

INVESTIGATION OF GASTROINTESTINAL
MUCOSAL IMMUNITY AND INFLAMMATION
IN CHILDREN

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Dedication

This thesis is dedicated to Gita without whose love and support this would never have been completed.

Declaration

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

13/3/97

Acknowledgements

First and foremost I must thank my supervisor Professor Anne Ferguson for her help, support and enthusiasm throughout the three years that I spent working in the GI laboratory at the Western General Hospital.

My deepest gratitude goes to all the staff in the GI laboratory. The friendly, helpful and open attitude made my time there an enjoyable and stimulating period of my working life.

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Abstract

In this thesis I have introduced a new technique, whole gut lavage (WGL), for the study of gastrointestinal secretory immunity in children. Initially I arranged to collect specimens from children, undergoing whole gut lavage prior to colonoscopy or surgery, at the Royal Hospital for Sick Children, Edinburgh. Whole gut lavage has been used to treat severe constipation and so I organised a study to look at the effectiveness of this, intending both these groups of children as immunologically normal controls. Analysis of specimens from the first four severely constipated children showed that the total IgA levels in all were very low. I went on to examine some possible reasons for these low IgA levels including mucosal IgA deficiency, dilution or degradation by other factors in the bowel lumen. Having collected specimens from control children I then arranged a study of intestinal secretory immunity and inflammation in children with cystic fibrosis (CF). This was stimulated by a paper in the Lancet suggesting that CF children, taking high dose pancreatic enzyme supplements, had developed strictures of the ascending colon possibly due to direct toxic effects of these medications. As CF children have chronic lung infections I then studied the possible influence of respiratory secretions on assays of whole gut lavage fluid by measuring concentrations of immune factors in sputum. With the data from these patient groups I was able to analyse, in some detail, the clinical aspects of whole gut lavage in children.

Although I had established that WGL could be an ethical and useful method for research in children it was clear, for clinical reasons, this could not be used for the study of acute diarrhoeal illness, one of the most common paediatric problems involving the gastrointestinal mucosal immune system. With the help of adult patients I directly compared outputs of immune factors in faeces and whole gut lavage. This showed that faeces had very much lower daily outputs than found in coresponding WGL, but in patients with active inflammatory bowel disease (and thus diarrhoea) the daily outputs in faecal material were more representative. I went on to compare outputs of immune substances in faecal material from adults with ileostomies, as a

model of diarrhoea. The recovery of immunoglobulins A and M in ileostomy effluent, as a percentage of the output measured in whole gut lavage, was shown to be more representative than the groups in the previous study, and seemed to increase the higher the water content of the ileostomy effluent.

Following this I collected acute watery diarrhoeal specimens from children in Sierra Leone, West Africa. By measuring the rate of passage of diarrhoea it was possible to estimate the daily secretion rate of immune factors in the diarrhoeal specimens. These were compared with daily secretion rates, measured in whole gut lavage, in UK control children, control African children (using data collected previously by Dr Mary Hodges and collated by myself), and the UK children with cystic fibrosis. These are the first figures to estimate the daily intestinal secretion rates of immune factors in children.

During the course of the thesis I introduced a new assay for eosinophil cationic protein, to allow study of the influence of eosinophils in these and other gastrointestinal mucosal disorders.

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Abbreviations

A1AT	Alpha-1 anti-trypsin
AGA	Anti-gliadin antibodies
Alb	Albumin
BLG	Beta-lactoglobulin
BSA	Bovine serum albumin
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
DEA	Diethanolamine
DIOS	Distal intestinal obstruction syndrome
E.Coli	<i>Escherichia coli</i>
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
GE	Granulocyte elastase
GI	Gastrointestinal
Hb	Haemoglobin
HDES	High dose enzyme supplements
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-8	Interleukin-8
LDES	Low dose enzyme supplements
LREC	Local Regional Ethical Committee
OD	Optical density
Ova	Ovalbumin
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
QC	Quality control
RANTES	<u>R</u> egulated on <u>a</u> ctivation, <u>n</u> ormal <u>t</u> cells, <u>e</u> xpressed and presumably <u>s</u> ecreted
RHSC	Royal Hospital for Sick Children (Edinburgh)
RIA	Radioimmunoassay
SL	Sierra Leone
TNF	Tumour necrosis factor
Tween	Polyoxyethylene sorbitan
UK	United Kingdom
WGH	Western General Hospital (Edinburgh)
WGL	Whole gut lavage
WGLF	Whole gut lavage fluid

l	Litres
ml	Millilitres
μ l	Microlitres
kg	Kilograms
mg	Milligrams
μ g	Micrograms
ng	Nanograms
pg	Picograms
cm	Centimetres
mm	Millimetres
μ m	Micrometres
u	Units
BPU	BP units

Presentations and Publications Based upon this Thesis

Gut inflammation in children with cystic fibrosis on high-dose enzyme supplements

N M Croft, T Marshall, A Ferguson

Lancet 1996 347, 327 (letter)

Gut inflammation in children with cystic fibrosis on high-dose enzyme supplements

N M Croft, T Marshall, A Ferguson

Lancet 1995 346, 1265-1267

Direct assessment of gastrointestinal inflammation and mucosal immunity in children with cystic fibrosis

N M Croft, T G Marshall, A Ferguson

Postgraduate Medical Journal 1996 72 (supplement 2), S32-S36

Technical report: results of immunological tests on faecal extracts are likely to be extremely misleading

A Ferguson, K A Humphreys, N M Croft

Clinical and Experimental Immunology 1995, 99, 70-75

Presentations/Posters

Daily intestinal secretion rates of immunoglobulins A and M in British and African children

N M Croft, T G Marshall, M Hodges, L H Kabba

ESPGAN Young Researchers Forum, January 1996

Evidence for gastro-intestinal mucosal immune activation in children with cystic fibrosis whilst taking high dose pancreatic enzyme supplementation

N M Croft, T Marshall, A Ferguson

Proceedings of British Paediatric Association Annual Meeting 1995; Vol 67:43

Factors which influence the degradation or survival of IgA in the distal gut

N M Croft, N Caputo, K Humphreys, L H Kabba, A Ferguson

International Congress of Mucosal Immunology, San Diego, July 1995

INTRODUCTION

Background

Antibodies were detected in the faeces of rabbits by Besredka in 1919. These copro-antibodies were not accompanied by similar antibodies in the corresponding serum. Accordingly, the concept of the gastrointestinal mucosal immune system as a distinct and separate entity from the humoral immune system was established and has since gained wide acceptance.

Acute and chronic diarrhoeal illnesses are among the most serious problems in international child health, and diarrhoea is also a common cause of morbidity and referral in UK paediatric practice. Understanding gastrointestinal mucosal immunity is of fundamental relevance to many of these conditions.

However research in this field has always been hampered by the fact that the organs of interest are not readily accessible to direct investigation.

Current ways to investigate gastrointestinal immunity in humans require the collection of a variety of specimens including serum, saliva, jejunal fluid, jejunal or colonic segmental perfusion specimens, mucosal biopsies and whole gut lavage fluid (WGLF).

All these methods have practical and/or theoretical problems:

1. some techniques provide information on a localised area or segment of the gut;
2. samples may be of regions or mucosal organs remote from the bowel;
3. most are invasive - particularly relevant to studies in children, for which ethical issues have also to be addressed; and
4. the baseline or background material or reference (e.g. time) against which to relate and standardise the substance being measured, may be difficult to define.

Whole Gut Lavage

Whole gut lavage (WGL) is a method of clearing the bowel of all retained faeces by per-oral administration of a non-absorbable, isosmotic fluid. This technique is widely

used in adults and children for preparing the bowel prior to gastro-intestinal surgery or colonoscopy. Elson adapted WGL in order to collect intestinal secretions from mice for immunological studies (Elson *et al* 1984), and Gaspari then applied this method to adult use (Gaspari *et al* 1988). Research staff in the GI Laboratory at the Western General Hospital in Edinburgh have, in recent years, used this technique in many studies of gastrointestinal immunity including research on inflammatory bowel disease, coeliac disease, *Salmonella* infection and vaccine (O'Mahony *et al* 1990; O'Mahony *et al* 1991a; O'Mahony *et al* 1991b; Choudari *et al* 1993b; Ferguson & Sallam, 1992).

The use of WGL offers a number of potential advantages. It is possible to study the entire gastrointestinal tract including relatively inaccessible areas such as the majority of the small bowel. Transit of fluid is rapid, and degradation and loss of molecules of interest (e.g. from exposure to pancreatic or bacterial proteases) can be prevented or reduced by treating the resulting effluent with anti-proteases immediately after being collected. Recent studies by Dr Jamal Sallam and Mr Gordon Brydon in the GI laboratory have shown that when the procedure is supervised by an experienced nurse, with a standard protocol, WGL functions as a whole gut perfusion system (Sallam, 1995). Rates of production of a variety of substances, such as IgA, can be calculated and results obtained are similar to those reported with the invasive, closed loop, segmental perfusion systems.

The main disadvantage is that the procedure can take several hours, and requires the patient's cooperation. It would be classified as invasive when a naso-gastric tube is required. These are significant considerations in children, especially when the procedure may be being considered for use in a research study rather than when there is clinical need. It is important to note that though immune abnormalities can be detected using WGL, other methods are needed to locate the site of the problem within the bowel.

Aims and Methods

My aim in this work has been to explore the use of whole gut lavage for the investigation of intestinal mucosal immunity and inflammation in children. Specific aims were:

1. To consider ethical aspects of using whole gut lavage for research purposes in children in the United Kingdom.
2. To study the practicalities of whole gut lavage in children, both for clinical need and research purposes.
3. Using whole gut lavage fluid as a reference material, to examine whether tests on faeces can give information on gut mucosal events. For this aspect of my work I used adult volunteers.
4. To study aspects of intestinal immunity in children with cystic fibrosis and acute diarrhoeal disease.

My predecessors in the GI laboratory have developed and evaluated assays for many different immune substances in WGLF. There have been no previous studies of eosinophils and so, in view of the importance of these cells in gut allergy and tissue inflammation, I explored methods to study eosinophils using one of their products, eosinophil cationic protein (ECP).

Practical Aspects of the Clinical Investigation Technique

At the start of my studies WGL was rarely used in the RHSC, Edinburgh. Preparation for colonoscopy or surgery involved three days of clear fluids and laxatives prior to the examination. WGL had never been used in the treatment of constipation.

I established a system for collecting and processing specimens from children undergoing WGL for established clinical reasons. The surgeons at the RHSC agreed to use WGL for the preparation of some children undergoing colonoscopies. I also read that WGL could be a useful treatment for severe constipation and so set up a study in the RHSC looking at the use of this. I was thus able to introduce the

technique to paediatricians in Edinburgh and also collect and process some specimens. Specimens from both these sets of children were intended as control specimens.

Cystic Fibrosis

A specimen collected from a child with cystic fibrosis who was having WGL as treatment for distal intestinal obstruction syndrome (DIOS) had grossly abnormal immune parameters. Four months later reports of a new problem of intestinal strictures in children with cystic fibrosis, taking high dose pancreatic enzymes, were published. The local regional ethical committee approved an application to use WGL to investigate GI mucosal immunity and inflammation in CF children whilst on high dose and then low dose pancreatic enzyme supplements. This is the first time that WGL has been approved for research in children in the United Kingdom.

One problem that has not been encountered in previous WGL studies is the possibility that the significant differences were due to ingested respiratory secretions. By collecting sputum from some of the cystic children I planned to estimate the amount of sputum that would have to be swallowed per hour to cause the concentration of a substance in the WGL specimen. This allowed rational judgement to be made on the potential influence of ingested sputum.

Diarrhoeal Disease and Analysis of Watery Faecal Specimens

It became clear that despite the advantages of WGL the problems of administration were going to preclude the use of this technique in certain groups of children. By far the most important of these on a world-wide basis is the situation of acute gastroenteritis where the child is likely to have associated nausea and vomiting, which would make WGL both practically and ethically impossible.

Initially, in work published in collaboration with Professor Anne Ferguson and Kenneth Humphreys, we found that analysing normal faeces did not reflect IgA and specific antibody production as measured in WGL. However it seemed possible that the collection of acute watery diarrhoea is a similar situation to WGL. The difficulty of performing WGL in patients (adults or children) with acute diarrhoea and

associated symptoms such as vomiting, led to a study to compare the output of immune substances in WGL to that measured in output from ileostomies. This is a similar situation to acute diarrhoea in that it has a short transit time and an increased water content. Evidence confirming that ileostomy fluid is comparable to WGL would lend support to the theory that diarrhoeal fluid can also be representative of results found in WGL.

I then arranged to collect samples of acute watery diarrhoea from Edinburgh children. Unfortunately specimen collection was not as straightforward as expected as the delay from the time of the initial symptoms to referral to the infectious disease ward meant the patients had usually recovered. Thanks to contacts in Freetown, Sierra Leone I was able to set up a new collaboration with Dr Kabba at the National Rehydration Training Centre. In this centre I was able to collect freshly passed, watery diarrhoeal specimens, process them as in lavage specimens, and then aliquot and store them for transport back to Edinburgh. I also measured over a timed period the output of diarrhoea so that estimates of the daily output of factors could be made.

Whilst there on my first visit I collated data on the practical aspects of WGL in African children who had participated in a research project prior to my starting. I also took the opportunity to ask the mothers and children what they felt about undertaking WGL for research which will be discussed in the chapter on ethics.

Eosinophils in GI Diseases

Eosinophils have been implicated in number of gastrointestinal diseases including parasitic infestations, allergies, and the strictures found in children with cystic fibrosis. I proceeded to investigate the role for eosinophils in groups of patients which included African children with parasites and acute diarrhoea, children with cystic fibrosis and control children. I was able to do this by adapting an assay for eosinophil cationic protein (ECP) for lavage and diarrhoeal specimens.

Structure of this Thesis

In the first section of this thesis intestinal mucosal immunity is introduced and current and past methods of investigation in both adults and children are reviewed. Ethics of research in children are also considered here. Literature reviews relevant to specific studies such as cystic fibrosis and faeces versus whole gut lavage are included at the start of the respective chapters.

The second section discusses the practical details of whole gut lavage in children and then the collection, processing and analysis of WGL and other specimens used in this thesis. Technical developments of the assays used including ECP are included in this section.

Section three describes the results of WGL analyses in control children and results of the studies in children with cystic fibrosis.

Section four describes the comparison of outputs of immune substances in faecal and ileostomy specimens with WGL. This includes the chapter summarising the estimated daily outputs of factors in different groups of children including those with acute watery diarrhoea.

Finally I discuss the general implications of this work.

***SECTION I: Background Literature Review and Ethics of
Research in Children***

Chapter 1: Background Literature Review

Investigation of GI Mucosal Immune System

Historical Overview

The first person to realise that the secretory immunity of the gastro-intestinal mucosa was a distinct immunological entity to serum was Besredka who demonstrated that rabbits, after oral immunisation with killed *Shiga* bacillus, were protected against fatal dysentery by coproantibodies that appeared irrespective of the serum antibody titre (Besredka, 1919). Three years later Davies was able to show agglutinins against the dysentery bacillus in faeces several days before they were detectable in serum (Davies, 1922).

In the 60's Tomasi demonstrated that the predominant immunoglobulin in external secretions is secretory IgA (Tomasi, Jr. *et al* 1965). It is since the sixties that the field of mucosal immunology has developed as a distinct subset of immunology and major advances have been made.

In children a large proportion of the most common diseases involve an interface between a mucosal surface and pathogens or antigens. In infections of the gastrointestinal and upper and lower respiratory tracts, the first line of defence is the mucosal surface and it is the failure of this surface to prevent infection that ultimately leads to symptoms and illness. Other common diseases that involve the gastrointestinal mucosal surface include food allergies/intolerance (such as coeliac disease) and inflammatory bowel disease (Crohn's disease and ulcerative colitis).

Immunological mechanisms are an integral aspect of the defence system however there are also non-immunological factors that are important. These include indigenous gastrointestinal tract flora, mucus layer, peristaltic motion, lactoferrin, lysozyme and bile salts (Hill & Meier, 1986).

Despite the mucosal surfaces being of great importance and interest in these illnesses, investigation of them is hampered by the problems of directly collecting suitable

specimens. Thus improvement of techniques to study how the mucosal immune system works and fails is essential for the understanding of the aetiology and pathogenesis of these illnesses.

Methods for Investigation of GI Mucosal Immunity

A number of different methods have been used for the investigation of the GI mucosal immunity, all have advantages and disadvantages. No techniques answer all the potential problems and so it is essential to consider these when reviewing results.

These problems can be categorised into

1. Invasiveness of the technique
2. Remote/direct collection of specimens
3. Standardisation

I will now discuss the various methods that have been used to assess gastrointestinal mucosal immunity, with regard to each of these three categories.

Serum/plasma

Blood is easy to collect and standardise although it is an invasive method. Many assays can be performed on these specimens including immunoglobulins and antibodies, cytokines and antibody secreting cells.

The main problem of blood samples is that they are remote specimens and how they relate to what is happening at the mucosal level is difficult to ascertain.

A large number of papers have attempted to use the serum immunoglobulin and antibody response as a measure of the local mucosal immune response (La Brooy *et al* 1982; Kelly *et al* 1991; Grimwood *et al* 1988; Losonsky *et al* 1988; Stoll *et al* 1986; McLean *et al* 1980; La Brooy *et al* 1980; Johnson *et al* 1992; Kuitunen & Savilahti, 1994; Siddons & Chapman, 1995). These can be of use in following the course of GI infections (Bernstein *et al* 1989; Hjelt *et al* 1986) or responses to oral vaccines (Jertborn *et al*, 1986), however a number of studies have found disparate results between serum samples and the findings of directly collected specimens (Kelly *et al*

1991; Stoll *et al* 1986; O'Mahony *et al* 1991a; Coulson *et al* 1990; La Brooy *et al* 1982; Jertborn *et al* 1986).

More recently, circulating antibody-secreting cells have been shown to demonstrate both a polyclonal and specific response to bacterial diarrhoea (Kantele *et al* 1988). The cells are circulating plasma cells that are thought to have been exposed to antigen in the gut associated lymphoid tissue and are migrating back to both intestinal and non-intestinal mucosa.

Saliva

Saliva, although part of the GI tract, has been found to have disparate responses to directly collected specimens from other parts of the GI tract (Kelly *et al* 1991; Ward *et al* 1992; Jertborn *et al* 1986). It is possible to collect saliva from children, but can be difficult in the younger age groups. Standardisation is also a problem as the secretion of salivary fluid varies depending upon whether the subject is fasting.

Small Bowel Aspirates

Collection of secretions from the small bowel is a frequently used method for investigation of GI mucosal immunity (La Brooy *et al* 1980; Hjelt *et al* 1988; Zinneman & Kaplan, 1972; Lebenthal & Clark, 1981; Lebenthal *et al* 1980; Savilahti, 1972). This involves passage of a tube to the small bowel, which is invasive. The area collected from is localised to the area from which the fluid is obtained. No assessment can be made of the bowel distal to the end of the sampling tube.

Biopsies (small/large bowel)

Biopsies of tissues can involve the counting of cell types such as lymphocytes (Ferguson & Murray, 1971), plasma cells (Savilahti, 1972), and antibody secreting cells (VanCott *et al* 1994). This is relatively easy to standardise using image analysis and counting the number of cells per unit area. However the collection of the specimens is invasive, requiring intubation of the bowel to gain the biopsy. Biopsies

also only look at a very localised area of the bowel so that in illnesses where abnormalities are patchy, it is possible to get false negative results.

One study failed to find any correlation between the number of IgA secreting cells found in a biopsy and the concentration of IgA in locally collected intestinal juice suggesting that findings in biopsies do not necessarily equate to what is secreted (Savilanti, 1972).

Balloon Perfusion Techniques (small bowel/large bowel)

Double and single balloon segmental perfusion techniques (Knutson *et al* 1989) have been developed over the last few years as methods of collecting localised secretions and preventing exposure to degradative substances. Both small (Jonard *et al* 1984; Colombel *et al* 1990) and large (Prigent-Delecourt *et al* 1995) bowel studies have been performed using this technique, measuring the secretion of a variety of substances such as immunoglobulins, antibodies, plasma proteins (Jonard *et al* 1984; Colombel *et al* 1990), eosinophil cationic protein, hyaluronan, histamine and myeloperoxidase (Knutson *et al* 1993; Hallgren *et al* 1989).

These are the best accepted methods for quantifying rates of secretion of substances from the GI mucosa, however this is both invasive and only applies to a localised area. When calculating secretion rates for the whole gut, assumptions have to be made as to the length of the bowel and the uniformity of the secretion rates throughout. These techniques are good for direct challenges of food antigens which can be administered in a controlled way looking for immediate immune reactions.

Washings (large bowel)

Another method for investigating intestinal mucosal immunity is the collection of intestinal washings (Marcucci *et al* 1985). Fluid is administered in the same way as an enema and left in the rectum/colon for a fixed length of time. It is then aspirated and stored for analysis. This method is both localised and invasive.

Faeces/Diarrhoea

Faecal and diarrhoeal specimens have the major advantages that they are easy to collect, non-invasive and are made up of substances originating from the entire gastro-intestinal tract. However immunological (and other) analyses of faecal material are complicated by a number of variables:

1. Degradation within the gastro-intestinal tract.
2. Transit time of secreted substances through the bowel.
3. Reference standard against which to express the results.
4. Whether single stool concentrations are representative of secretion throughout the day.
5. Extraction of substances to be analysed from faecal material.

1. Degradation

Substances of interest are proteins and can be degraded to a greater or lesser extent in the GI tract either by acidity in the stomach or by proteolytic enzymes from the pancreas or bacteria in the colon (Haneberg & Aarskog, 1975).

