL screw-capped bottle. Add pyrogen-free water, 10 mL at a time, mix thoroughly.

5. **Electrode buffer. 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3.**

6.057g Tris (hydroxymethyl) methylamine (BDH Analar), 28.827g glycine (BDH chromatographically homogeneous). 2g sodium lauryl sulphate (SDS) (BDH especially pure). Pyrogen-free water to 2000mL.

6. **Separating gel buffer (double strength). 0.75 M Tris/HCl pH 8.8, 0.2% SDS.**

90.885 g Tris (hydroxymethyl) methylamine (BDH Analar)

2g sodium lauryl sulphate (SDS) (BDH especially pure)

5 M and 1M hydrochloric acid (BDH Analar)

Pyrogen-free water to 500 mL.

7. **Stacking gel buffer (double strength). 0.25M Tris/HCl pH 6.8, 0.2% SDS.**

15.142g Tris (hydroxymethyl) methylamine (BDH Analar)

1 g sodium lauryl sulphate (SDS) (BDH especially pure)

5 M and 1M hydrochloric acid (BDH Analar)

Pyrogen-free water to 500 mL.

8. **Sample buffer (single strength). 0.0625 M Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.001% bromophenol blue.**

0.757g Tris (hydroxymethyl) methylamine (BDH analar), 2g Sodium lauryl sulphate (SDS) (BDH especially pure), 12.6g glycerol (BDH analar) (=10% v/v glycerol)

1 mL 2 mercaptoethanol (BDH), 2mL Bromophenol blue (0.05% aqueous solution) (BDH water soluble), 1 M HCl. Pyrogen-free water to 100mL.

[SDS-free gel buffer is prepared as above but omitting the SDS]

9. **Sample buffer (double strength). 0.125 M Tris/HCl pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.002% bromophenol blue.**

1.514g Tris (hydroxymethyl) methylamine (BDH Analar).

4g sodium lauryl sulphate (SDS) (BDH especially pure). 25.2 g Glycerol (BDH analar) (=20% v/v glycerol). 2 mL 2-mercaptoethanol (BDH). 4mL Bromophenol blue (0.05% aqueous solution) (BDH water soluble). 1 M HCl. Pyrogen-free water to 100mL.

Silver stain reagents

1. **Fixative**. 25% propan-2-ol, 7% acetic acid.

500mL propan-2-ol (BDH GPR)

140 mL glacial acetic acid (BDH Analar)

1360 mL distilled water.

2. **Oxidizer.** 0.7% periodic acid in dilute fixative.

1.05g periodic acid (BDH Analar)

4mL of fixative and 150mL of distilled water.

Dissolve the periodic acid in the water and add the fixative.

3. **Silver stain (Ammoniacal silver nitrate).**

a) Ammonia solution (BDH Analar, SG 0.88).

b) 0.36% sodium hydroxide solution (BDH Analar)

3.6g NaOH, 1000mL distilled water. Dissolve the NaOH in the water in a glass screw-capped bottle.

c) 19.4% silver nitrate solution

19.4g silver nitrate (BDH Analar)

Distilled water to 100mL

Dissolve the AgNO3 in about 70 mL distilled water and make up to 100 mL with distilled water.

Complete stain (Ammoniacal silver nitrate) (work in a fume cupboard). In a 100mL flask kept specifically for this reagent, mix 21 mL 0.36% NaOH and 1.4 mL ammonia solution. Then add dropwise with continual mixing 4mL 19.4% silver nitrate solution. A dense brown precipitate forms on adding the silver nitrate, but the mixture becomes colourless on mixing.

4. **Developer**. 0.005% citric acid in 0.019% formaldehyde solution.

1mL of formaldehyde solution (BDH Analar, 38-40%)

2000 mL distilled water.

The complete developer is made up as follows:

10mg citric acid (BDH)

200 mL 0.19% formaldehyde solution.

Reagents for Coomassie blue staining

Reagent No	Coomassie brilliant R-250	Propan-2-ol (GPR)	Methanol (GPR)	Acetic acid (Analar)	Distilled water
1	1g	500 mL	-	200 mL	1300 mL
2	100mg	200 mL	-	200 mL	1600 mL
3	48 mg	-	-	200 mL	1800 mL
4	-	-	800 mL	200 mL	1000mL
5	-	-	-	200 mL	1800 mL

ECP Reagents

Included in the kit

- ECP (human) Standards 0,2,5,15,100,200 μg/L
- ECP ^{125} I
- Anti-ECP
- Decanting suspension (Sepharose ^{R} anti-rabbit IgG raised in sheep)

The Lavage solution

Commercially available as " Klean- Prep" from Norgine Ltd, Oxford, UK.

One sachet to be made up to one litre by adding tap water which contains:

- Polyethylene glycol (PEG), molecular weight of 3350 - 59g/L tap water (Osmolality 260 mosm/L)
- Sodium chloride (BP 1.4g)
- Sodium bicarbonate (BP 1.63g)
- Sodium bisulphate (BP 5.68g)
- Potassium chloride (BP 0.75g)

2. Instrumentation

- Centrifuge:

Mistral 3000I (MSE,UK)

- ELISA Reader:

Dynatech MR 500 (Dynatech)

- Freeze Dryer:

Heto CT 60E (HETO Lab Equipment)

- Shaker:

Denley Wellmix 1

- Spectrophotometer:

Pye Unicam PU 8610 UV/VIS (Philips)

- Vortex Mixer:

Rotamixer Deluxe (Hook & Tucker)

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