Immunoglobulins

In vitro, secretory IgA is resistant to tryptic digestion but not to peptic digestion (Samson *et al* 1973). Increased resistance to proteolysis (compared with non-secretory IgA) is felt to be partly due to the presence of secretory component (Lindh, 1975). Using radio-immuno-diffusion (RID) for the estimation of IgA in intestinal fluid leads to an overestimation of the concentration of IgA measured, because of the reduction in size of the fragments being measured (Samson *et al*, 1973). Analysis using ELISA, as will be shown in chapter 5, shows a reduction in the measured concentration following degradation in intestinal fluid.

Both secretory and dimeric IgA, the most prevalent forms of IgA in the GI tract, have been shown to be more resistant to proteolysis than monomeric IgA (Lindh, 1975; Underdown & Dorrington, 1974).

In one study, faeces was stored at 20⁰C for up to 96 hours without any significant reduction of total IgA in extracts (Haneberg & Tonder, 1973). It is possible that this apparent lack of degradation is because most of the digestion of the IgA has happened within the colon prior to the stool being passed and the first extract being prepared.

Studies have attempted to estimate the survival of ingested IgA during passage through the gastrointestinal tract in neonates and infants. The estimated amount recovered has varied from 70% in neonates (Ogra *et al* 1977) to 30% in older infants (Prentice *et al* 1989). A possible reason for this difference is that the neonates, who had the study performed within 72 hours of birth, had not developed the system of protein digestion found in older infants (Britton & Koldovsky, 1989). In the study of older infants (Prentice *et al* 1989) the suggestion by another group that breast feeding can directly stimulate IgA secretion in the GI tract (Koutras & Vigorita, 1989) could have led to an overestimate of the ingested IgA recovered. Prentice also found that daily output of IgA in faeces increased with increasing stool frequency and concluded that the majority of breakdown occurs in the large intestine (Prentice *et al* 1989). Five children with acute diarrhoea also had very high daily outputs of IgA.

Gaspari *et al*, when developing the technique of whole gut lavage for research into the secretory immunity of the gastrointestinal tract, found that delaying the addition of protease inhibitors for two hours reduced the measured IgA content (using ELISA) by between 0 and 62 % (mean 31%) (Gaspari *et al* 1988). They comment that longer delays, with or without refrigeration, 'resulted in even greater losses of IgA'.

In the GI laboratory at the WGH, O'Mahony found that there was a loss of IgA content of between 0 and 92% by delaying the addition of protease inhibitors to nine lavage specimens kept for up to 2 hours (O'Mahony *et al* 1990). The graph in the paper demonstrates that 6/9 specimens reduced by less than 10 %, well within the range of error for the assay. He also does not note the initial values for the IgA, if these had been very low in the specimens with the highest degradation then the effects of delaying addition of the protease inhibitors, expressed as percentages, may have been exaggerated.

In chapter 5 I have investigated further the rate of degradation of IgA in WGLF kept at 37°C and found that this was similar in all five specimens from 100% at 0 hours, to 82% at 2 hours, to 44% at 8 hours to 22% at 24 hours.

In summary secretory IgA, which constitutes the majority of the IgA in lavage fluid, is susceptible to peptic degradation however is less so to tryptic degradation. In lavage 80% of the original concentration can be detected after 2 hours, however rapid processing to reduce protease levels can only help preserve the molecules.

IgG and IgM have been found to be degraded by both trypsin and pepsin which would also lead to overestimation using RID for analysis (Samson *et al* 1973). They have also been found to be degraded in human small intestinal fluid (Plaut & Keonil, 1969).

IgM incubated in intestinal fluid at 37°C is broken down from the 19S size molecule to a primarily 9S molecule over 24 hours. At 4 hours 76 % of the original IgM remains, at 24 hours 53% remains (Richman & Brown, 1977). Almost all secretory component attached to the IgM molecule is removed within 30 minutes (Richman & Brown, 1977). In this study, although they showed smaller IgM molecules after incubation, they did not show whether this affected the measured concentration (using double antibody RIA). In chapter 5 I found that the concentration of IgM (using ELISA) went from 100% at 0 hours to 100% at 2 hours, 58 % at 8 hours and 28 % at 24 hours.

Antibodies

When analysing faecal material for antibodies the most important point is usually the presence or absence of the antibody, as the aim of the research performed is often to establish whether the specific antibody (e.g. rotavirus) is detectable. I have been unable to find any studies that have assessed the degradation of antibodies, analysed by ELISA, in faecal or lavage specimens.

In vitro, a reduction in virus-neutralising activity of bovine colostrum has been shown after incubation with both pepsin at pH 2 and pancreatic enzymes (Petschow & Talbott, 1994). They also reported evidence for proteolysis in this two stage system, although how this would affect ELISA analysis of antibodies is not known. It is

possible that ELISA could still detect fragments of the original antibody.

In chapter 5 I will show that in WGLF at 37°C, anti-Ovalbumin IgA degrades at a similar rate to total IgA with 100% at 1 hour reducing to 81% at 2 hours, 47% at 8 hours to 27% at 24 hours.

Alpha-1-antitrypsin and albumin

A1AT is resistant to proteolysis in faeces (Davidson & Lonnerdal, 1990) however can be susceptible to peptic breakdown in an acidic environment (Davidson & Lonnerdal, 1990; Moran *et al* 1995).

Unpublished work in the GI laboratory, WGH, has shown that albumin is very susceptible to proteolytic breakdown in WGL fluid that has not been treated with protease inhibitors (G.Brydon, PhD Thesis, University of Edinburgh 1996).

2. Transit Time

When considering the degradation of molecules in gastrointestinal fluids the duration of exposure to degradative processes must be considered. Exposure within the bowel will vary considerably, depending on the time for materials to travel through the GI tract. The whole gut transit time (WGTT) is reduced in diarrhoeal disease and increased in constipation. Whole gut transit time also varies widely within the normal healthy population. In normal faecal specimens immunoglobulins secreted in the small bowel could have been exposed to degradative enzymes within the gastrointestinal tract for 24 or more hours. Antibodies secreted in the bowel thus may be degraded so that they are not detectable in faecal material. However in the acute diarrhoeal state the duration of exposure within the bowel to degradative enzymes may be considerably reduced so that antibodies can be detectable in diarrhoeal extracts. This issue, that the presence of antibodies may depend upon the transit time through the bowel (and the form of the stool) has never been investigated.

3. Reference Standard

When attempting to interpret results of other studies one fundamental problem is the presentation of results and their reference ranges. Studies have tended to present their

results either as milligrammes per gramme of faeces (wet weight) (Koutras & Vigorita, 1989; Moran *et al* 1995), or per gramme of faeces (dry weight) (Haneberg & Tonder, 1973). Variables such as the amount that the subjects eat or drink is likely to affect the weight of faeces without necessarily altering the amount of the immune substance being secreted in the GI tract. Other methods of expressing results of antibody or immunoglobulin assays include as a ratio over total immunoglobulin (La Brooy *et al* 1982), or total protein (Bull *et al* 1972).

The simplest means of expressing results to allow direct comparisons between different types of specimens from the gastrointestinal tract is to estimate outputs (in fixed units such as microgrammes) over a fixed period of time (Prentice *et al* 1989; Schanler *et al* 1986). The exception to this is in perfusion studies where direct comparisons can be made of the concentration of the substance in the perfusion fluid if the rate of administration of the fluid is the same between patients. In this situation the output over a particular time can also be calculated.

4. Single stool versus output

It has been shown that analysing single stool specimens correlates in a linear fashion with estimation of output of IgA (in faeces) in three day collections (Haneberg & Aarskog, 1975). Thus single specimens of faecal material can be used for estimation of IgA outputs. In this study they also found that there was little variation in the faecal IgA concentrations from day to day in the same individuals.

5. Extraction of substances to be analysed

The extraction of immunoglobulins from faecal material varies between different studies. It has been found that the majority of IgA is extracted in a single extraction of faeces by using a 1/11 extraction (weight by volume, i.e. 1 gramme of faeces plus 10 ml of phosphate buffered saline) (Haneberg & Tonder, 1973). However stools with a higher water content such as diarrhoeal specimens may be equally well extracted with larger weight to volume ratios such as 1/5.

Table 1.1: Summary of disadvantages of techniques for investigating the GI mucosal immune system.

Specimen	Disadvantage		
	Invasive	Difficult to Standardise	Localised / Remote
Serum/plasma	Yes	No	Yes
Saliva	No	Yes	Yes
Small Bowel Aspirate	Yes	Yes	Yes
Small/Large Bowel Perfusion	Yes	No	Yes
Biopsies	Yes	No	Yes
Faeces/Diarrhoea	No	Yes	No
Whole Gut Lavage	Yes*	No	No

* If using naso-gastric tube.

Whole Gut Lavage for Clinical and Research Use

History of WGL

In 1973 Hewitt et al described a method for preparing patients for large bowel surgery using whole gut irrigation (Hewitt *et al* 1973). The major reason for their interest in this technique was the high incidence of wound sepsis after bowel surgery in their hospital. This was based on a method that had been described for investigating intestinal ion flux in patients with cholera (Love *et al* 1968). The solution that they used was a combination of water, sodium chloride, potassium chloride and sodium bicarbonate. They had recognised the problems of fluid absorption in the patients (estimates range between 1.5 litres per hour (Davis *et al* 1980) to 2.4 litres over 3 hours in healthy volunteers (Love *et al* 1973)), and excluded patients with impaired heart or kidney function. The large quantities required (10-12 litres) in these forms of bowel irrigation (Keighley, 1993) and the salty taste meant that the solutions were always given by naso-gastric tube.

The problems of the fluid shifts by using the above solutions were approached by adding osmotic agents to the irrigation fluid such as mannitol (Keighley, 1993; Davis *et al* 1980) and polyethylene glycol (Davis *et al* 1980). Mannitol as a fermentable compound could theoretically be a source of explosive gases such as hydrogen and so an inert compound such as PEG is felt to be safer (Davis *et al* 1980).

PEG based Lavage Solutions

Polyethylene glycols are group of compounds with a wide range of molecular weights (400-4000) (Ma *et al* 1990; Brady *et al* 1986). The size used as the osmotic agent in intestinal lavage solutions is most commonly PEG 3350 which is excreted virtually unchanged in the urine and is minimally absorbed during whole gut lavage (Brady *et al* 1986). Since their first description (Davis *et al* 1980) PEG based solutions have become widely available and accepted for clinical use in cleansing the bowel for a variety of indications. There have been minor alterations in recent years with regard to

the sodium content (Fordtran *et al* 1990) and flavouring (Froehlich *et al* 1991) in order to make the solutions more palatable for the patients.

Use of PEG solutions in adults

Clinical

PEG based whole gut lavage solutions are used for a range of indications in adults. These include preparation for surgery (Keighley, 1993; Beck & Fazio, 1990; Beck *et al* 1985), colonoscopy (Cohen *et al* 1994; Rösch & Classen, 1987), and barium enema (Ernstoff *et al* 1983), and as a treatment for constipation (Andorsky & Goldner, 1990). Whole gut lavage has also been used for the treatment of drug ingestion (Smith *et al* 1991; Makosiej *et al* 1993; Hoffman *et al* 1990; Hassig *et al* 1993; Burkhart *et al* 1990; Buckley *et al* 1993).

Diagnostic

In diagnostic use WGL has also been found to be useful in assessing intestinal bleeding (Brydon & Ferguson, 1992) and the assessment of active inflammatory bowel disease by measuring plasma derived proteins (Brydon *et al* 1993). Recent papers have found that it may be possible to use cytology of WGL fluid in the diagnosis of colonic carcinoma (Gordon *et al* 1991; Wuerker *et al* 1993; Brandt & Greenebaum, 1989). In one study, out of 33 patients being investigated for carcinoma of the colon, 15 were confirmed at colonoscopy. 14/15 of these patients had positive lavage cytology (Rosman *et al* 1994).

Research

Whole gut lavage, using a saline based solution for immunological research, was first used in Bangladesh (Stoll *et al* 1986). However there were considerable problems of fluid absorption, taste and the large volume required. With the development of polyethylene-glycol based electrolyte lavage solutions, fluid absorption and the volume required to collect appropriate specimens have been greatly reduced.

PEG based lavage solutions were first used for immunological research in mice by Elson (Elson *et al* 1984), later Gaspari (Gaspari *et al* 1988) adapted the use of these for adults. Since then most published work using this technique for immunological research have been performed in Professor Ferguson's department based in the Western General Hospital in Edinburgh (O'Mahony *et al* 1990; Brydon *et al* 1993; O'Mahony *et al* 1991a; Brydon & Ferguson, 1992; O'Mahony *et al* 1991b; Handy *et al* 1995).

Work performed by Dr J.A.Sallam (Sallam, 1995) has confirmed that whole gut lavage is a perfusion system with regard to total IgA, specific IgA (anti-*salmonella typhi* (LPS)), total IgM, total IgG, albumin, and alpha-1-antitrypsin.

Research by other groups include the assessment of intestinal responses to oral vaccines and gastrointestinal infections (Jertborn *et al* 1986; Stoll *et al* 1986; Ahren *et al* 1993) and in studying immune responses in inflammatory bowel diseases (Levy *et al* 1995; Hommes *et al* 1995a).

Advantages

The potential advantages of WGL for immunological research into GI mucosal immunity can be summarised as follows:

1. WGL assesses the whole GI tract rather than a localised area.
2. WGL is a perfusion system and substances measured in the fluid remain at a steady state once the fluid is clear of all faecal material (O'Mahony *et al* 1990; Sallam, 1995). The secretion rate of substances over a fixed time can thus be calculated.
3. Degradation of substances is reduced as much as possible by;
 - rapid processing of the effluent immediately it is passed,
 - minimising the whole gut transit time and thus exposure to intra-luminal degradation.
4. If taken orally it is non-invasive.

Disadvantages

1. Drinking the lavage fluid can be difficult due to the large volume required, which is a major problem in children.
2. It is invasive if a naso-gastric tube is required.

Practical Details

In adults the time taken to clear the bowel of all faecal material can vary, depending upon the rate of the ingestion of the fluid and the indication for the lavage. The recommended rate for ingestion of the lavage fluid in adults is 250 ml every 10-15 minutes (ABPI Data Sheet Compendium, 1994-5) and the time taken for the lavage to be completed is generally less than 5 hours (Ernstoff *et al* 1983). The solution is usually taken orally but adults who have problems drinking the fluid can have the solution administered by naso-gastric tube.

Acceptability and Efficacy

When compared with standard methods of clearing the bowel PEG solutions are effective and preferred by adults to standard methods involving clear fluid diets, laxatives and enemas (Ernstoff *et al* 1983; Burke *et al* 1988).

In a randomised blinded study the view at colonoscopy was better in the group prepared with a PEG based electrolyte solution. For barium enemas the resulting examination was as good as in the non-PEG group (Ernstoff *et al* 1983).

The volume required to be drunk (up to 4 L) is one of the biggest problems with whole gut lavage. Some studies have found that it is possible to effectively prepare the bowel (for colonoscopy) with a lower volume of the solution by either taking 2 litres of the solution (Rösch & Classen, 1987) or a dose of senna (Iida *et al* 1992) the night before, and then drinking 2 litres on the morning of the examination. In both studies these methods were found to be more acceptable than drinking 4 litres of the solution on the day of the examination.

Whole gut lavage has also been found to preserve normal mucosal histology when compared with a standard preparation consisting of a clear liquid diet, magnesium citrate, and senna (Pockros & Foroozan, 1985). However PEG may interfere with ELISA detection of tumour associated antigens (Tobi *et al* 1991).

Side Effects

The major side effects found are those of nausea, bloating and vomiting. These problems settle with reducing or stopping the rate of ingestion of the fluid.

There are case reports of unusual complications including Mallory-Weiss tears (Santoro *et al* 1993; Raymond, 1991), urticaria (Brullet *et al* 1992), and anaphylaxis (Schuman & Balsam, 1991). It was not certain whether the anaphylaxis was due to the PEG based solution.

There is no increase in the number of colonic flora or explosive colonic gases, a potential hazard with mannitol (Ambrose *et al* 1981; Strocchi *et al* 1990). Fluid absorption is minimal (Davis *et al* 1980; Fordtran *et al* 1990), the latter study finding absorption rates in adults of 50 ml/hour.

Use of PEG based lavage solutions in children

Indications

Although not licensed for use in children in the United Kingdom, polyethylene-glycol based lavage solutions are used both in the UK, and abroad, for a similar range of indications to the adult population. This includes as a preparation for surgery (Tuggle *et al* 1987; Tuggle *et al* 1989; Konings, 1989), colonoscopy (Tolia *et al* 1984; Bichet-Sicard *et al* 1988; Sondheimer *et al* 1991) and barium enema (Bichet-Sicard *et al* 1988), and as treatment for constipation (Ingebo & Heyman, 1988; Millar *et al* 1988; Tolia, 1988; Gleghorn *et al* 1991; Tolia *et al* 1993), drug ingestion (Everson *et al* 1991) and distal intestinal obstruction syndrome in children with cystic fibrosis (Koletzko *et al* 1989).

Route of Administration

In children, because of the large quantities required, administration is more commonly performed using a naso-gastric tube. However oral administration is possible in older children (Tolia *et al* 1984). Children with severe constipation, because of the large volumes required, have always required a naso-gastric tube (Ingebo & Heyman, 1988; Millar *et al* 1988).

Rate of administration

In most studies the rate of administration of the fluid is calculated in ml/kg/hr, and the faster that the fluid is given the faster the lavage is completed (Millar *et al* 1988). The rate ranges from 14-40 ml/kg/hr. However the faster rates of administration are also associated with increasing frequency of side effects such as vomiting (Sondheimer *et al* 1991; Millar *et al* 1988) particularly above 30 ml/kg/hr.

In one study using lavage preoperatively at a rate of 25 ml/kg/hr, all the patients completed the lavage and 1/20 vomited (Tuggle *et al* 1987), the mean time for the lavage was 4 hours (range 3-7). A study of 20 patients pre-colonoscopy, using a rate of 40 ml/kg/hr, took a mean of 2.6 hours (range 1.5-5.8) in this study 4/20 vomited (Sondheimer *et al* 1991).

The ideal rate is the one that minimises the time taken and avoids side effects however there is no single rate suitable for each child undergoing WGL.

Duration of Lavage

The time required depends in part upon the indication for the lavage. Children with severe constipation and encopresis had a mean duration of lavage, to produce clear effluent, of 22.5 hours (range 7-33) (Ingebo & Heyman, 1988) and in another study 6-10 hours (Millar *et al* 1988). The most likely reason for this difference is probably the severity of constipation in the children. By comparison, in the two studies mentioned in the previous paragraph, of children pre-colonoscopy and pre-operatively, the mean times taken were 2.6 and 4 hours.

Volume Required

In constipated children the total volume required ranged from 230 ml/kg (Millar *et al* 1988) to 574 ml/kg (Ingebo & Heyman, 1988) probably a sign of different severity of the constipation in the latter study. Children having lavage pre-operatively or pre-colonoscopy required between 15.6 and 183.3 ml/kg (Sondheimer *et al* 1991), this inversely correlated with the age and weight of the patient. Another study found that children undergoing cleansing pre-operatively and pre-colonoscopy required 220 and 210 ml/kg respectively (Millar *et al* 1988).

Side Effects

The major side effects in children include nausea, bloating and vomiting. Vomiting seems to be more frequent in the studies where rates of administration greater than 30 ml/kg/hr are used (Millar *et al* 1988; Sondheimer *et al* 1991). In these studies 20-25% of patients vomit whereas in another study where lower rates are used there is a lower frequency of vomiting of 5% (Tuggle *et al* 1987). Nausea and bloating or distension are also frequent (Koletzko *et al* 1989) again related to the speed at which the lavage fluid is administered.

There have been no studies that have found any changes in biochemical parameters (Tolia *et al* 1984; Tuggle *et al* 1987; Tolia, 1988) other than mild metabolic acidosis after a prolonged lavage (Millar *et al* 1988), and clinically insignificant decreases in serum potassium, glucose (Sondheimer *et al* 1991) and urea (Lieberman *et al* 1988).

Fluid is not significantly absorbed by the patient during lavage. One study showed no change in weight before and after the lavage (Tuggle *et al* 1987), another an increase of 0.5% (Sondheimer *et al* 1991).

There is one case report of pulmonary oedema following ingestion of one litre of PEG based electrolyte solution (Paap & Ehrlich, 1993). Aspiration rather than fluid absorption and consequent fluid overload was thought to be the most likely reason for this complication.

Acceptability

No studies have compared the acceptability of lavage in children with alternative methods of cleansing the bowel. Millar stated that lavage has been ‘enthusiastically endorsed by the nursing staff’ (Millar *et al* 1988).

PEG solutions for constipation in children

When treating children with severe constipation it is essential to completely clear the bowel of all faecal material, particularly in children with large faecal masses. This is most commonly done using a combination of oral laxatives and enemas (Levine & Bakow, 1976; Saraban *et al* 1982), frequently requiring admission for a week or more. Enemas are not only a traumatic experience for the child, parents and the nursing/medical staff but are also not very reliable at clearing the bowel (Miller, 1975).

When reviewing the literature it became clear that whole gut lavage is a feasible alternative to this standard treatment, with the possible advantages:

1. Of being sure that the child is completely clear of all faecal material.
2. Reducing the length of the in-patient stay.
3. Avoiding the need for enemas.

Although there have been studies published that have demonstrated that whole gut lavage with PEG based electrolyte solutions for the treatment of constipation is safe and effective, none have compared the efficacy and acceptability when compared with standard modes of treatment.

Whole Gut Lavage at the RHSC Edinburgh

At the beginning of this study whole gut lavage was used occasionally in the surgical ward pre-colonoscopy. The more usual preparation involved three days of clear diet, laxatives and enemas. Whole gut lavage had never been used in the treatment of constipation.

Ontogeny of the Gastrointestinal Immune System

When studying the intestinal secretory immunity in children it is essential to have some knowledge of the development of the mucosal immune system. Most work in this field has involved looking at immunoglobulins mainly IgA.

Biopsies

A study by Savilahti showed that the number of IgA secreting cells in small bowel biopsies from children aged 3 months to 2 years was 80% of the levels found in 2-16 year olds (Savilahti, 1972). IgM cells were already at peak levels by 3 months of age. He later found that in neonates aged up to 12 days there were no immunoglobulin secreting cells and that IgM secreting cells were greater in number over IgA secreting cells up to 1 month of age (Perkkiö & Savilahti, 1980).

This data suggested that all immunoglobulin secreting cells are absent up to 12 days of age, with IgM cells appearing first and peaking at three months but IgA cells increasing in number up to adolescence.

Faeces

In faeces IgA is absent at birth (Koutras & Vigorita, 1989), consistent with the above data. The same study found that the rate of increase of IgA in faeces, over two months, was faster in breast fed babies than in formula fed babies. This was not felt to be due to the IgA in breast milk as the level measured in the milk tends to decrease over the same time period and the increase in faecal IgA levels was six fold. Increased intake of breast milk over this period could not explain this rise. The authors postulated that breast milk has a stimulatory role in the development of the IgA secretory system. The increase in faecal levels of IgA in the first few months agreed with an earlier study by Haneberg (Haneberg, 1974) and a more recent study in very low birthweight infants (Schanler *et al* 1986). Another study of immunoglobulins in faeces (Haneberg & Tonder, 1973) showed that IgA and IgM was present in infants greater than one month old with the levels of IgM reducing between 5 months and 2 years of age.

Small Bowel Aspirates

Savilahti reported higher concentrations of IgM and lower levels of IgA in duodenal juice of infants less than 2 years compared with older children (Savilahti, 1972).

In duodenal fluid no changes were found in the levels of IgA, IgM, and IgG, expressed as mg/g protein, measured between 2 weeks and 19 years (Lebenthal *et al* 1980). Similar results were found by Hjelt *et al* who showed no age related change in the immunoglobulins except that IgG decreased with age through childhood and the proportion of secretory IgA rose from 55-94% to >97% in adults (Hjelt *et al* 1988). They suggested that this is due to a reduction in plasma leakage from the gut mucosa with age.

Serum

Serum IgA is 25% of adult levels at 1 year, 50% at 3.5 years and reaches adult concentrations at 12 years of age (Allansmith *et al* 1968). IgM is 50% of adult levels at 4 months and adult levels by 2 years (Allansmith *et al* 1968). At birth serum IgG is at adult levels but drops over the first 2 weeks. From then the concentration gradually rises, reaching adult levels at the age of 7 years (Allansmith *et al* 1968).

Malnutrition and the Mucosal Immune Response in Children

Protein-energy malnutrition is a significant problem especially in the developing countries. The relationship of malnutrition to illnesses such as diarrhoea is difficult to establish as both are frequently present concurrently. In a review of available data Briend found that there is strong evidence for malnutrition leading to diarrhoeal illness (Briend, 1990). There was however, little good evidence for the converse, that is diarrhoea leading to malnutrition.

The method for classifying malnutrition using anthropometric data has most often been expressed as percentage of the weight for age of the reference population. Gomez then suggested a classification system; >90% weight for age being normal, 75-90% being mild or grade 1 protein energy malnutrition (PEM), 60-75% being moderate or

grade 2 PEM and <60% being severe or grade 3 PEM (Gomez *et al* 1956). Shann, in an editorial in the Lancet, commented on the use of Z scores for the expression of anthropometric data as recommended by the World Health Organisation (WHO) (Shann, 1993). Z scores are standard deviation scores calculated by subtracting the weight of the child from the median of the reference population at the same age and dividing it by the standard deviation. These Z scores are comparable between ages as -4.0 would equal the same deviation (four standard deviations below the median) for children irrespective of their age. In this thesis, when expressing anthropometric data, I have primarily used the weight for age Z score (WAZ) calculated using Anthro, anthropometric software available from the WHO.

Animal Studies

In mice deliberate protein restriction led to reduced intestinal IgA in intestinal washings but an increase in serum levels of IgA compared to controls (Mcgee & McMurray, 1988). These changes were reversed by renutrition. The response to oral immunisation was only reduced in the most severely protein restricted mice.

Humans

Malnutrition has been shown to be associated with a lower levels of IgA in tears and saliva but an increase serum IgA was found (McMurray *et al* 1977).

Another study also showed decreased levels of IgA in duodenal fluid, saliva and tears with a slightly increased serum IgA levels in severe malnutrition (Reddy *et al* 1976) but no differences in the concentration of IgM. The duodenal fluid and salivary IgA concentrations increased with renutrition (Reddy *et al* 1976).

Nasal secretory IgA has been shown to be reduced in malnutrition with increase in the serum IgA (Sirisinha *et al* 1975).

Nasopharyngeal antibody responses to immunisation have also been found to be reduced in malnourished children (Chandra, 1975), in the same study serum responses were not significantly different.

In Gambian children with PEM a decrease in the number of IgA and IgG plasma cells in intestinal biopsies (both large and small bowel) and an increase in IgM secreting cells has been reported (Green & Heyworth, 1980). This study used previous data from Savilahti as the control data. Savilahti's data was from Finnish children who may well have different levels of IgA secreting cells to normal African children given the different exposure to pathogens in their day to day lives. The same study biopsied Gambian children with gastroenteritis who had increased IgA and dramatically increased IgM plasma cells over the Finnish controls (Green & Heyworth, 1980).

In summary malnutrition appears to be associated with a reduction in the mucosal secretion of IgA or numbers of IgA plasma cells, and an increase in serum concentrations of IgA. Whether these alterations are due to malnutrition is not known as the children studied often have other conditions including diarrhoeal disease.

Eosinophils in Gastro-intestinal Mucosal Diseases

Eosinophils are granulocytes that originate in the bone marrow and migrate to the tissues (Weller, 1991). They have wide ranging functions, some protective and some pathological. Within the cells are granules that contain storage proteins which can be released into endocytic vacuoles or secreted to the outside of the cell (Weller, 1991). One of the proteins contained within the matrix of the crystalloid granules, is eosinophil cationic protein (ECP). ECP is a cationic polypeptide which has been shown to have bactericidal and helminthotoxic properties as well as being toxic to host cells (Weller, 1991). Others include major basic protein, eosinophil neurotoxin and arylsulphatase B (Weller, 1991).

Activated eosinophils have been associated with a number of diseases relevant to the gastrointestinal mucosa. These include coeliac disease (Colombel *et al* 1992; Talley *et al* 1992), allergic colitis in infancy (Machida *et al* 1994), food hypersensitivity with or without atopic dermatitis (Businco *et al* 1993; Knutson *et al* 1993; Sampson, 1987; Wahn *et al* 1993; Majamaa & Isolauri, 1995), inflammatory bowel disease (Berstad *et al* 1993; Walsh & Gaginella, 1991; Levy *et al* 1995; Benfield *et al* 1990; Choy *et al*

1990), and parasitic infections including helminths (Davidson, 1985; Sanderson, 1992; Newsome & Ebeigbe, 1991). In a study of specimens from various inflammatory fibrotic sites (retroperitoneal fibrosis, sclerosing cholangitis, sclerosing mediastinitis and pulmonary fibrosis) the authors found eosinophilic infiltration which was not found in non-inflammatory fibrosis (Noguchi *et al* 1992). Whether eosinophils caused the fibrosis in these cases is not known but may be of relevance to other potentially fibrotic conditions such as strictures in children with cystic fibrosis which will be discussed in chapter 8.

Eosinophil cationic protein has been found to be raised in faeces (Majamaa & Isolauri, 1995), serum (Wahn *et al* 1993) and jejunal perfusion fluid (Knutson *et al* 1993) in patients with food hypersensitivity. The latter two studies showing increases after food challenges. In inflammatory bowel disease increased levels of ECP have been shown in faeces (Berstad *et al* 1993) and whole gut lavage fluid (Levy *et al* 1995). In atopic dermatitis high ECP has been shown in serum (Kapp *et al* 1991). A study looking at eosinophil and neutrophil proteins in jejunal perfusion specimens showed an eight-fold increase in coeliac patients and two-fold increase in Crohn's disease (Hallgren *et al* 1989). In biopsies the same study found significant extracellular deposition of ECP in the lamina propria.

Chapter 2: Ethics of Research in Children

Definition and Introduction

Ethics, originating from the Greek word *ethos*, is defined in Dorland's Medical Dictionary (27th edition, W.B.Saunders, Philadelphia, 1988) as 'the manner and habits of man and animals'. Medical ethics from the same source is defined as 'the values and guidelines that *should* govern decisions in medicine'. The italics are mine, however as this definition makes clear the motivations behind decisions made in medicine are not absolute.

In clinical research the researcher's enthusiasm may not always be purely for the increase in understanding of the illness, hopefully leading to improvements in treatment. There may be financial inducements such as in drug therapy research, alternatively the researchers may instead be looking for personal gain from presentations and publications. All researchers should have an interest in performing good clinical research and publishing the results, but when this enthusiasm overrides the interests of the subjects being researched this is not ethical.

In attempting to use whole gut lavage for the study of children with potentially abnormal gastrointestinal mucosal immunity it was essential to assess the ethics of using this technique as it had never previously been used for research in children.

History

Although the Hippocratic Oath is only now recited and read by a minority of medical students on graduation, the central tenets still apply. There is no specific mention of medical experimentation or research however two sentences relate to clinical research in any situation: 'I will use treatment to help the sick according to my ability and judgement, but never with a view to injury and wrong-doing'; and 'I will abstain from all intentional wrong-doing and harm, especially from abusing the bodies of man or woman, bond or free'.

Some of the earliest medical experiments to be recorded in children involved attempts at vaccination in the 18th century towards smallpox and measles (Grodin & Glantz, 1994). Clearly children were at the greatest risk of illnesses such as smallpox and had lack of prior exposure to the disease. Jenner vaccinated his one year old son and later vaccinated an eight year old boy whom he subsequently challenged with smallpox material.

Claude Bernard, a French physiologist, has been described as the father of modern clinical investigation (Nicholson, 1986). He was firmly in favour of the need for experiments on man in order to improve understanding in physiology and clinical medicine. He wrote 'It is our duty and our right to perform an experiment on man whenever it can save his life, cure him or gain him some personal benefit'. He also realised that there were limits, saying in 1865; 'The principle of medical and surgical morality, therefore, consists in never performing on man an experiment which might be harmful to him in any extent, even though the result might be highly advantageous to science, i.e. to the health of others. But performing experiments and operations exclusively from the point of view of the patient's own advantage does not prevent their turning out profitably to science'.

One of the first organisations to attempt to introduce firm guidelines for the approach to research in humans, including children, was the German Ministry of the Interior in 1931 who advised that 'application of a new treatment must be considered particularly carefully if it involves infants or adolescents of less than 18 years', and 'experimentation on infants or persons of less than 18 years is forbidden even if it will only expose them to a very slight danger' (Anonymous 1980). They affirmed the experimenter's primary duty to his subject, the need for animal experimentation and the need for results to be published accurately.

These guidelines were sadly ignored in the so-called research performed on prisoners by Nazi doctors, including Mengele, during the second World War. The Nuremberg Trials in 1947, convicting a number of Nazi doctors of atrocities in the name of 'medical experimentation', led to the Nuremberg Code in which the informed,

voluntary consent of the subject was considered essential and emphasis was placed on the need to protect the rights of every individual involved in medical research.

In 1958 part of a study was published detailing the deliberate exposure to hepatitis virus of subjects at a school (Willowbrook) for severely mentally handicapped children (Ward *et al* 1958). The researchers' rationale was to observe the natural history of the disease in children that were highly likely to be exposed to the virus, which was endemic in the school. In this study parental consent was obtained, however if the parents agreed the children were assured a more rapid admission to this special unit. The dubious ethics of deliberately infecting children created grave public concern and widespread debate.

In one of the first public documents on the ethics of research in the UK, the Medical Research Council, in their 1962-1963 annual report made a statement 'Responsibilities in investigations on human subjects' (Medical Research Council, 1964), that addressed some legal and ethical problems of clinical research. In this statement they cautioned against research on minors that offered no direct benefit to the subject as consent gained solely from the parents/guardians was felt to have no basis in law.

In 1964 the Declaration of Helsinki by the World Medical Association followed on from the Nuremberg Code by recognising a place in medical research for experimentation in special populations and called for some protection for these groups, including a process of surrogate decision making. They also drew the distinction between research that is purely therapeutic and research which is essentially scientific, with no benefit to the person involved. This declaration has since been revised in 1964, 1975, 1983 and 1989.

In 1966 Beecher drew attention to 22 research projects where patients had been put at considerable risk in what he felt to be improperly conducted studies (Beecher, 1966). One year later Pappworth, in his book "Human Guinea Pigs", described 500 examples of studies that he felt were unethical (Pappworth, 1967). This included a chapter specifically dealing with 'experiments' in children. He went on to recommend

peer review for all research projects, including the presence of lay members on the ethical committee. The high media profile resulting from these two publications have contributed to the improvement in the ethical review procedures by bringing the debate to public attention and forcing hospital boards, researchers and national institutions to carefully assess the ethics of studies being performed under their authority. As both were doctors who faced opposition from their peers, the public image of doctors was improved as it was from within the profession that these critical reports originated.

The first formal requirement for ethical review of research studies was made by the Surgeon-General of the United States Public Health Service, who insisted in 1964 that before any grants could be made in support of research, that the project should be reviewed by a committee of the researcher's associates to assess the methods used to gain informed consent, the rights of the subject and the risks and benefits of the research.

Soon after this it was pointed out, in a letter to the President of the Royal College of Physicians, London, that ethical review committees were being established in British institutions that received grants from the US Public Health Service, in order to satisfy the above regulations whereas there was no such requirement for other sources of funding from within the UK (Royal College of Physicians, 1973). This anomaly led to the College committee in 1967 advising that ethical review should occur in all UK medical establishments.

Ethical Guidelines for Children

Other than the MRC statement in 1964 (Medical Research Council, 1964), which suggested that no non-therapeutic research could be performed in children, there were no other clear guidelines referring to the ethics of research in children. In 1977 a commission in the United States (National Commission for the Protection of Human Subjects of Biomedical and Behavioural Research, 1977) and in 1980 the British Paediatric Association (British Paediatric Association, 1980) published reports specifically looking at the ethics of research in children. Both said that therapeutic and

non-therapeutic research can be ethically acceptable, providing that there is approval of an ethical committee, consent with the parents and that there is risk/benefit assessment.

Serious and comprehensive discussions of the ethics of research in children as a special group have been more recent (Nicholson, 1986; Grodin & Glantz, 1994; Consent Panel Task Force, 1992). The book edited by Nicholson (Nicholson, 1986), was the publication of a report by a working group from the Institute of Medical Ethics, on the ethics of clinical investigations in children.

In 1990 a document from UNICEF, 'The Rights of the Child' also states that 'the child has the right to express his or her opinion freely and have that opinion taken into account in any procedure affecting the child'.

Latest guidelines upon which I will base my discussion of the ethics of this thesis include recent statements from the MRC (Working Party on Research in Children, 1991), BPA (Ethics Advisory Committee, 1992) and the National Council for Bioethics in Human Research (NCBHR) in Canada (Consent Panel Task Force, 1992). The Canadian report was produced in response to concerns from the paediatric community about a previous report from the Canadian MRC in 1987 (Guidelines on Research Involving Human Subjects) which were found to be confusing and unnecessarily restrictive in regard to research in children. The Canadian Paediatric Society, in a letter to the NCBHR, said of the report that "the printed recommendations were unacceptable and would be the end of research in children if applied as recommended". Thus a task force was set up by the NCBHR and the above report, with recommendations, produced.

Concepts

When assessing the ethics of research in children it is essential to clarify a number of the basic concepts.

Is it ethical to perform research in children ?

The first question to be asked is whether it is ethical to do **any** research in children. The consensus in the above recommendations is that research should be done in children. It has been argued in the Archives of Diseases in Childhood that it would be unethical not to perform research in children, thereby depriving them the benefit of improved knowledge of illnesses and treatment specific to children (Editorial 1978).

Importance of the study planned

When organising research in children the hypothesis being examined should be important and the research should never be performed if the same studies could be done in adults or animals. However the different range and severity of illnesses in adults and children mean that it is frequently not appropriate to extrapolate results between the two groups. The same is true of animal work, where the problems of finding an appropriate disease model often make animal work impossible.

Consent/Assent

Fundamental to the concept of research in children is the issue of consent or assent. In this there are both moral and legal issues to consider.

Informed consent is taken to mean a full understanding of the reasons for, implications of, and personal involvement required in participating in a research project. However as Ingelfinger pointed out in 1972 it is very unlikely that any subject, whether adult or child, truly understands all of these when agreeing to research (Ingelfinger, 1972). Almost certainly in a large number of cases the subjects agree to participate partly because they trust the researchers involved. An interesting unpublished study that suggests this is a follow up questionnaire that a colleague of mine, Dr Ben Stenson, sent to parents of premature infants that had been involved in a study comparing two doses of a surfactant for the treatment of respiratory distress syndrome. Although all had signed consent forms a minority of the parents knew what the study had actually been for, however the majority said they would happily be involved again.

In younger children consent is not feasible whereas *assent*, defined as 'to agree, voluntarily, in the absence of full comprehension, to the action or suggestion of another' (Consent Panel Task Force, 1992), can be obtained.

The legal aspects of research in children revolve mainly around the issue of consent and in particular whether parents can give consent to a procedure that will not benefit the child concerned. In practice this question has never been tested in the courts and the debates largely rely upon expert legal opinion. Dworkin reviewed this subject with regard to non-therapeutic research in 1978 (Dworkin, 1978) and concluded that '...it appears that it is lawful to conduct non-therapeutic research procedures on infants and young children provided that the following requirements are strictly observed: a) the design, details, and ethical criteria of the research are approved by the appropriate ethical committee; b) there is voluntary, informed, parental consent; c) there is no, or a minimal risk.'

The age at which children start to develop autonomy is from the age of about 7 years. Nicholson suggests that from this age the assent of the children is not mandatory, especially if there is parental consent to a therapeutic procedure (Nicholson, 1986). By the age of 14 their views and considerations should be respected as much as their parents. Similar divisions have been published in the Canadian report (Consent Panel Task Force, 1992) where they categorise the children into three groups: less than 6/7 years where parents agreement is essential, 7-14 years where parental agreement is required and assent is also required from the children, and >14 years where informed consent is required from the child concerned.

Koren et al however pointed out that many children between the ages of 10-15 years are used as baby-sitters in Canada, not necessarily with their full consent, involving a large number of adult responsibilities (Koren *et al* 1993). They compare this with the research situation where the children less than 14 years are felt to not be able to give consent on their own behalf. The authors feel that this is inconsistent and that "ethical standards may be divorced from reality and, as a result, deprive minors of important

rights.” The authors’ interest in this subject stemmed from the difficulties of arranging research into sexual and drug behaviour in adolescents without involving the parents.

The age at which children are thought to be competent to consent to a procedure can also be looked at from a legal point of view. In Scotland, the Age of Legal Capacity (Scotland) Act, 1991, says that a child under the age of 16 may give a valid consent provided that, in the opinion of a medical practitioner, he or she is capable of understanding the nature and possible consequences of the procedure or treatment. The converse is also true in that if a child in the same situation refuses consent then the proposed procedure should not be performed. In practice if the child is refusing necessary (and proven) treatment then the courts can almost certainly overrule the child. For research purposes the child’s refusal is legally valid and cannot be overruled by the parents consent, although this has never been tested in court.

The situation in England and Wales regarding consent of a child to undergo treatment was decided at the House of Lord’s, in *Gillick versus West Norfolk RHA 1985*. The decision was that treatment could be given to a child under 16, against the wishes of the parents, providing that the child understood the nature of the of the proposed treatment. The treatment under consideration was the oral contraceptive pill.

Therapeutic/Non-therapeutic Research

Many people consider therapeutic research to imply that the subject will receive potentially beneficial treatment. Nicholson (Nicholson, 1986) defines *therapeutic research* as ‘research consisting in an activity which has also a therapeutic intention, as well as a research intention, towards the subjects of the research’, and *non-therapeutic research* as research ‘...which has not also a therapeutic intention.’

Therapy was defined as ‘all elements of a doctor’s duty of care to a patient’. This includes working up to the diagnosis with history taking, examination, investigations, making a diagnosis and assessing the prognosis.

If research involves some potential benefit for the children, assuming that it is safe and not going to cause more harm than benefit, it would be considered ethical. Over the years the biggest debate has been over non-therapeutic research in children. The most

recent guidelines agree that non-therapeutic research can be ethical in children if certain criteria are met. The BPA say that there should not be more than a minimal risk (Ethics Advisory Committee, 1992), the MRC guidelines “..no more than ‘minimal’ or ‘negligible’ risk” (Working Party on Research in Children, 1991). Healthy children who are included as controls should be considered in the non-therapeutic category when assessing the risk/harm/benefit ratio.

Invasive/Non Invasive

Research is considered to be invasive if there is ‘an entrance of any sort into a body’ (Nicholson, 1986).

Risk/Harm/Benefit

There is no widely accepted objective method of assessment of risks/harm/benefit in medical treatment or research.

Risk is “the probability of occurrence of hazard or danger” (Consent Panel Task Force, 1992), and **benefit** is “...a good or an advantage, that which promotes well-being (physical, intellectual, psychological, social, spiritual...)” (Consent Panel Task Force, 1992). Benefit can be applied to an individual child, children as a whole or specific groups of children.

Harm has been defined as “physical or mental ‘hurt’; an unwanted and unpleasant disturbance of the person” (Consent Panel Task Force, 1992). This included pain, anxiety and injury as aspects of hurt.

To assess the risk of a procedure one method is to compare the risk of the procedure to the risks of every day life. All children, and their parents on their behalf, take risks every day in crossing the road, when walking to the park, cycling etc. Nicholson details a number of these in his book in a chapter on risks and benefits in research on children (Nicholson, 1986). For instance, he quotes the risk of fatality to people travelling by bicycle, per 100 miles travelled as 14 per million. He also says that there is a 1 in a million risk of death from simply being a boy, aged 12, for one day.

Alternatively a risk of death has been estimated at 1:1,000,000 for all childhood immunisations (Papadatos, 1989).

Nicolson also estimated that the risk of severe complications from insertion of a nasogastric tube is less than 10 per million (no definitive data was available), although there is almost always some discomfort when the tube is passed.

For non-therapeutic research in children, the Scottish Office guidelines (Home and Health Department, 1992) for Local Research Ethics Committees state that the child should be exposed to no more than minimal risk as a results of the research. However they do not define exactly what minimal risk is.

Nicholson compiled a table of British and American definitions of risk (table 2.1 below) (Nicholson, 1986). Using this arithmetic model allows some objective assessment of risk especially when compared to the risks of everyday life as shown above. The main problem with this method is the lack of data available for new treatments or diagnostic tests to calculate the frequencies of complications.

Table 2.1: British and American Definitions of Risk (Nicholson, 1986)

British Definition	Negligible	Minimal	More than Minimal
American Definition	Minimal	Minor increase over minimal	Greater then minor increase over minimal
	<u>Risks (per million)</u>		
Risk of Death	<1	1-100	>100
Risk of Major Complications	<10	10-1,000	>1,000
Risk of Minor Complication	<1,000	1,000-100,000	>100,000

The recent BPA guidelines describe risks in terms of being minimal, low or high (Ethics Advisory Committee, 1992).

- Minimal includes questionnaires, collection of urine (not by aspiration), and assays of blood from a sample that has been taken as part of a treatment.
- Low includes procedures that cause brief pain or tenderness and small bruises or scars.
- High include procedures such as liver biopsies, arterial puncture, cardiac catheterisation.

More than minimal risk is said not be ethical if the child was not going to gain any benefit from the procedure. However higher risks are acceptable when the children are enduring very harmful disease. These guidelines also give some advice how to assess the potential harms and benefits but without giving any clear instructions how to apply these to the ethical assessment.

The National Council on Bioethics in Human Research report from Canada gives guidelines based upon careful comparison of the magnitude of harm and magnitude of benefit (Consent Panel Task Force, 1992). By doing this they avoid the need for a definition of therapeutic or non-therapeutic research, solely relying on the comparison of harm and benefit. They then issued four recommendations, the first of which applies to children who will receive no benefit from the research. "Research involving children be allowed when a negligible magnitude of harm or a negligible probability of harm is estimated, even when there is a negligible probability of benefit to the individual child, to children in general, or to specific groups of children as long as there is appropriate consent or authorisation, and the REB (Regional Ethics Board) has approved the research." The risks are divided into negligible, minimal, moderate, substantial. The subsequent three recommendations gradually increase the level of harm permissible along with the potential level of benefit required.

Ethics of Research Overseas

When organising research in other countries the ethical considerations depend upon the legal and cultural values and attitudes in that country. This can be a problem when

trying to set up collaborative work in more than one country, with different perceptions of what is ethical.

In the situation of a developed country organising research in a developing country, the question of whether it is right to impose your own ethical guidelines upon a foreign country has been discussed in a number of editorials (Taylor, 1979; Ijsselmuiden & Faden, 1992; Angell, 1988). One concluded that you should impose the same standards as you would expect in your own country, however one should be sensitive to the local cultural values (Angell, 1988). The ideal situation is for the developing country to have their own standards and practices for the approval of ethical applications (Ijsselmuiden & Faden, 1992). Large international organisations sponsoring research, such as the WHO or MRC, have their own ethical boards and require ethical approval for any collaborative studies to apply to both countries. However for small innovative projects, not funded under the auspices of one of the above organisations, it is very difficult to apply for formal ethical approval if the country in which the research to be performed does not have an ethical committee. LREC's are able to consider proposals outwith the NHS, however this usually refers to other local health organisations such as private hospitals, rather than other countries.

Summary

Questions to be asked when assessing the ethics of a research procedure include:

1. is the question being asked an important one for children or the child concerned ?
2. could the same work be performed in adults or animals ?
3. is the research therapeutic or non-therapeutic ?
4. what is the risk/benefit ratio for the subjects ?
5. is there ethical committee approval ?
6. is there suitable means of gaining consent /assent ?

Assessment of Ethics

The above questions can be reviewed in a number of stages. First and foremost will be that of the researcher himself which will inevitably involve subjective opinions. Next come the ethical committees relevant to where the research will take place. Finally (retrospectively) is the acceptance of the study for presentation at meetings and publication in peer reviewed journals.

Personal Opinion

The most important question that a researcher should ask themselves is whether they would be happy for their own family or friends, if they were in the same situation as the potential subjects, to undergo the research that they are proposing. If the answer is no, then it is impossible to see how a researcher could morally proceed with the research he is planning. A number of people have used their own children in their research, such as Jenner. It is difficult to believe that the researchers at the Willowbrook School would have considered deliberately exposing their own children to the hepatitis virus as they did the subjects of their research.

Local Regional Ethical Committees (LREC)

It is currently a requirement for Health Authorities to have LREC's to assess research proposals. Their task is to advise the NHS body (such as a hospital) their opinion of the ethical basis of that research (Home and Health Department, 1992), it is then up to that body whether or not the project should go ahead. Approval by one of these LREC's and the NHS authority are the first objective assessment of a research proposal. In Edinburgh the LREC responsible for paediatric proposals is combined with reproductive medicine and obstetrics.

Grant Funding Authorities

Institutions such as the MRC or the WHO have their own ethical committees to review projects under their authority and if the study is felt to be unethical then will refuse funding.

Presentation

The next assessment of the ethics of research studies is in reactions to presentation to peer groups at national or international meetings. The organisation of the meetings themselves usually have clear ethical guidelines for papers to be presented, and at the presentation itself, the audience have the opportunity to question and criticise the researchers.

Publication

It is felt that the editors of medical journals have a role to play by insisting on including details that ethical proprieties have been observed (Beecher, 1966). Whether to publish scientifically important studies, where ethics have not been observed, is debatable (Beecher, 1966) but if they are published then it is essential for some comment or editorial to be included (Ingelfinger, 1975).

Ethics of WGL for Research in Children

A number of potentially dangerous techniques have been used for investigation in children including broncho-alveolar lavage, intestinal biopsies, exercise ECG's, liver biopsies, and renal biopsies which have yielded useful information. One example is food challenges which are potentially fatal, even if given in a controlled environment in hospital. In the US, to confirm that a new milk formulation is 'hypoallergenic', the company has to prove that it causes less allergic reactions. To do this, clinical investigators recruited by the commercial companies give challenges to children known to be allergic to milk (Prof. A Ferguson, personal communication). This seems to be very dubious on ethical grounds, particularly as there are likely to be milk allergic adults who could volunteer for such studies, understanding and accepting the theoretical risks.

The clinical use of WGL in children in Edinburgh

In Edinburgh WGL was only occasionally used by the surgeons prior to colonoscopy. They agreed that it would be possible to collect specimens from any of their patients undergoing WGL prior to colonoscopy for immunological analysis.

When reviewing the literature I found a few reports describing the use of WGL for severe constipation. This had never been used in Edinburgh. However as a clinician I was aware of the difficulties in treating severe constipation in children as they generally required protracted admissions simply to clear them of all faeces and often had to undergo repeated enemas. The possibility of a treatment that could clear them out in one day, and did not require any enemas, seemed to be a good alternative. No publications had compared the use of WGL with alternative treatments so I organised a study in children requiring hospital admission for treatment of their constipation, to compare the use of WGL with the currently used treatments.

These children were all having lavage for clinical reasons and I gained ethical approval to collect WGL specimens for immunological analysis. However this did not answer the question as to whether WGL could be used purely for immunological research purposes in children.

The use of WGL for research in children

WGL was at the time being extensively used for research in adults in the GI unit at the WGH. However there had never been any publications using WGL for immunological research in children.

Africa

At the start of this thesis Dr Mary Hodges, in collaboration with Professor Ferguson, was using WGL as part of a project to assess GI mucosal immunity and inflammation in African children with parasitic infestations. This was in healthy children and the question arose whether this could be considered ethical. On my first visit to Sierra Leone the initial part of the project had been completed and specimens were ready for

transport to Edinburgh. I was very interested to review the ethics of the study during my stay there.

The problem that Dr Hodges proposed to research was that of the immune response to parasitic infestations. Having performed epidemiological studies Dr Hodges had found that parasitic infestation was hyperendemic in the children, however there were a significant number who were not infected despite living in the same conditions and with apparently the same exposure as the infected children. The question was whether they had a different mucosal immune resistance to the children who were infested. This question is an important one as parasitic infestation is widespread.

Attempts had been made by Professor Ferguson to apply for ethical approval from a British based authority (the Local Regional Ethical Committee (LREC) in Edinburgh and the Institute of Child Health, London) both of which felt it was not appropriate for them to consider the proposal as it was outwith their remit. Initially there was no ethical committee in Sierra Leone, however one year after the study had first been proposed a committee was set up by the Department of Health. Dr Hodges then applied to this ethical committee in Freetown to collect WGLF specimens from children from a local community, in whom she had performed previous epidemiological studies. This was approved by the committee with the only concern being the possibility of the watery stools leading to dehydration. Dr Hodges was able to reassure the committee, with published data, that the children are not dehydrated during WGL.

Consent was obtained from the parents and assent from the children. The families had no dependence on Dr Hodges, and her previous studies had been performed with their agreement and approval. No rewards were offered to the children prior to the study. Those that attended were given small gifts afterwards. The solution was taken orally by all the children. Further details of these children are included in chapter 3.

In Freetown I visited the community from which the children were recruited. I spoke to 10 of the mothers (of 17 of the children involved). All the mothers were happy with the study, and said that the children had not had any complaints of the procedure. I

also suggested that some people overseas in the UK may not have allowed this study to go ahead. The mothers were quite insulted at this suggestion replying that it was 'none of their business', they and their children were happy to be included.

Some of the data acquired from this study has recently been published (Hodges *et al* 1994), after a year of debate about the ethics between the authors, referees and editors of the journal. There was an accompanying supportive editorial from Professor Milla at the Institute of Child Health (Milla, 1994). If there had not been an ethical committee in Sierra Leone (which there was not at the time that the study was being planned) then it would not have been possible to gain ethical approval for this study.

United Kingdom

Having gained ethical approval to collect specimens from children undergoing WGL for clinical reasons I proposed to use these as the control specimens and compare the data obtained with that acquired from healthy adult controls to see if there were age related differences.

In July 1993 I collected a specimen from one child with cystic fibrosis, taking high dose enzymes supplements, who had WGL as treatment for distal intestinal obstruction syndrome. The resulting effluent had grossly abnormal immune parameters as will be discussed in more detail in chapter 8. Having noted these interesting results I awaited further patients being treated for the same condition. In December 1993 reports of strictures in children, possibly related to high dose enzyme supplements, led me to consider the possibility that the abnormalities found in his child could be due to the high dose preparations that the child had been taking. This was the basis for the ethical application for using WGL to assess the GI mucosal immunity in children with cystic fibrosis.

The hypothesis that I proposed to study was whether children with cystic fibrosis, taking high-strength pancreatic enzyme supplements, had inflammation and immune activation of the mucosa of the GI tract and if they did, did this recover subsequent to the children changing to low-strength enzyme preparations.

When considering this study the factors that I took into account were:

1. That this was an important problem as the children who developed strictures required major surgery and often had months of related problems prior to diagnosis.
2. High strength enzymes were taken by the majority of children with cystic fibrosis, and were considered a major advance by the children and their parents as much fewer tablets had to be taken with each meal or snack. Thus the change to the low-strength enzymes was a retrograde step.
3. Given the wide usage of these enzymes there was the potential for a large number of children to present with this problem.
4. This problem has never been seen in adults with CF. Therefore it was not relevant to use adults for this study although later my colleague Dr Nives Caputo collected some specimens from adults with cystic fibrosis. There is no animal model for this complication.
5. This research was therapeutic as we were trying to diagnose gastro-intestinal inflammation due to HDES. The safety of the enzyme preparations is of considerable therapeutic importance.
6. I already had experience of the risks and discomfort of WGL in clinical usage, and this allowed me to judge the risks/benefits in relation to the new knowledge that the project could generate. Insertion of a naso-gastric tube causes discomfort but major adverse effects are rare. The lavage itself is safe, the most likely problems are abdominal distension or nausea. These rapidly settle by slowing the rate of administration. Careful observation of the child including measurement of the abdominal circumference minimises the risks. Thus I judged that the risks could be considered as minimal.

An objective assessment of my ethical application was made by the Lothian Paediatrics and Reproductive Medicine Ethical Committee who gave the study approval without any problems being identified. This was then approved by the

management of the Royal Hospital for Sick Children, where the research was due to take place.

Twelve patients were recruited for this study with the consent of the parents/guardians and the assent of the children. All of the subjects completed their lavage successfully, all had one or more abnormality found in the resulting specimen.

Five of the original 12 CF children taking high dose enzyme supplements repeated the lavage. Reasons for the other seven not repeating the lavage are described on page 154.

Work from this study has since been presented at the Annual Meeting of the British Paediatric Association (Croft *et al* 1995b). There was considerable discussion of the study, however there were no points made questioning the ethics.

A paper based upon this research has been published by the Lancet (Croft *et al* 1995a). In the comments from the referees there were none questioning or criticising the ethics of the study.

Discussion

This is the first time in the UK (and outside Sierra Leone) that WGL has been used in children purely for research purposes. I have shown that use of this technique for research in children can be ethical. A number of common and serious childhood illnesses such as food allergy or intolerance, chronic diarrhoea, inflammatory bowel disease, with appropriate consideration of the above ethical points, can be studied using this method.

SECTION II: Methods

Chapter 3: Clinical Aspects Of WGL In Children

Introduction

In chapter 1 I have described the theoretical reasons that whole gut lavage may be a useful means to study gastro-intestinal mucosal immunity in children as well as the literature relevant to the development of whole gut lavage in the clinical setting. This chapter describes the children studied in this thesis and the practical details of using whole gut lavage in these children.

Aims

1. To compare and contrast the practical use of whole gut lavage for different indications in children.
2. To compare whole gut lavage with standard treatment for children needing in-patient treatment for constipation, with particular regard to efficacy, safety and acceptability.

Children Studied in this Thesis

The children studied in this thesis can be divided into five groups. Clinical details of these children are summarised in table 3.2.

Group 1: Disease Controls

Eight children undergoing whole gut lavage for clinical reasons (see table 3.1). These children were notified to me by ward staff at the RHSC, I collected details on the practical aspects of the lavage as well as collecting, processing and storing the resulting effluent for immunological analysis later.

Patient 3 was a boy aged 13.8 years who was small for his age and had recurrent oral ulcers and was clinically suspected as having Crohn's disease. There has been no colonoscopic or radiological confirmation of this diagnosis.

Table 3.1: Indication for Lavage and Diagnoses in Group 1

Patient No.	Reason for Lavage	Indication	Result
1	Pre-colonoscopy	Rectal bleed	Normal
2	Pre-colonoscopy	Rectal bleed	Normal
3	Pre-colonoscopy	Oral ulcers, small ?Crohn's	Normal
4	Pre-colonoscopy	Rectal bleed	Juvenile polyp
5	Pre-colonoscopy	Rectal bleed	Normal
6	Pre-colonoscopy	? Ulcerative colitis	Macroscopically normal. Biopsy - inflammation with eosinophils
7	Pre barium enema	Soiling, not constipated	Ba Enema - large sigmoid colon, no mucosal abnormalities
8	? GI inflammation	Cystic hygroma, gastrostomy, failure to thrive	Normal

Patient number 6 was a 13.4 year old girl with asthma and eczema who had previously had diarrhoea with blood. She had a macroscopically normal colonoscopy, however biopsy showed evidence of large numbers of eosinophils and inflammation. She has since been confirmed as having ulcerative colitis.

Patient 7 was a 9 year old boy child preparing for a barium enema for the investigation of soiling. He was not constipated as he neither had palpable abdominal masses nor rectal faecal masses.

Patient 8 was a 13 month old boy who has a large cystic hygroma and required gastrostomy tube feeds. Despite adequate caloric intake he had poor weight gain and the possibility that he was malabsorbing due to inflammation of the gastrointestinal mucosa arose. The lavage was performed to see if there were any signs of mucosal inflammation, a method regularly used in adults with inflammatory bowel disease at the Western General Hospital in Edinburgh. Biopsy of his small bowel had been normal.

Group 2: Constipated Children

Sixteen children with severe constipation needing in-patient treatment were notified to me in Edinburgh (n=10) or Dr Peter Gillet in Victoria Hospital, Fife (n=6) by the consultants in charge. The need for in-patient treatment for constipation was decided by the consultant or his junior staff in the out-patient clinic. All had reduction of frequency of bowel movements, and either palpable abdominal faecal masses or rectal faecal masses (or both). Children thought to have Hirschsprung's disease or other obstruction causing the constipation were excluded from the study.

Originally it was planned to randomise the constipated children to receive either standard treatment in current use on the ward or whole gut lavage. However the consultants in charge, when contacting us, specifically requested the lavage because enemas had been used previously and they, the parents and the children were keen to avoid them. This became more pronounced as the initial lavages were found to be a successful method of treatment. Thus it proved impossible to perform a randomised study and so the data we were able to collect was based upon the practical details (time taken, volume, rate administered), side effects (vomiting, nausea, abdominal pain) and biochemistry (sodium, potassium, urea) before and after the lavage.

From four of these children I collected whole gut lavage specimens with the intention of using them as immunologically normal controls. As I will discuss in chapter 7, all these specimens were found to have low total IgA but the remainder of the parameters were within the adult normal range. One of these four children had a repeat WGL, as treatment for severe constipation, later in the study which revealed

normal total IgA. In view of these unexpected low total IgA results I did not collect further lavage specimens from this group.

Group 3: Children with cystic fibrosis

Seventeen children with cystic fibrosis underwent whole gut lavage as part of the study into intestinal mucosal immunity or as treatment for distal intestinal obstruction. A total of 23 lavages were performed with specimens collected for immunological analysis. This group are described in detail in chapter 8.

Group 4: Sierra Leonean Children (who underwent WGL)

Forty seven African children had whole gut lavage as part of a study on intestinal mucosal immunity in children, the ethics of which I have discussed in chapter 2. Of these nine failed, 2 developed pyrexia and had to be stopped, the remaining seven refused to drink the fluid. The fevers were due to malaria which is hyperendemic in the region. Two of the children who refused to drink the fluid returned another day and so 40 lavages were completed. Some of these children have since had results of assays of their lavages published (Hodges *et al* 1994).

In two visits to Freetown, Sierra Leone I have been able to collate more data on the route administered, length of time taken, volume required and side effects of the lavage. It seemed likely that these children, because of differing dietary habits, would be less constipated than both UK disease controls (group 1) and UK constipated children (group 2) and take a shorter time to complete the lavage. The people that arranged and performed the lavage in these children were Dr Mary Hodges and Renee de la Haye (a nurse seconded by VSO to the St. Andrew's Clinic for Children) in collaboration with Professor Ferguson in Edinburgh.

Group 5: Sierra Leonean children with acute watery diarrhoea

The fifth group consists of 14 African children with acute watery diarrhoea. These children, and the study involving them are discussed in detail in chapter 11.

Table 3.2. Clinical details of the groups of children included in this thesis

	Whole Gut Lavage				Watery Diarrhoea
	Controls <i>Group 1</i>	Constipated <i>Group 2</i>	Cystic Fibrosis <i>Group 3</i>	Sierra Leone <i>Group 4</i>	Sierra Leone <i>Group 5</i>
Number of Patients	8	16	17	40	14
Median Age (Months)	99	89	107	80	12
Median Weight (kg)	24.3	22.7	25.3	20	6.7
Weight for Age*	-0.5	-0.5	-0.5	-1.03	-2.03
Weight for Height*	-0.3	-0.7 (n=5)**	0.2	-2.0	-2.0

* All results expressed as Z scores

** Five had their height recorded at the time of the lavage

Methods for WGL in Children

Preparations used

Two PEG based electrolyte solutions were used in this thesis.

Klean-prep (Norgine, Oxford, UK) a commercially available preparation, was used for all the patients except for the constipated children based at the Royal Hospital for Sick Children. This group had Movie-Col, a new preparation under development by Norgine, Oxford, UK. The fundamental differences in Movie-Col and Klean-prep are shown in the table below. One other difference is the flavour. Klean-prep is flavoured with a vanilla essence, Movie-col is flavoured with a citrus flavouring and has a reduced sodium content.

Table 3.3 : Concentration of Substances in Reconstituted Polyethylene Glycol Based Lavage Solutions

	Klean-Prep	Movie-Col
PEG 3350 USNF (g/l)	59	65
Sodium (mmol/l)	125	65
Potassium (mmol/l)	10	5.4
Sulphate (mmol/l)	40	0
Chloride (mmol/l)	35	53
Bicarbonate (mmol/l)	20	17

Both come in the form of a dry powder and are reconstituted with tap water. Klean-prep comes in sachets that are mixed with 1 litre of water, Movie-Col comes in smaller sachets requiring the addition of 125 ml water. Children drinking the solution were allowed to flavour the solution with cordial, and it was chilled to improve the palatability.

Route Administered

The solution was either administered orally or by naso-gastric tube.

If given by naso-gastric tube (size 8/10 Vygon, Ecoen, France) this was inserted on the ward and the position in the stomach was confirmed by demonstrating a pH of less than 3 in aspirated fluid using litmus paper.

Rate Administered

The children were started at a rate of 10-15 ml/kg/hour which, if tolerated, was increased up to 20 ml/kg/hour after 1-2 hours. These rates are similar to those used in adults (1 litre per hour = 15.4 ml/kg/hour assuming an adult weight of 65 kg).

When drinking the fluid orally the children were supervised and encouraged by their parents/carers and the nursing staff on the ward. They drank from 250 ml cups and were told how many cups they needed to drink per hour. The completion of each cup was recorded.

When using a naso-gastric tube the rate of administration was controlled by infusion pumps. These were either the Kangaroo 324 Pump (Sherwood Medical, Crawley, UK), the Frentamat Pump (Fresenius AG, D-Bad Hamburg, Germany) or a Neomate IVAC 565 (IVAC Corporation, San Diego, California, USA). The first two are feeding pumps for the administration of naso-gastric feeds, the Kangaroo pump has a maximum speed of 300 ml/hour, the Frentamat 450 ml/hour which was less than the intended rates if the child was more than 15 or 22.5 kg respectively. IVAC pumps can be used if faster rates are required.

Monitoring of the Children

The children undergoing lavage were monitored for side effects including vomiting, abdominal pain and distension hourly during the lavage.

In the latter stages of the study abdominal girth was measured hourly, an increase of 20% and a taut belly being an indication for reducing (or stopping) the rate of administration for one hour.

Over the course of the study experience gained in the lavages led to a sheet giving guidelines for the administration of whole gut lavage for severely constipated children (see appendix 1). The principles of this sheet also apply to children undergoing lavage for other reasons.

End Point

The end point aimed for was when effluent was passed clear of all faecal staining (ideally a clear yellow colour). However because of the prolonged duration of the lavage in the constipated children the lavage could be stopped once the effluent was clear of lumps of faeces and abdominal palpation was clear of any solid faecal material.

Data Collected

Details of the rate and method of administration, time taken, volume required, side effects (nausea, vomiting, abdominal pain, distension) were taken at the time of the lavage in all the children.

Serum electrolytes including sodium, potassium, urea and creatinine before and after the lavage were performed in the constipated children.

Children having lavage pre-colonoscopy had their biochemistry checked routinely after the lavage. None of these had any clinically significant abnormalities.

We also asked older children in the constipated group at the time of the lavage what they felt about the lavage treatment. This was particularly relevant in the children who had previously undergone admissions requiring laxatives and enemas as treatment for constipation.

The notes of the constipated children were reviewed 6-12 months later to see what their medium term outcome had been.

Results

Table 3.4 tabulates the results for the practical details of the lavage in the four groups of patients who underwent whole gut lavage. The medians for groups 1 and 3 are very similar and when discussing the results below I will refer to them together as the non-constipated children, except when discussing the route that they take the fluid.

Table 3.4: Practical details of the children who underwent WGL.

	Control	Constipated	Cystic Fibrosis	Sierra Leone
	Group 1	Group 2	Group 3	Group 4
Number of Lavages	8	16	23	40
Time (hours)	8 (3.7-16)	12 (3.5-40)	7.6 (4.8-23)	4.3 (2.2-7.3)
Volume (l)	3.0 (2.4-4.3)	3.0 (0.7-6.0)	3.1 (1.6-10.5)	1.6 (0.6-3.0)
Volume (ml/kg)	123 (83-154)	141 (69-291)	111 (74-375)	84 (34-149)
Rate (ml/kg/h)	13 (11-34)	13 (6.3-27)	16 (10.9-23)	20 (7.3-38)
Oral/Total	0/8	3/16	6/23	40/40
Vomiting	3/8	6/16	2/23	0/40
Pain	1/8	2/16	0/23	11/40
Distension	1/8	5/16	2/23	2/40

Medians and ranges of the continuous variables are shown.

Groups 1 and 3 comprise the non-constipated group in the following discussion.

Success/Failure

All the UK children successfully completed the lavage. It is important to point out that completion in the constipated children included patients who had cleared all palpable abdominal masses without necessarily passing clear effluent, whereas the remaining three groups all passed clear effluent. Thus for the constipation group to reach the identical end-point they would have taken longer and a larger volume than the results indicate.

In Sierra Leone there were a total of 49 lavages attempted of which 40 ultimately produced a clear specimen. The mean age of the nine children who initially failed to complete the lavage was 4.7 years (range 2.8-6.1), younger than the remainder (6.7 years). Two of these children later returned to repeat the lavage and completed it without further problems.

Age, Weight and Weight for Age Z Score

There were no significant differences in any of these parameters between the three groups.

Route of Administration

In the CF group in 6/23 lavages the fluid was taken orally. This was a higher proportion than the two therapeutic groups (controls (0/8) and constipation (3/16)). This higher proportion is partly because some of the CF children would not have participated if they had required a naso-gastric tube. Others participated because they were familiar with insertion of naso-gastric tubes as these were used for overnight feeding, thus allowing them to insert the tube rather than attempt to drink the fluid. The pre-colonoscopy group on the surgical ward are routinely given naso-gastric tubes for lavage, hence none of them took the fluid orally.

The constipated group were given a choice of the oral or naso-gastric tube, however because of the large volumes required oral administration was difficult. The three children that were able to manage oral administration were aged 9.8, 13.8 and 14

years, the latter two being the oldest in the series.

All the children in Freetown took the fluid orally, and there was a good overall success rate for this group. The failures are described above. There are two reasons for the successful administration orally in the African children. First of all they all had an experienced, dedicated nurse, continuously present to help the children to continue drinking until completion. This was not the case on the wards in the RHSC Edinburgh, where the staff involved will also be looking after other children in the ward and so cannot devote as much time to individual children. Secondly, as I will show below, the children needed to drink less fluid (per kilogramme body weight) leading to a shorter duration of lavage than the UK children.

Time and Rate of Administration

One major problem for the children was the time taken for the lavage to be completed as they became bored and hungry.

Non-constipated children compared with adults

The usual rate given to adults is 1 litre per hour. If one assumes an average adult weight of 65 kilogrammes this means a rate of 15.4 ml/kg/hour close to the median rate of 13 ml/kg/hour that the lavage fluid was administered to the children.

The non-constipated children from the UK (disease controls and CF), with a median of 8 hours, took very much longer than the 3-4 hours that would be expected in adults at the same rate of administration.

One possible reason for this discrepancy is that children with cystic fibrosis have previously been shown to have slower small intestinal transit time. However this would not explain the length of time taken in the disease control group being similar to the CF group.

Another possibility is that the method of calculating the rate of administration as ml/kg/hour is inappropriate. One factor that is likely to affect the length of time for the lavage to be completed is the total length of the bowel. This, in post mortem specimens, is said to be correlated with the height of the subject but not the weight or

age (Gray's Anatomy, 1989). Thus calculating the rate of administration of the fluid with reference to the height of the patient may be more appropriate.

If this is done for the groups above the non-constipated children have a mean rate of administration of 3.3 ml/cm/hour, the constipated children have a mean rate of 2.7 ml/cm/hour and adults (assuming 1 litre per hour and a height of 165 cm) 6.1 ml/cm/hour. Thus adults, using these calculations, have the fluid administered at approximately double the rate in children, which could explain the very much faster completion time. Doubling the rate of administration in these UK children above would lead to rates (calculated by weight) of approximately 30-35 ml/kg/hour.

Non-constipated children compared with constipated children

As expected the constipated children took significantly longer than the non-constipated group ($P < 0.05$). The difference would have been greater if the end-point achieved for the constipated children had been clear effluent as it was in the non-constipated children.

Sierra Leone children

The Sierra Leone children were significantly faster than both the non-constipated children ($p = 0.001$) and the constipated children ($p = 0.001$). The median time of 4.3 hours was similar to the time taken in adults in the UK. There are two factors that could be involved. Either the rate at which they take the fluid could be greater, or the volume they require could be less than in the UK children, I will discuss these below.

The use of whole gut lavage was a new technique and all new techniques have a learning curve. At the outset of the study in the UK I was cautious with the rate of administration of the lavage in order to minimise the side effects. However this may have meant that the lavages were not completed as quickly as possible. Figure 3.1 below demonstrates the time taken for the lavage in the non-constipated children versus the date of the lavage. Despite the wide scatter the trend line suggests a

gradual reduction in the time taken. Figure 3.2 shows how for the same lavages over the same time period there was a trend to increasing the rate of the lavage.

Figure 3.1: Duration of individual lavages over the duration of the study

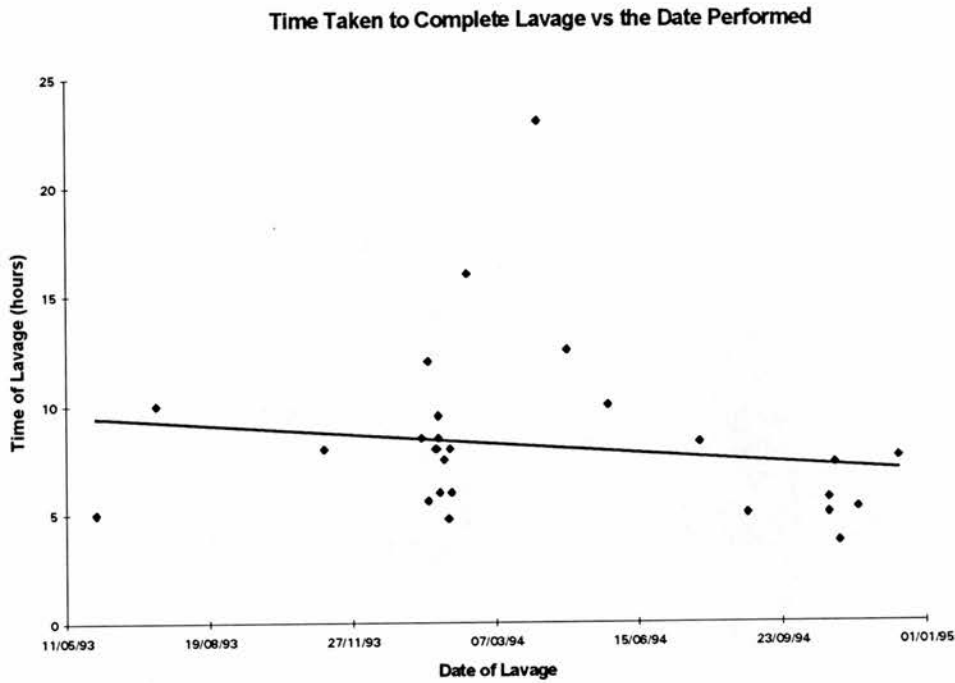
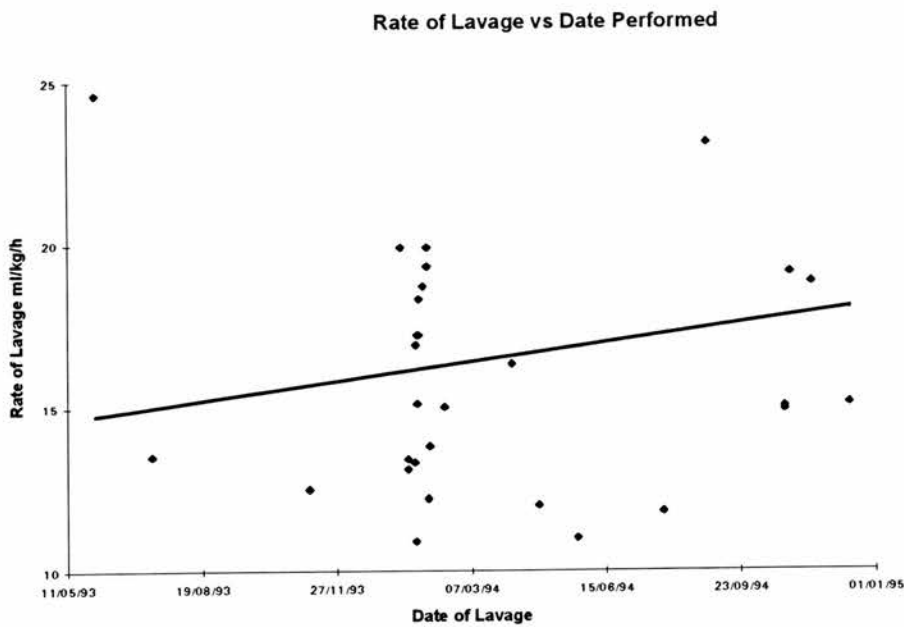


Figure 3.2: Rate of administration of the lavage over the duration of the study.



The constipated children had a slightly slower rate than the non-constipated children ($p=0.15$) but were significantly slower than the Sierra Leone children.

The Sierra Leone children took their fluid significantly faster than the non-constipated ($p=0.014$) and the constipated UK children ($p=0.006$) this may partly explain their shorter time to completion.

Volume Required

I will discuss this with reference to the volume in ml/kg.

The constipated children required a median of 141 ml/kg which was not significantly ($p=0.15$) higher than the non-constipated UK children (123 ml/kg). The fact that most of the constipated children had their lavages completed at an earlier end point than the non-constipated children suggests that these children would, to reach clear effluent have required a greater volume of fluid.

The African children required a considerably lower volume (84 ml/kg) than both the non-constipated children ($p=0.006$) and the constipated children ($p=0.001$). This is almost certainly because their dietary and bowel habits lead to less build up of faecal material within the gastro-intestinal tract.

Vomiting or Pain

Overall the children tolerated the lavage well. Although a number did vomit these tended to be small amounts and did not prevent the children from completing the lavage.

Of the 23 lavages in the children with CF, only 2 vomited during the procedure, both of whom took the fluid by naso-gastric tube. In group 1 3/8 vomited.

The constipated children vomited in 6/16 lavages. This was one of the factors that meant that the children did not complete the lavage to a clear specimen. The six that vomited had a slower rate of their lavage (8.9 ml/kg/h) than the other nine that did not (16.5 ml/kg/h, $p=0.002$). The slower rate found in the children that vomited is explained by the reduction in the rate of the lavage after the children vomited. The

reason for this high frequency of vomiting is likely to be a combination of large amounts of faecal material in the bowel and slower transit time related to the constipation. During the course of the lavage the children with severe constipation would gradually become distended until they started to pass stool. If the child continued to distend without passing stool and their belly became taut then the child was more likely to vomit. Once the child started to pass stool then they could tolerate increased rates of the lavage again.

Pain

Pain was rarely a problem in the UK, however in the African children it was a frequent complaint. There is no obvious reason for this from a clinical point of view. It is possible that this is a cultural feature of the African children. In Sierra Leone complaint of pain is a common way to describe or express unease or discomfort, either physical or non-physical. Thus the African children may have been expressing pain due to unease rather than true physical pain. All these children would have previously had diarrhoeal illness with abdominal pain and so once passing fluid stool during the lavage associated that with pain as in previous illnesses.

Biochemistry

In the constipated children there were no clinically significant changes in the sodium, potassium or urea.

One constipated child aged 10 months (the youngest in the series) who had whole gut lavage lasting 15 hours developed asymptomatic hypoglycaemia (1.4 mmol/l). After this case regular monitoring of the blood sugar in all children aged under 18 months was recommended. For prolonged lavages maintenance intravenous glucose is advisable.

Acceptability in the Constipation Group

All the children, parents and nurses felt that the use of lavage was acceptable both before and after the procedure. The children did not like the naso-gastric tubes,

however of four older children (more than 8 years) who had previously required in-patient treatment with enemas, all said that they preferred lavage with a naso-gastric tube.

Short and Medium Term Results of Constipation Group

All the children showed improvement in the short term after the lavage with increased frequency of bowel movements and reduction in soiling as detailed in the notes of their outpatients visits.

Three of the children have had three or more lavages since the first one in preference to enemas as treatment for recurring problems.

Six of the children now have what the parents consider to be normal bowel habits and are on no or minimal doses of laxatives. Four of these had, prior to the WGL, had considerable problems with repeated enemas and evacuations under general anaesthetic. Since the bowel washout they have had no recurrence of the original problem. This is in accordance with a publication describing children with faecal impaction who dramatically improve after whole gut lavage (Tolia, 1988).

Conclusions

Whole gut lavage is safe, effective and can be used for treating children with severe constipation.

The major problem is that the time taken for lavage is too long and needs to be reduced. There are four possible methods to help do this in the UK setting.

1. There should be a dedicated nurse who is experienced in the procedure of whole gut lavage who is able to look after the child during the procedure. This is probably best done in a day unit, also reducing pressure on the ward beds.
2. When administering the fluid the fastest rate that the child can tolerate should be used. This requires close observation by an experienced nurse. A rate of at least 20 ml/kg/hour should be aimed for, except in severely constipated children prior to starting to pass stool.

3. Once the child starts to pass stool the rate should be increased as much as tolerated.
4. Further studies using fractionated methods, i.e. giving smaller amounts of the lavage fluid over two or more days, need to be performed. This may also mean that the use of naso-gastric tubes can be reduced.

As a result of my experience in administering whole gut lavage I have written an advice sheet (see appendix 1) for medical or nursing staff planning to use this technique. Although intended for children with severe constipation it is also applicable to all children undergoing lavage.

Lavage in Sierra Leonean children is well tolerated and completed faster than non-constipated UK children. The major reason for this difference is the much smaller volume that is required to completely clear the bowel.

Chapter 4: Sample Collection, Preparation and Analysis

Collection and Preparation of Whole Gut Lavage Fluid

Sample Collection

In subjects undergoing lavage, samples were collected once clear of all faecal material. The subsequent filtering and processing is performed immediately the fluid is passed.

Filtering and Processing of Lavage or Faecal/Ileostomy Extracts

References (Gaspari *et al* 1988; Brydon *et al* 1993).

Filtering:

WGLF or faecal/ileostomy extract is filtered through GF/A (Whatman) glass fibre filters.

Processing:

To the filtered fluid the following reagents were added, with mixing after each addition (final concentrations in brackets):

- soya bean trypsin inhibitor in phosphate buffered saline (PBS) (80 µg/ml)
- sodium ethylene diamine tetraacetic acid (15 mM) in PBS (chelating agent for calcium and magnesium ions which are required for the activation of protease enzymes)
- phenyl methyl sulphonyl fluoride (2 mM) in 95% ethanol (protease inhibitor),
- sodium azide (1 mM) (bactericidal agent),
- newborn calf serum (5% v/v) (to provide an alternative substrate for any remaining enzymes).

Aliquots of the processed extract or WGLF were stored at -70°C for later analyses.

Nomenclature for WGLF Samples

For the assays used in this thesis, specimens prepared in different ways are used, below is a key to the different types of preparations.

UF/UP = Unfiltered and unprocessed

UF/P = Unfiltered and processed, i.e. not filtered but processing agents added

F/UP = Filtered and unprocessed

F/P = Filtered and processed

Collection and Preparation of Faecal Extracts

All faecal specimens were frozen immediately at -20 °C and transferred to a -70 °C freezer within 48 hours.

Faecal samples were removed from the freezer and a portion of approximately 1g was thawed, accurately weighed, homogenised in saline (9 ml per gramme of faeces), centrifuged at 25,000 g for 15 minutes at 4°C, and aliquots were stored at -70°C.

"Processed" faecal samples were prepared by treating the above extracts of faeces with the same series of agents as processed WGLF.

Collection and Preparation of Ileostomy Extracts

An aliquot of the volunteer's ileostomy output (less than 1 hour old) was collected from their bag. This was immediately mixed 1/5 (weight by volume) in PBS at 4°C, and centrifuged at 3000g and 4°C. The resulting supernatant was then filtered and processed as in WGLF specimens and stored at -70°C.

Collection and Preparation of Sputum

Reference: (Virchow, Jr. *et al* 1992).

Expectorated sputum was collected and immediately frozen at -70°C.

1. Sputum was removed from the freezer and defrosted.

2. Placed in a pre-weighed container, and reweighed to establish wet weight of the sputum.
3. Extracted 1/5 weight by volume with phosphate buffered saline (PBS) (1g sputum plus 4 ml PBS).
4. Vortexed until the solution was homogeneous.
5. Centrifuged at 3000g, 4°C, 30 minutes.
6. The resulting supernatant was then separated and processed exactly as in lavage specimens. The specimens were not filtered.

Sonication of WGLF Specimens

Sonication is a means of disrupting cell walls in order to measure intra-cellular constituents.

The technique for sonicating lavage specimens is as follows.

1. Add 1 µL Triton x100 (a detergent that disrupts the cell wall) to an Eppendorf 1.5 ml tube.
2. Add 500 µl of the specimen to be sonicated.
3. Vortex three times
4. Inside the sonicator lower the tube into a beaker of ice and water
5. Sonicate three times for one minute each time, allowing the tube to cool in ice, with the cap closed, between each sonication.

Fluid Loss During Sonication

Despite immersing the tubes into ice and water the sonication process heats the fluid, and it is possible that fluid loss may occur leading to falsely high concentrations of substances in the sonicated fluid, I estimated the water loss during this process by weighing the tubes before and after the sonication.

In 14 specimens the loss of water during sonication was a mean of 2.8 % (range 1.3-3.4%).

Analyses

Enzyme Linked Immunosorbent Assays (ELISA) for Immunoglobulins or Antibodies

Principle of ELISA

A double antibody 'sandwich' technique was used for total immunoglobulin A, G, and M quantification using purified secondary standard material (Gaspari *et al* 1988; O'Mahony *et al* 1991a; 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 ovalbumin, gliadin and beta-lactoglobulin (O'Mahony *et al* 1991a), cholera-toxin B subunit (Gaspari *et al* 1988) and *salmonella typhi* LPS (Sallam, 1995). Essentially the assays are the same except for the initial coating of the solid phase.

1. Class specific antihuman immunoglobulin or pure antigen is bound in excess to a solid phase overnight and then washed. In these methods, a 96 well ELISA plate is the solid phase - different plate types were used as they have different binding characteristics.
2. The plates are incubated with a protein containing solution to "block" any non-specific binding sites.
3. Standards and samples for total immunoglobulin and antigen-specific antibody quantitation are added to the plates in serial doubling dilutions, in order to cover the wide range of concentrations found.
4. Samples are incubated and then washed. Binding takes place between the antihuman globulin and the immunoglobulin being quantified or between the specific antigen and corresponding anti-antigen immunoglobulin.

5. A class specific antihuman antibody conjugated with alkaline phosphatase is added to the plate, incubated and then washed. This conjugated antibody binds to the complex bound to the solid phase.
6. The substrate for alkaline phosphatase is p-nitrophenyl phosphate in a diethanolamine buffer at pH 9.8. The substrate is added to the plate and the reaction:

p-nitrophenyl phosphate (colourless) \Rightarrow p-nitrophenol (yellow) takes place.

7. The colour development takes place within an hour and the optical density at 405 nm is read on a dedicated ELISA reader (Dynatech MR5000) when the top standard or reference standard has reached 1.0.
8. For total immunoglobulins a standard curve is plotted using the log-transformation of the concentration on the x-axis against the optic 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. In the latter stages of the study I used a curve fitting programme (Prism) to plot the standard curve and calculate the results without having to transform them. Again the correlation coefficient had to be > 0.99 for the standard line to be acceptable.

Specific antibodies are plotted as the optic density against the concentration without the log transformation.

9. The concentration of the specimen is calculated by averaging two or three of the doubly diluted concentrations that lie within the standard line.
10. 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 (other than the standards) and instrumentation are detailed in appendix 2.

Test Procedure

1. Coat plate with appropriate antigen

Using an 8 channel multichannel pipette, dispense 125 μ l coating solution including the antigen to each well of the ELISA plate.

Total Immunoglobulins

Use Immulon 1, flat bottomed 96 well plates.

anti-human IgA	1/2500
anti-human IgG	1/5000
anti-human IgM	1/5000

Specific Antibodies

Use Immulon 2, flat bottomed 96 well plates (which adsorbs coating antigen more strongly than Immulon 1 plates).

Make up following antigens in coating buffer:

Ovalbumin	5 μ g/ml
Gliadin	5 μ g/ml
Beta-lactoglobulin	5 μ g/ml

For the **Cholera toxin** assay there is a two stage coating phase, the first coats monosialoganglioside to the well. This is a constituent of cell walls to which cholera toxin binds strongly:

Stage 1: Monosialoganglioside (GM ₁)	10 μ g/ml in coating buffer, incubate overnight at 4 °C,
Stage 2: Cholera Toxin	5 μ g/ml in coating buffer.

2. Incubate

Cover plate with a plastic lid, place in a moist box and incubate at 4°C overnight.

3. Block Plate

Wash the plate x 3 and then add 250 µl Diluent. Leave for a minimum of 1 hour.

4. Samples and standards

All are 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. Purchased from Dept. of Immunology, P O Box 894, Sheffield S5 7YT.

Top standard for IgG assay = 1000 ng/ml.

Top standard for IgM assay = 1000 ng/ml.

Total IgA

- **Human IgA** - purified immunoglobulin from colostrum (Sigma Chemical Co., Cat No I-2636).

Top standard for IgA assay = 1250 ng/ml.

2. Specific Antibodies:

Quantitative Assays: For **food antibodies** a serum sample from a patient (Main) with high levels of specific food antibodies was used. For calculations I have allocated arbitrary units to this standard.

	Arbitrary Units (units/ml of undiluted standard)	Dilution of Top Standard	Conc. of Top Standard (units/ml)
Ova-A	1000	1/50	20
Ova-M	100	1/100	1
Gli-A	300	1/30	10

For **Cholera toxin** antibodies the standards used were from volunteers, lavage for CT-A, serum for CT-M.

	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

Samples

Total Immunoglobulins A, M, and G

Make up the samples in ELISA diluent for the top row to start at the dilutions shown below. Once the dilution has been made add 125 µl of the ELISA diluent to all the rows except the top row. Then add 125 µl of the sample to the top and second rows of the plates. Thus the second row is a 1/2 dilution of the first row.

	WGLF	Faecal/Ileostomy Extract
Total IgA	1/100	1/200
Total IgM	1/25	1/100
Total IgG	1/25	1/100

Specific Antibodies

Add 125 µl of sample to 125 µl of diluent in the top row i.e. 1/2 dilution.

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

5. Incubate

Incubate at 4°C overnight.

6. Conjugate

Prepare alkaline phosphate conjugated antisera dilutions in ELISA diluent following dilutions in appendix 2.

Wash plate as above, then using a multichannel pipette dispense 125 µl conjugate to each well of the ELISA plate.

7. Incubate

Cover plate with a plastic lid, place in a moist box and leave at room temperature for 3 hours.

8. Colour Development

Prepare alkaline phosphatase substrate 30 minutes before it is required.

Wash plate as above, then using an 12 channel pipette dispense 125 µl substrate to each well of the ELISA plate. Leave on the bench for 5 minutes as the colour starts to develop, then shake on Denley Wellmix 1.

9. Reading

Monitor the colour development manually on the Dynatech MR5000 Microplate reader and as the top standard reaches its endpoint of approximately 1 read with the filter set to 405 nm and the reference filter set to 630 nm.

10. Calculate

Calculate the concentrations of the samples as described above.

Alpha 1-antitrypsin and albumin in whole gut lavage fluid

Reference: (Brydon *et al* 1993).

Principle of Method

When human alpha-1-antitrypsin (A1AT) and albumin reacts with its specific antibody, precipitating immunocomplexes are quickly formed in the presence of polyethylene glycol. If the antibody is present in large excess, these precipitates produce a turbidity which is related to the concentration of A1AT or albumin in the sample. The turbidity is photometrically measured at the wavelength 340 nm.

Absorbance readings obtained by assaying calibration standards are used to generate a standard curve, from which the concentration of A1AT or Albumin in the sample is derived.

Standard Material

Standard serum: SPS-01, concentration of albumin = 40.5 g/L, A1AT = 1.52 g/L (varies between batches).

A1AT Standards: Dilute standard serum with Diluent to give the following range of standards : 0,10,20,50,100,200 µg/ml.

Albumin Standards: Dilute standard serum with diluent to give the following range of standards : 0,10,20,50,100,200 µg/ml. Prepare fresh on day of assay.

Procedure

1. Bring PEG and diluent reagents to room temperature.
2. Blanks: In duplicate, dilute 50 µl diluent (B1), standards (SB), and test samples (TB), with 0.95 ml PEG reagent in 2 ml polystyrene tubes.
3. Tests: In duplicate, dilute 50 µl diluent (B2), standards (S), and test samples (T), with 0.95 ml antibody reagent in 2 ml polystyrene tubes.

4. Read Blanks and Tests after 15 - 20 minutes at 340 nm using the spectrophotometer. Read corresponding blanks and then tests for each sample which minimises any errors caused by baseline drift.
5. Calculate $B_2 - B_1 = \text{OD value for } 0 \mu\text{g/ml A1AT and albumin standards.}$
 $S - SB = \text{OD values for } 10 - 200 \mu\text{g/ml A1AT and albumin standards.}$
 $T - TB = \text{OD values for test samples.}$
6. Plot a graph of OD 340 values for standards and read test sample results from this. Calculate and report mean values of the duplicated assays.

Haemoglobin in whole gut lavage fluid

Reference: (Brydon & Ferguson, 1992).

Principle of Method

Haemoglobin is converted to fluorescing porphyrins by the removal of Fe. Total haemoglobin is determined by reaction with heated oxalic acid: FeSO_4 reagent which converts haem to porphyrin without loss of preformed porphyrins. A three step purification procedure eliminates other interfering fluorescent materials which may be present.

Instrumentation

Fluorescence is measured using the LS-5B Luminescence Spectrometer (Department of Medicine). Set excitation wavelength to 402 nm and emission wavelength to 600 nm.

Standard Material

Cyanomethaemoglobin Standard: Dissolve 10 mg haemoglobin (Sigma - H 7379) in 100 ml Drabkins reagent. Leave at room temperature for 15 minutes.

To calculate actual haemoglobin concentration read OD at 540 nm against Drabkins reagent (blank).

Quality Control Material

Take 10 mg haemoglobin (Sigma - H7379) and dissolve in 100 ml Klean-prep. Add sodium azide to final concentration 0.02 g/100 ml.

Method

1. Lavage fluid samples should be frozen at -70°C before assay to allow haemolysis of any intact red cells. After thawing these should be centrifuged at 2000 rpm (Mistral 3000i), and the supernatant used for assay.
2. Add 0.4 ml of the oxalic acid reagent to 0.1 ml lavage fluid supernatant, quality control, haemoglobin standard, and blank (Klean-prep) in a 30 ml stoppered quickfit tube. Mix thoroughly and heat at 100°C for 30 minutes in a water bath. Remove stoppers and cool for 2 minutes.
3. Add 1 ml of the 3M potassium acetate reagent, followed by 3 ml ethyl acetate/acetic acid reagent. Vortex mix for 30 seconds. This extracts the porphyrin analytes into the upper organic phase.
4. Transfer 2 ml of the upper organic phase to a 30 ml stoppered quickfit tube and add 0.8 ml butanol and 6.0 ml of 3M potassium acetate in 1M KOH. Mix for 30 seconds. This extracts coproporphyrin and other porphyrins not derived from haemoglobin haem into the lower alkaline aqueous phase.
5. Transfer 1 ml of the upper organic phase to a 10 ml stoppered quickfit centrifuge tube, and add 3 ml phosphoric acid/acetic acid reagent. Mix for 30 seconds. Remove the top layer which contains chlorophyll. Read the fluorescence of the lower acid extract.

Reference values for lavage haemoglobin in adults range from 0 - 5 $\mu\text{g/ml}$ (Brydon & Ferguson, 1992).

Determination of polyethylene glycol levels in WGLF

Reference: (Malawar & Powell, 1967).

Introduction

This is a turbidimetric assay based on the development of an oil-in-water emulsion of PEG when exposed to trichloroacetic acid in the presence of barium ions.

Standards and Samples

PEG 3350 Standard 10 g/litre of distilled water,

diluted in distilled water to 200, 400, 600, 800 mg/ml

WGLF samples (UF/UP)

Method

1. Samples and standards were diluted 1/10 into duplicate 20 ml test tubes.
2. Add:
 - 1 ml barium chloride (10% weight/volume)
 - 2 ml Barium Hydroxide
 - 2 ml Zinc Sulphate (5% w/v).
3. Mix vigorously for 1 minute, stand for 10 minutes.
4. Filter (through double thickness Whatman no. 42 ashless filter paper)
5. Take 1 ml of the filtrate.
6. Add:
 - 3 ml gum Arabic solution
 - 4 ml TCA/BaCl₂.
7. Stand for 60-90 minutes, read standards and test samples on spectrophotometer (Pye Unicam PU 8610, Phillips) at 650 nm.
8. Construct standard curve and extrapolate concentration of the test solution

CHYMOTRYPSIN (CT)

Reference: (DelMar et al 1979)

Principle

The inactive precursor chymotrypsinogen is secreted by the acinar cells of the pancreas and hydrolysed by trypsin in the small intestine to the active peptidase, α -chymotrypsin (α -CT). α -CT is quantified by the hydrolysis of a synthetic polypeptide labelled with p-nitroaniline. The production of the p-nitroaniline is measured kinetically in a spectrophotometer at 405 nm.

Instrumentation

1. *Pye Unicam 8600 UV/VIS* spectrophotometer programmed with the following settings.

Mode	Kinetic
Wavelength (nm)	405
Factor	212
Delay (seconds)	0.5
Time (seconds)	1
Cycles	2
Temperature ($^{\circ}$C)	37
Volume (ml)	0.8

Test procedure

1. Remove from the freezer 1 aliquot of QC material and 1 aliquot of substrate per test or QC to be assayed.
2. Weigh an empty universal container.
3. Transfer 100 μ l of WGLF into the universal container
4. Reweigh the universal container.

5. Add solvent to WGLF in ratio 100:1, i.e. add 10 ml solvent to a 100 μ l sample.
6. Dissolve enzyme in solvent by shaking. If necessary centrifuge samples at 1000 g for 5 min to precipitate particulate material.
7. The supernatant is stable for 24h at 4°C or 3h at room temperature.
8. Switch on the spectrophotometer, check programme parameters and allow temperature stabilisation.
9. Assay water to check cycle function and blank.
10. Pipette 1.0 ml substrate into a 3 ml plastic assay tube. Add 0.05 ml supernatant or 20 μ l of neat WGLF to the substrate, vortex mix and measure absorbances in the spectrophotometer using the settings above.
11. Repeat with control material.

Results

The spectrophotometer printout gives the absorbance of the sample, the calculated enzyme activity for each of the two cycles and the mean activity of the two cycles in U/g.

Determination of Non-Specific Protease Activity (Gaspari et al 1988)

1. Azocoll (Sigma A 9409) is suspended in PBS.
2. 0.5 ml aliquots are transferred to the polystyrene reaction tubes.
3. Bring to 37°C in water bath.
4. Add 50 μ L test sample.
5. Stop reaction after 30 minutes by transferring tubes to iced water for 5 minutes.
6. Centrifuge at 2000 rpm at 4°C for 5 minutes.
7. Add 0.1 ml of the supernatant to 0.9 ml PBS and mix.
8. Read OD of processed samples and hence the percentage inhibition by comparison with the unprocessed specimen.

Cytokines

The cytokine assays are commercially available ELISA kits that have been adapted for analysis of WGLF. IL-1 β and IL-8 were the first to be introduced into the GI laboratory, I then introduced RANTES with methods identical to those introduced for the IL-8 kit from the same manufacturer.

IL-8 and RANTES

IL-8 - R & D Catalogue No. D8000

RANTES - R & D Catalogue No. DRN00

Principle

These kits use the 'sandwich' type ELISA similar in principle to that described for the immunoglobulin and antibody assays. Monoclonal antibody to IL-8/RANTES is coated onto the microtitre plate provided. Standards and samples are added and any IL-8 or RANTES present is bound to the solid phase. After washing, an enzyme-linked polyclonal antibody is added to the wells to attach to the chemokine bound previously. Following a further wash a substrate solution is added to the wells and the colour develops in proportion to the amount of 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 - Klean-prep (Norgine, Oxford UK) + processing agents for WGLF.

Methods

Samples and Standard

Standard:

Reconstitute standard with standard diluent to make a stock solution of 6000 pg/ml for IL-8 and 2000 pg/ml for RANTES.

Then make serial double dilutions to make concentrations of 2000 to 31.25 pg/ml for IL-8 and 1000 to 15.63 pg/ml for RANTES.

Add to the microtitre plates diluted 1/2 in processed Klean-prep within the wells (e.g. 100µL standard added to 100 µL processed Klean-prep).

WGLF Samples

These are not diluted prior to addition to the microtitre plate where they are diluted 1/2 in the standard diluent (e.g. 100µL sample added to 100 µL processed Klean-prep already in the wells).

Sputum Extracts

Sputum was pre-diluted 1/10 in the standard diluent.

These dilutions were then added to the wells 1/2 in processed Klean-prep.

Assay procedure

1. Add specimens and standards as above.
2. Incubate 2 hours at room temperature.
3. Aspirate and wash x3.
4. Add 200 µL of IL-8 or RANTES conjugate.
5. Incubate 1 hour at room temperature.
6. Repeat wash.
7. Add 200 µL substrate solution, incubate for 20 minutes at room temperature.
8. Add 50 µl stop solution.
9. Read optic density on the spectrophotometer at 450 nm with a reference filter at 570 nm.
10. Calculation of results:

Using a curve fitting programme in Prism software, an r value of > 0.99 including all the points was required in the standard curve. Concentrations of the samples were then extrapolated from their optic densities.

Interleukin-1 β

High sensitivity Interleukin-1 β ELISA kit - Cistron Biotechnology 03-HS96

Principle is the same as the sandwich ELISAs described above for immunoglobulins, specific antibodies, IL-8 and RANTES.

Standards and Specimens

Standards - diluted in distilled water to 300, 150, 100, 50, 20, 10, 5 pg/ml

Samples - dilute 1:2 by adding 50 μ L of standard diluent (from the IL-8/RANTES assays) to wells and then add 50 μ L of the lavage.

Assay Procedure

1. Add 100 μ L of each standard, and sample, into the wells.
2. Incubate at 37 $^{\circ}$ C for 1 hour.
3. Wash x3.
4. Add 100 μ L polyclonal IL-1 β anti-serum to each well.
5. Incubate at 37 $^{\circ}$ C for 20 minutes.
6. Wash x3.
7. Add 100 μ L of conjugate.
8. Leave at room temperature for 20 minutes.
9. Mix solutions A & B in equal volumes to make substrate
10. Wash wells again.
11. Add 100 μ L of substrate.
12. Incubate at room temperature for 20 minutes.
13. Stop with 50 μ L of 4N sulphuric acid.
14. Read at a wavelength of 450 nm.
15. Calculate by constructing standard curve and extrapolating optic density readings.

Quantitation of IgA producing Plasma Cells in the lamina propria of rectal biopsies using image analysis

Specimens

Biopsy specimens were collected by surgeons at the RHSC during investigation of the children for severe constipation and possible Hirschsprung's disease. These were collected by the pathology department at the RHSC and fixed in formalin. Dr J Keeling, consultant histopathologist at the RHSC, kindly supplied these sections for IgA cell counting.

Principle of Staining

Streptavidin biotin peroxidase method for immunocytochemistry

Reference: (Arranz *et al* 1992).

Formalin fixed paraffin sections are dewaxed and then rehydrated with alcohol, followed by methanol containing hydrogen peroxide. This removes unwanted endogenous peroxidase that is present in eosinophils and red blood cells. Sections are treated with trypsin to unmask the antigenic sites which have been blocked due to the cross linking of proteins during formalin fixation.

Non specific antibody interaction is blocked by treating the section with diluted normal serum of the host producing the secondary antibody. Polyclonal antibody directed against the specific immunoglobulin is placed in contact with the tissue section and allowed to react.

The secondary biotinylated antibody which is directed against the bound monoclonal antibody is then placed on the tissue section and allowed to react. Finally the streptavidin biotin peroxidase is added and this binds to the biotinylated secondary antibody. The procedure is then visualised histochemically by reacting the peroxidase with the peroxidase substrate diaminobenzidine.

Image Analysis

Counting of the brown stained cells was then completed using the image analyser (Leitz (TAS plus, Bosch). The microscope was set to Kohler illumination to obtain optimum resolution and the slides were examined under x40 objective. The number of IgA cells within the lamina propria were counted in up to 25 fields and the area in which they were seen was measured to exclude Peyer's patches, and intestinal glands such as the crypts of Lieberkühn. From this the mean number of cells per square mm of lamina propria could be calculated.

Eosinophil Cationic Protein

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 and samples

Standards and samples are all assayed in duplicate.

Standard:

As included in the kit in a range of dilutions.

Samples:

WGLF: No pre-dilution necessary.

Sputum: Diluted 1/4 in PBS prior to analysis (including the extraction step, the sputum has been diluted a total of 1/40 before assay).

Procedure

1. Add 50 μ L of standards and sample to polystyrene centrifuge tubes.
2. Add 50 μ L ECP¹²⁵I.
3. Add 50 μ L anti-ECP.
4. Mix and incubate for 3 hours at room temperature.
5. Add 2 ml decanting suspension.
6. Incubate for 1/2 hour at room temperature.
7. Centrifuge 10 minutes at 1500 x g. Decant immediately and let stand for 30 seconds upside down on absorbent paper.
8. Determine the radioactivity.
9. Calculation of the results.
 - Express mean counts (B) for the standards and samples as a percentage of the mean counts of the zero standard (B₀)
 \Rightarrow % activity bound = 100 x B/B₀.
 - Plot percentage values obtained for the standards against the concentration on a linear-log paper and construct a standard curve.
 - Read the concentration of the unknown samples from the standard curve.

Software Used in this Thesis

Data was stored using **Access version 2.0 (Microsoft)**.

The word-processing package used in this thesis was **Word for Windows v6.0 (Microsoft)**.

Statistics were calculated using **Minitab for Windows v10 (Minitab statistical software)**.

Storage of references and preparation of the bibliography were performed using **Reference Manager for Windows v 6.01 (Research Information Systems)**.

Graphs were produced using **Prism v1.03 (GraphPad)** or **Excel v 5.0 (Microsoft)**.

Statistical Analyses in this Thesis

As mentioned above these were performed using the software Minitab for Windows.

Where data was normally distributed student's 't'-test was used to compare two independent groups (paired t-test). The Pearson correlation coefficient 'r' was used to examine the relationship between two variables.

For non-parametric data results were expressed as medians and ranges (either interquartile or complete). Wilcoxon signed-rank test was used for comparison of paired data and 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.

Chapter 5: Technical Developments And Trouble Shooting

Total IgA ELISA in WGLF

In order to confirm that PEG containing electrolyte solutions do not interfere with the total IgA assay I made up the IgA standard in four different solutions:

- water.
- water plus processing agents.
- Klean-prep.
- Klean-prep plus processing agents.

Plotting the optic density of each dilution showed that the standard curves for all these four solutions were identical. Thus there is no evidence to suggest that processing agents or Klean-Prep interfere with the total IgA assay. This is important as in chapter 10 and 11 I will be comparing faecal extracts with lavage specimens, only the latter containing polyethylene glycol.

IgA: Quality Control

As I have indicated most analytical methods used were established in the laboratory for regular diagnostic and research work. For these I followed standard protocols, the responsibility for the functioning of these assays were shared among staff.

IgA and IgM had both had assays set up for occasional specific projects where all the assays could be run at the same time. As I knew I would be running these assays over a two to three year period for this project I introduced the use of a quality control (QC) for these analyses. The QC used is a lavage specimen, collected from a patient undergoing WGL for clinical reasons.

In December 1994 I found that the measured concentrations of the IgA QC were lower than they had been over the previous 14 months (dropping from about 75 µg/ml to 47 µg/ml). This is illustrated in figure 5.1 on the next page. One of my colleagues

running a batch of IgA for a separate study found the same problem. This change occurred as the original QC had nearly run out, but also at about the same time as a new batch of standard material had arrived. Regrettably parallel runs of old and new standard had not been performed by any of the research staff who were making use of the IgA assays. I then proceeded to investigate the reason for this change and if possible establish a correction factor for assays run at the time of the high or low QC results.

The assay technique, which is described in detail in the previous chapter, involves doubling dilutions of the purified colostrum IgA standard supplied by Sigma Chemicals alongside which the specimens are also diluted in parallel. The concentration of IgA in the standard was accepted as the concentration quoted by the manufacturer with the batch supplied. Each plate had the standard, the QC, and up to four specimens all assayed in duplicate.

There were two possibilities for the change in the results for the QC.

1. Deterioration in the QC or the original standard during storage in the -70°C freezer over the period studied.
2. The value for IgA concentration quoted by the manufacturer was not correct in one of the standards.

The trend line in figure 5.1 is for the old QC and old standard results. Although this does suggest a slight deterioration up to November 1994, this was not significant and is insufficient to explain the drop from more than 70 to less than 50 in November 1994.

The line is the trend line for the old QC using the old standard up to November 1994. At about the same time as the new standard arrived the quality control material was almost finished. In order to ensure that I could reliably compare the new and the old QC I ran them concurrently on 10 separate plates using the new standard. Figure 5.2 shows that the two QCs gave almost identical results. The one very low level of the new QC is likely to have been a technical error on my part. The mean results for the old QC on these 10 plates was 47.3 $\mu\text{g/ml}$, for the new QC was 45 $\mu\text{g/ml}$.

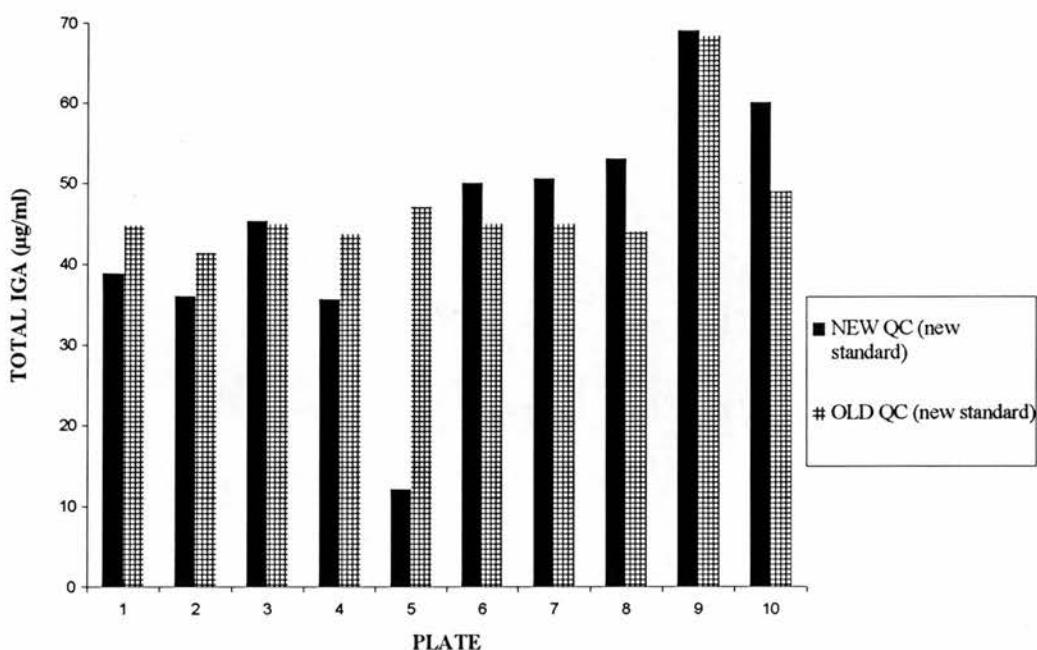


Figure 5.2: Comparison of old and new IgA QCs on the same plates

Following this, using the new QC, I compared the new and the old standards. Figure 5.3 below shows that the old standard gives a concentration of total IgA of approximately 76 $\mu\text{g/ml}$ whereas the new standard gives a result of about 45 $\mu\text{g/ml}$. These two figures are very close to the mean IgA concentration in either QC for all the previous assays run using the old standard = 78 $\mu\text{g/ml}$ (n=23), and all the assays subsequently run using the new standard = 41.9 $\mu\text{g/ml}$ (n=21).

The first and third columns show the results using corresponding standards that had been defrosted once before. This did not make any difference to the total IgA measured in the QC.

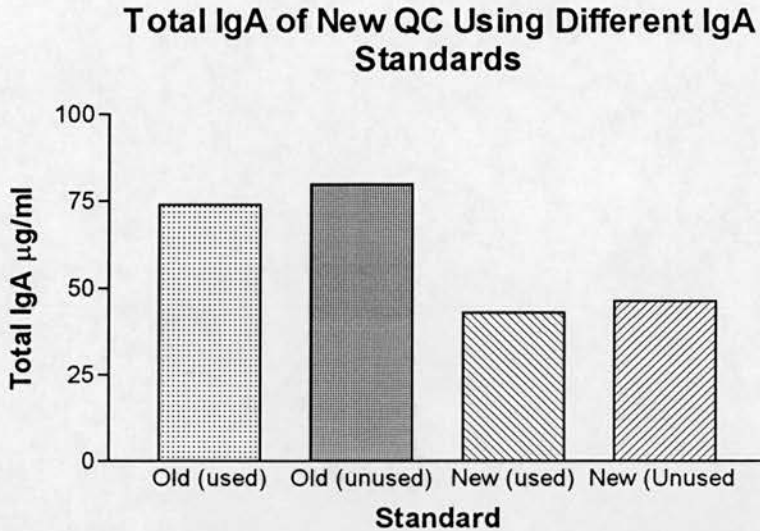


Figure 5.3: Comparison of total IgA in new QC

Using new and old standard as marked on the x-axis. Also compares standards that have been defrosted once and refrozen at -20°C (used), and fresh standard (unused).

Coefficient of Variation

The coefficient of variation for all the plates run during this study is 23 % at a concentration of $78\ \mu\text{g/ml}$. This is very close to the variation for the WGLF total IgG of 20 %, this latter assay is run on weekly basis as a diagnostic test.

Conclusions

In this section I have shown that the lower levels of IgA being recorded in the QC samples in the latter stages of the study was due to the new standard preparation.

I contacted the company that supplies the standard who said that although they assess purity of the protein, they do not guarantee the antigenic reproducibility for this sort of assay. This has shown the importance of using quality control specimens in regularly run assays. As it has not been possible to repeat all the assays done with the new standard (due to lack of the old standard and the lack of unused samples for

analysis). For all the assays done with the new standard I have multiplied the estimated result by a factor of $78/41.9 = 1.86$.

IgM: Quality Control

Using the same QCs I monitored the functioning of the total IgM assay over the period of the study (Figure 5.4). Although the trend line slightly increases over the time of the study this was not significant (rising from 10.8 to 11.8 $\mu\text{g/ml}$).

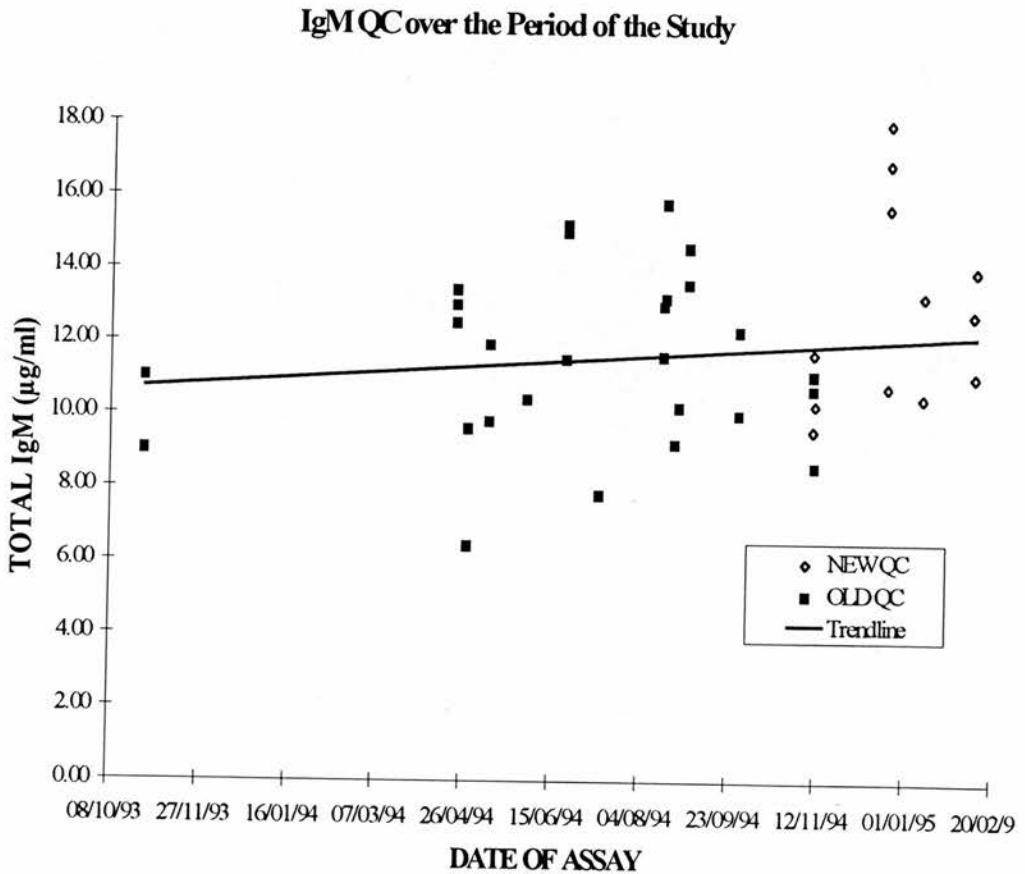


Figure 5.4: IgM QC over the period of the study.

Coefficient of Variation

The coefficient of variation for all the plates run analysing IgM was 21 %.

As with IgA I compared the old and new QC's on the same plate. Figure 5.5 demonstrates the there were no significant differences between the two QCs.

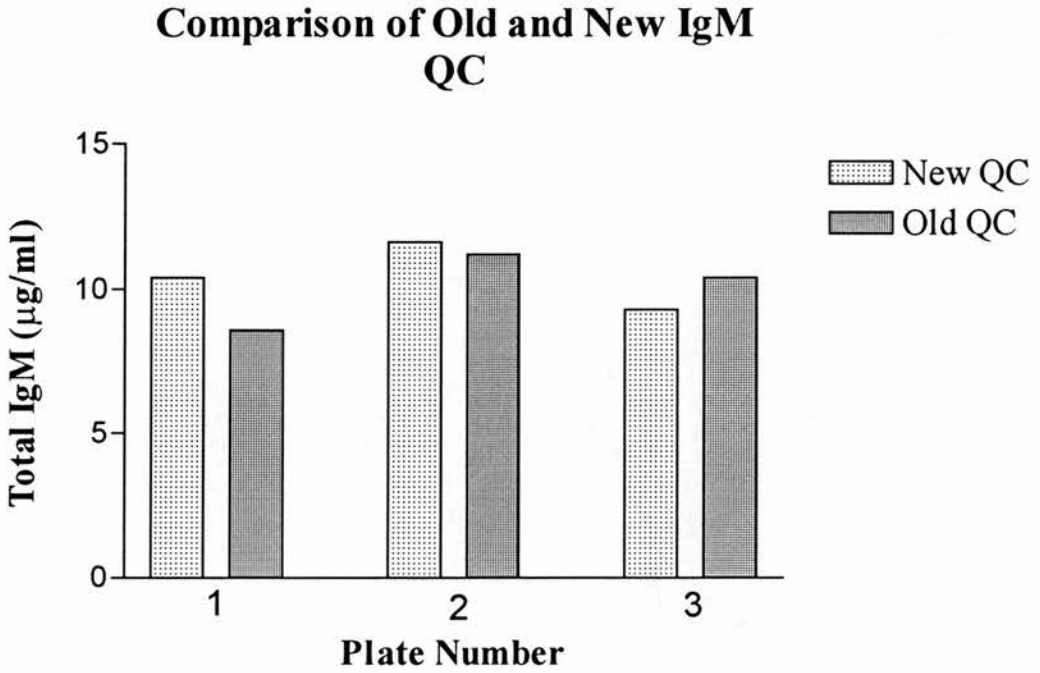


Figure 5.5: Comparison of old and new IgM QC on the same plates.

Degradation of Immunoglobulins and Antibodies

It was important to establish the rate of degradation in WGLF for two reasons.

1. To estimate the extent of breakdown that could occur in WGLF fluid within the GI tract.
2. To give an estimate how rapidly total immunoglobulins and antibodies are degraded in the presence of degradative enzymes throughout the GI tract.

In chapter 10 I will be discussing possible reasons for very much lower rate of recovery of total IgA in faecal/ileostomy output compared with that found in WGLF. Dr O'Mahony's previous work looked at the degradation of the immunoglobulin up to 2 hours (O'Mahony *et al* 1990). As most lavages continue for more than two hours I proposed to look at the degradation over a longer period.

Aims

To assess the rate of degradation of total IgA and IgM, and an IgA antibody (ovalbumin-A was selected) in whole gut lavage fluid over a 24 hour period.

Method

Five freshly passed lavage specimens were collected and kept in a water bath at 37°C for up to 24 hours. Specimens were removed, filtered and processed at 0, 2, 8, and 24 hours.

I analysed these for total IgA, IgM and Ova-IgA (an antibody that is almost always detectable in WGLF in adults).

Degradation of Total IgA

The five specimens all had an initial total IgA concentration of between 45 and 75 µg/ml and the parallel lines in figure 5.6 demonstrate homogeneity in the rate of degradation.

Degradation of Total IgA

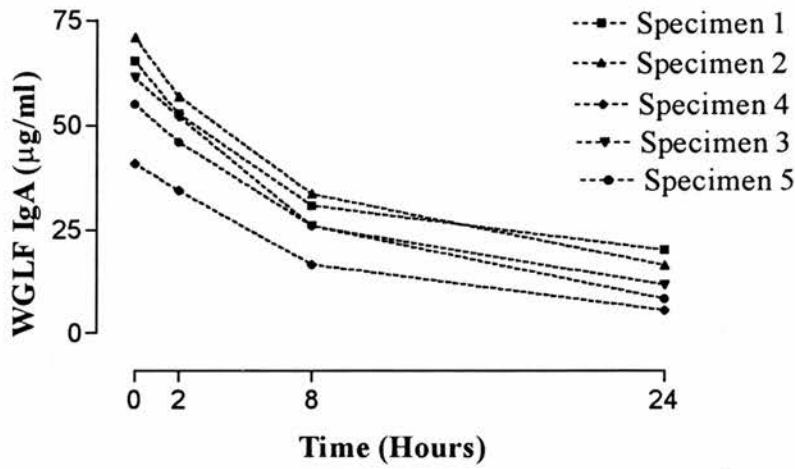


Figure 5.6: Degradation of total IgA in WGLF at 37°C over 24 hours.

For these specimens, taking their initial concentration as 100%, the mean rate of degradation was from 100 % (0 hours) to 82.3% (2 hours) to 44.4 % (8 hours) to 21.7% (24 hours).

Degradation of Ovalbumin IgA Antibody

Four of the above specimens were analysed for Ova A. Unfortunately two had levels below the limit of detection. The remaining two had very similar rates of degradation to that found in total IgA with means of 100% (0 hours), 80.7% (2 hours), 47% (8 hours), and 24.6 (24 hours).

Degradation of OVA-A

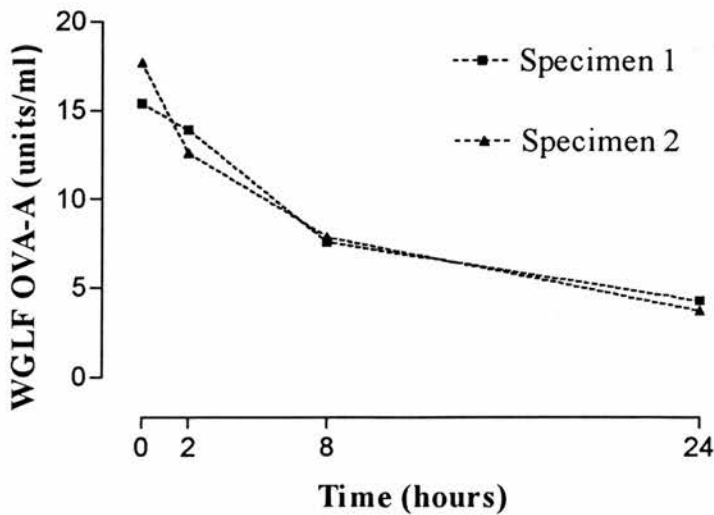


Figure 5.7: Degradation of Ova-A in whole gut lavage fluid at 37°C.

Degradation of Total IgM

In four specimens IgM showed a reduction of between 3 and 10 $\mu\text{g/ml}$ over the 24 hours period. The two specimens with the lowest levels of IgM were below the lower limit of the standard line at 24 hours.

Degradation of Total IgM in Lavage Fluid

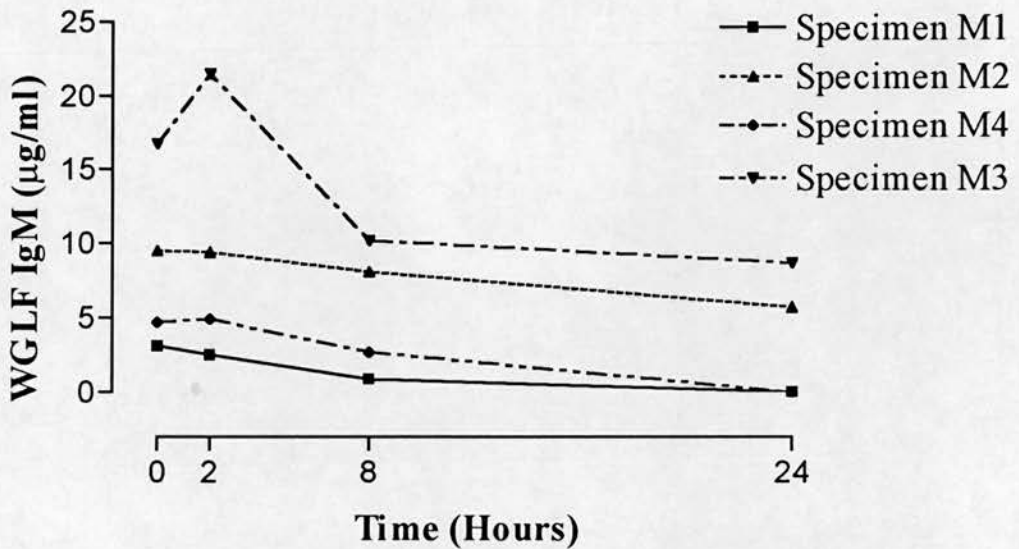


Figure 5.8: Degradation of Total IgM in WGLF at 37°C.

Expressing these results as mean percentages led to 100 % at 0 hours, 100 % at 2 hours, 58 % at 8 hours and 28 % at 24 hours. The two specimens with higher levels of IgM had 60 and 51 % at 24 hours. Thus there seems to be little reduction in the measured concentration of IgM in WGLF over two hours, up to 60% remains at 8 hours.

Discussion

This section has shown that when using ELISA to assay immunoglobulins and antibodies the concentration measured is reduced by delaying the processing of WGLF.

A previous study that investigated the degradation of colostral IgA (>90% secretory) in duodenal fluid over a period of 8 hours found that there was about 65% remaining at 8 hours, whereas only about 35% of serum (monomeric) IgA remained at 8 hours (Brown *et al* 1995). Previous work in our laboratory has shown that in patients with IBD 80% of IgA measured in WGLF is secretory (O'Mahony *et al* 1990). This accords with the relative stability of secretory versus monomeric IgA in GI fluids.

Thus the apparently slightly faster rate of breakdown to 44% over 8 hours in the WGLF specimens could be due to a higher proportion of non-secretory IgA. It is also possible that the presence of other degradative enzymes originating from other parts of the GI tract (e.g. from bacteria present in the large bowel) may increase the rate of degradation in the WGLF when compared to degradation in duodenal fluid.

For the total IgA ELISA the binding of both the coating anti-IgA antibody and the conjugate dependant upon the α chains of the immunoglobulin. The Ova-A ELISA differs in that the initial binding will depend upon the fragment antigen binding (Fab) end of the immunoglobulin. The similarity between the rates of reduction of concentration in the same degradative environment of both the total immunoglobulin and the antibody would suggest that this is less likely to be due to degradation of the Fab end of the molecules as the Fab binding in the total IgA ELISA is not an essential part of the binding. Thus it seems possible that the Fab is maintained in this system and the degradative process is more likely to affect the α -chain (Fc region) hence the similar rate of degradation. It has been found that enteric microbial enzymes produce an Fc fragment of total immunoglobulin A (Haneberg & Aarskog, 1975).

Development of the ECP Assay

Given the importance of eosinophils in conditions relevant to the gastrointestinal mucosa, including food hypersensitivity, parasitic infections and inflammatory bowel diseases I developed the ECP assay for whole gut lavage fluid, in which it had not been previously measured.

Recovery of ECP

Spiking of two filtered, processed lavage specimens demonstrated a recovery of 100% in both specimens. This showed that the processing agents do not interfere with the ECP assay.

Coefficient of Variation

Ten specimens had repeat assays from which the coefficient of variation equalled 6%.

Effect of Processing

Figure 5.9 shows that adding processing agents led to higher levels of ECP in all except one unfiltered WGLF specimen if processing agents were added prior to freezing. Thus for all my analyses I used processed specimens.

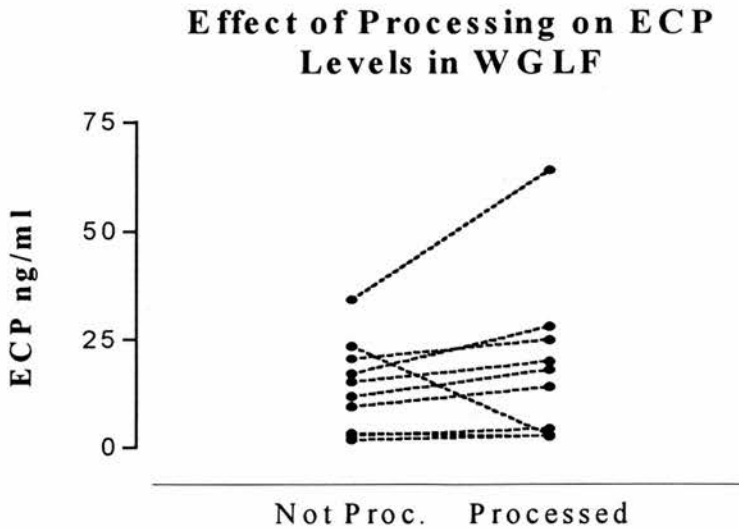


Figure 5.9: Effect of processing on the concentration of ECP in WGLF.

Effect of filtering

In 32 specimens I looked at the effect of filtration on the level of ECP in WGLF by analysing filtered and unfiltered specimens.

Figure 5.10 shows there is a significant reduction in the measured ECP in the filtered specimen.

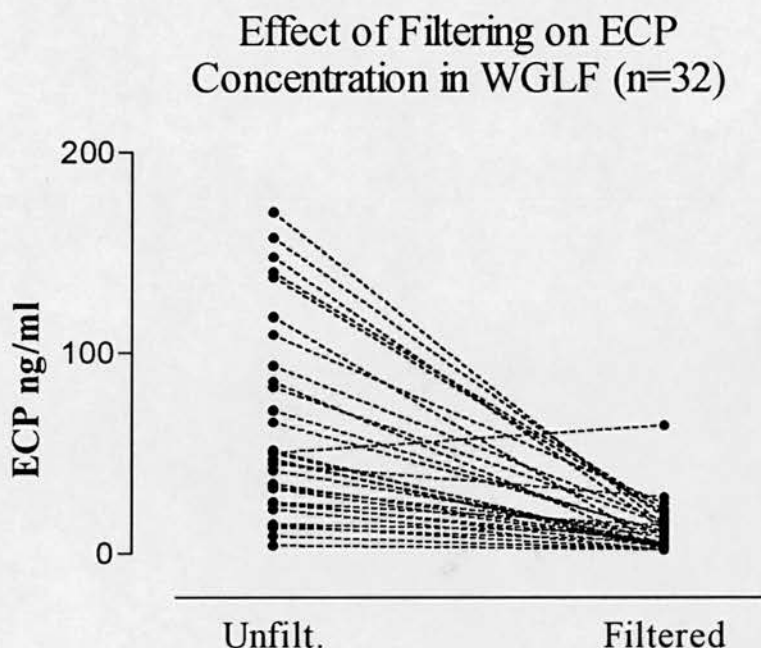


Figure 5.10: Effect of filtering on concentration of ECP levels in WGLF.

It is possible that ECP molecules in unfiltered specimens are attached to the surface of eosinophils or granules which have been released by the breakdown of whole eosinophils (due to freezing and thawing of the specimens). These granules are approximately 1 micron in size which is the pore size of the filter paper and would be filtered out.

The possibility that unfiltered and filtered ECP results were directly correlated in a linear fashion was not confirmed as the Pearson's rank correlation coefficient was 0.296, $p=0.1$.

Effect of centrifugation

If cells or cellular constituents to which ECP is attached are filtered out by the filter paper, then one would expect centrifugation to also reduce the level of ECP.

I examined this by measuring ECP concentration in seven specimens the first of which is unfiltered, processed and not centrifuged, then the same specimen centrifuged and finally the same specimen both filtered and processed.

Figure 5.11 shows that centrifugation clearly reduces the level of measured ECP, the resulting ECP concentration being slightly higher to that measured in the corresponding filtered specimen.

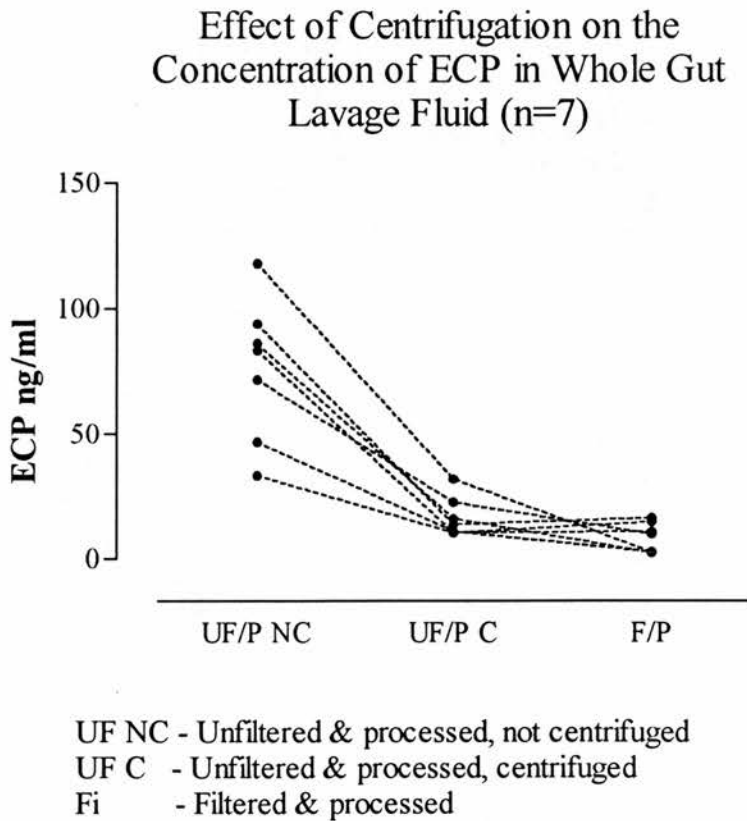


Figure 5.11: Effect of centrifugation on the level of ECP in WGLF.

Defrosting

The effect of defrosting specimens was examined by comparing the ECP in F/P specimens stored at -70°C with specimens that have been used once (defrosted to room temperature and then refrozen at -20°C). Ten of these paired specimens showed no deterioration in the measured level of ECP. This confirmed that it is possible to use specimens that have been defrosted once for ECP analysis.

Sonication

When analysing ECP it is possible that sonication could affect the measurement in one of two ways.

1. Whole eosinophils present, that contain ECP, will be broken down releasing intracellular ECP and hence increase the concentration in WGLF.
2. Sonication may denature ECP and lead to reduced concentration of ECP.

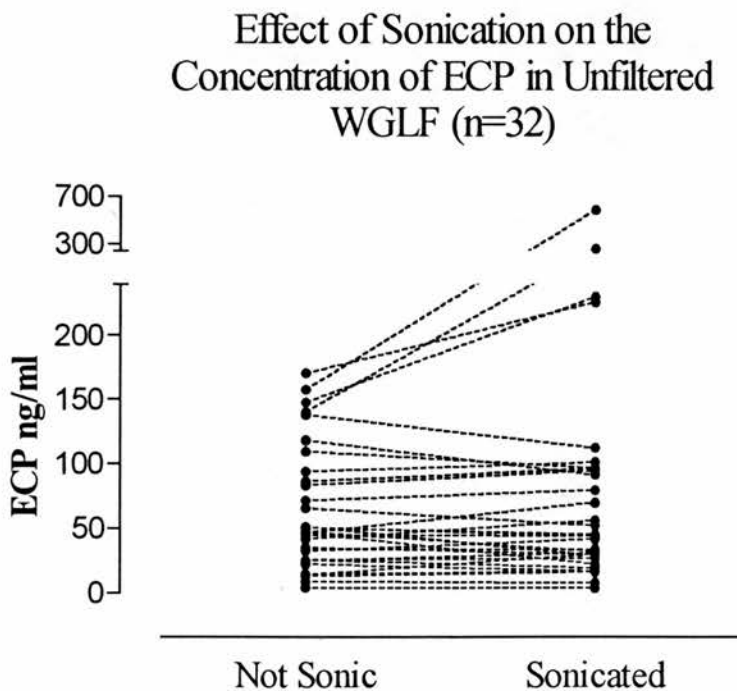


Figure 5.12: Effect of sonication on levels of ECP in unfiltered WGLF.

Figure 5.12 shows individual paired specimens with the concentration of ECP before and after sonication.

The small amount of water loss in the sonication process (2.8 %) (see description of sonication method in chapter 4) would not have any significant influence. These graphs show that, as a group, sonication does not significantly alter the measured level of ECP. However as you can see in figure 5.12 there are four specimens which do clearly increase with sonication. These are all at the top level in the 'not sonicated' group. This could be due to the presence of complete eosinophils in the lavage specimens which are broken up further by the sonication process, releasing ECP for assay.

Four specimens that were spiked with ECP with 100% recovery showed no reduction in the level of ECP with sonication. Thus showing evidence that sonication does not reduce the measurable level of the ECP standard.

Conclusions for Analysis of ECP in WGLF Specimens

In setting up the assay for ECP it was important to establish the most appropriate method of processing the specimens

I will refer to ECP measured in filtered specimens as 'free' ECP. ECP measured in unfiltered specimens is likely to be a combination of this 'free' ECP and ECP that is either still attached to the granules from the eosinophils or still attached to the cell, this will be referred to as 'total' ECP.

As there was no significant correlation between the filtered and unfiltered specimens I collected both when wishing to analyse ECP.

For measuring ECP in WGLF I analysed both **filtered, processed** specimens and **unfiltered, processed specimens**. These were not centrifuged or sonicated.

ECP in Serum and WGLF

It was also important to establish whether ECP actually originated from the gastrointestinal tract or could have been a result of plasma leakage.

For this I collected both lavage and serum from 10 patients undergoing lavage for clinical reasons.

Eight were adults with IBD. Five of these had active disease as defined by WGLF IgG >10 µg/ml (Brydon *et al* 1993), the remaining three were inactive. The remaining two were children, one had severe constipation the other cystic fibrosis (the former had normal total WGLF IgG, the latter a raised IgG of 20 µg/ml).

Concentrations of ECP were measured in WGLF (UF/P) and serum collected at the time of the lavage. The raw data of the concentration in the WGLF and the serum in table 5.1 clearly show that the ECP measured in the WGLF could not originate from the serum as the concentrations in WGLF are all greater than those measured in the corresponding serum.

During WGL in adults 20 ml/minute (1200 ml/hour) of the lavage fluid is given. For serum to be the cause of these ECP levels considerably more than 1.2 litres per hour of serum would have to be secreted into the GI tract. This is not possible as the patient would become rapidly hypotensive with fluid losses of this quantity.

Table 5.1: ECP in unfiltered WGLF and corresponding serum.

Patient	WGLF (UF/P) ECP (ng/ml)	Serum ECP (ng/ml)
1	45	41
2	78	21
3	38	41
4	>200	62
5	>200	23
6	>200	17
7	95	37
8	21	9.2
9	16	8.4
10	70	23

This confirms that the majority of ECP measured in the WGLF originated from within the GI mucosa as opposed to serum.

SECTION III: Analyses of Whole Gut Lavage in Children

Chapter 6: Analyses of Whole Gut Lavage in Control Children

Introduction

Using specimens collected from control children the first question that needed to be addressed was whether there were any fundamental difference between concentrations of substances in whole gut lavage fluid from control adults and control children.

The data for adults originates from publications from the gastrointestinal unit at the Western General Hospital in Edinburgh and as the methods are the same as I have used in this thesis the results are directly comparable with the data for the children.

In the section on practical details of lavage I have shown that the rate of lavage in the children studied, expressed as ml/kg/hour, is very similar to the rate in adults (approximately 15 ml/kg/hour). In this chapter I will refer to absolute concentrations in whole gut lavage fluid which, as the rates for adults and children are similar, can be comparable between the two groups. Later, in chapter 11 I will be discussing the daily rates of secretion of the substances.

Patients

The control children that I will be discussing in this chapter have been introduced in chapter 3. A total of 13 specimens were collected from 12 patients.

Included are the four children who underwent whole gut lavage for the treatment of severe constipation, one of these children had a second lavage performed four months later for the same indication.

Results

Analyses of WGLF can be divided up into groups of substances which look at different aspects of the gastrointestinal inflammatory/immune processes.

GI Inflammatory/Immune Process	Substances Analysed
GI Bleeding	Haemoglobin
Mucosal Inflammation / Plasma Leakage	Total IgG A1AT Albumin
Cytokines	IL-1 IL-8
Cellular Mediators	Eosinophil Cationic Protein Granulocyte Elastase
Secretory Immunoglobulins	Total IgA Total IgM

I will now discuss the results for the control children and compare them with normal ranges previously established in adults (summarised in table 6.1).

Gastrointestinal Bleeding

The normal range for adults is between 0 and 5 µg/ml (Brydon & Ferguson, 1992). The range of all the control children was from 0 to 4 µg/ml. Thus there was no evidence for GI bleeding in these children.

Mucosal Inflammation and Plasma Leakage

Total IgG

The normal range in adults is up to 10 µg/ml (Brydon *et al* 1993). In the control children the median and range were 2 (0-8) µg/ml. All the IgG results in the control children were ≤ 3 except in one child who had the level of 8. This child had short stature and is clinically suspected to have Crohn's disease but there is no radiological or pathological evidence to confirm this. Data in adults from the Western General Hospital now suggests that healthy control adults all have total IgG of less than 5

µg/ml (Sallam, 1995). Thus this one child may have had borderline evidence for gastrointestinal inflammation.

A1AT

The normal range in adults is up to 19 µg/ml (Brydon *et al* 1993). The control children had a median (range) of 7 (0-21) µg/ml. The one child with A1AT of 21 was a child being treated for severe constipation. He had no other results suggestive of GI inflammation.

Albumin

Normal concentrations for adults are up to 26 µg/ml (Brydon *et al* 1993). The median and range in the control children were 8 (0-18) µg/ml. Again the child with the highest level was the one suspected of having Crohn's disease.

Cytokines

Interleukin-1β

Healthy control adults have concentrations up to 18 pg/ml. The median (range) in the control children is 10.2 (2.2-84.2). The child with suspected Crohn's disease had the highest level of 84 pg/ml which in adults would be considered abnormal.

Interleukin-8

The normal range for IL-8 in adults is less than 4 pg/ml. The median and range in control children is 0 (0-14). The two highest concentrations were 12.6 and 14 pg/ml in the child suspected of having Crohn's and the child subsequently found to have ulcerative colitis.

Cellular Mediators

Granulocyte Elastase

The normal range in adults is <100 kcat/ml (Ferguson *et al* 1994). The median and range of the control children were 0 (0-51) kcat/ml. Thus no children had results outwith the adult range.

Eosinophil Cationic Protein

There is no available normal data for adults for the ECP assay. The control children had concentrations of 24 (9-66) ng/ml. The highest result was in the child who had eosinophils seen in her biopsy specimen and was later found to have ulcerative colitis.

Secretory Immunoglobulins

Immunoglobulin A

In adult healthy volunteers total IgA in WGLF has been shown to have a median concentration of 73 µg/ml (range 10-173 µg/ml) (Sallam, 1995). In control children the median was 58 (range 3-167) µg/ml. It was noticeable that all four children undergoing lavage in the treatment of severe constipation had concentrations of IgA less than or equal to 10 µg/ml. If the constipated children are excluded then the median and range of total IgA in the remaining eight control was 69 µg/ml (21-167 µg/ml), very similar to the adult results.

The possibility that the children had a mucosal IgA deficiency associated with the constipation led me to study this in more detail which I will discuss in chapter 7.

Immunoglobulin M

Taking all 12 control children the median concentration was 2.4 (0.9-31.5) µg/ml. This is very similar to total IgM in adult healthy volunteers of 2.2 (0.1-9.5) µg/ml (Sallam, 1995).

Discussion

Taken as a group these children have results within the normal range for adults, see table 6.1 for summary. However the four with severe constipation have very low total IgA levels, their remaining parameters being within the adult normal data and similar to the remaining eight children. I will discuss further these low total IgA results in chapter 7.

Three of the remaining children also have distinctive results:

First is the child clinically suspected of having Crohn's disease. He was the child who had the highest levels of total IgG, albumin, IL-1 and the second highest level of IL-8. Only IL-1 and IL-8 are definitely outwith the adult normal range. All of these parameters can be raised in adults with Crohn's disease and these results would be consistent with this diagnosis.

Next is the child who has subsequently been found to have ulcerative colitis and had eosinophils in her biopsy at colonoscopy, despite being macroscopically normal, who had the highest concentration of ECP and IL-8 in her WGLF.

The child with possible malabsorption and a gastrostomy tube feed because of a large cystic hygroma had a concentration of IgM of 31.5 µg/ml. The next highest concentration in these children is 8.6 µg/ml. One other child who was the same age (11 months old) had an IgM concentration of 0.9 µg/ml, clearly different to this child. Thus this child appears to have a very high IgM concentration which may have some as yet unknown, clinical or diagnostic significance.

The three highest levels of total IgA were in the above three children. It is possible that these children have GI mucosal immune activation. In two this has not yet been identified (one of these could have Crohn's disease), the third has ulcerative colitis.

In this thesis I have collected specimens from children undergoing lavage for clinical reasons, thus they are disease controls. The children who underwent colonoscopy for rectal bleeding without finding any abnormalities (other than one rectal polyp) all had results within the adult normal range. In the future by collaborating with other

paediatric gastroenterologists I plan to collect more specimens from children undergoing lavage for this indication with these findings to be used as control data.

When discussing the control data in the remainder of this thesis I have included all the results except the total IgA concentrations in severely constipated children. This will tend to increase the medians for the control data as the three children discussed above all have parameters at the highest concentrations. My reason for following this policy is that at the outset of this thesis I planned to include as controls all children without a diagnosis of a gastrointestinal mucosal abnormality being made at the time of the whole gut lavage.

Table 6.1: Whole gut lavage fluid parameters in control children and the normal range in adults.

Please see above text for discussion and references.

Substance (Units)	Adult Normal Range	Control Children n=12 Median (Range)
Haemoglobin ($\mu\text{g/ml}$)	<5	0-4
Total IgG ($\mu\text{g/ml}$)	< 10	2 (0-8)
A1AT ($\mu\text{g/ml}$)	< 19	7 (0-21)
Albumin ($\mu\text{g/ml}$)	< 26	8 (0-18)
IL-1 β (pg/ml)	< 18	10.2 (2.2 - 84.2)
IL-8 (pg/ml)	< 4	0 (0 - 14)
GE (kcat/ml)	< 100	0 (0 - 51)
ECP (ng/ml)	Not available	24 (9 - 66)
Ig A	73 (10 - 173)	69 (21-167)*
IgM	2.2 (0.1 - 9.5)	2.4 (0.9 - 31.5)

* Excludes four severely constipated children, please see text for discussion.

Chapter 7: Low Total IgA in Severely Constipated Children

In the children who had a lavage for severe constipation all four had levels of IgA in their WGLF of ≤ 10 $\mu\text{g/ml}$. One later had a repeat lavage for the same indication with normal concentration of total IgA. All these children had their lavages early on in the study and were prolonged. The problems of continuing the lavage so that the effluent was completely clear of faecal material meant that these specimens were possibly not as clear as those collected later on in the study. Thus these lower levels of IgA could have been related to the fact that the specimens were not completely clear of faecal material.

Work by Dr O'Mahony has previously shown that unclear specimens can have lower levels of total IgA than subsequent clear ones (O'Mahony *et al* 1990). The reasons for this are not known.

Possible causes of low WGLF IgA in these children are:

1. Primary mucosal IgA deficiency being associated with severe constipation.
2. Dilution by the WGL fluid of intestinal secretions already present within the GI tract.
3. Degradation due to contamination with faecal material.
4. Binding of IgA to bacteria present in faecal material
5. Interference of the IgA assay by substances contained in the residual faecal material.
6. Inhibition of secretion of IgA from the GI mucosa by substances present in faeces thus there are truly lower amounts of IgA in the faecal material.
7. Recycling of IgA within the GI tract. This was suggested at a presentation made to a nutrition group where the comment was that there would be a large negative nitrogen balance if IgA (and other proteins) were secreted continuously in the normal state at the rate identified in WGL.

I will now discuss each of these possibilities with some experimental evidence for numbers 1-3.

Mucosal IgA Deficiency

Three of the four severely constipated children had had rectal biopsies as part of the investigation for the possibility of Hirschsprung's disease, four of the children undergoing colonoscopy also had rectal biopsies taken during the procedure. With the help of Dr J Keeling, consultant pathologist at the Royal Hospital for Sick Children, we were able to collect these biopsies and stain them for IgA secreting plasma cells. Using image analysis I then counted these specimens. Other control specimens were supplied by Dr Keeling for comparison. All were counted by me blind to the diagnoses of the patients.

Table 7.1 shows all the results of lavage total IgA and biopsy IgA plasma cell counts from these children. It shows that the constipated children did not have significantly lower numbers of IgA plasma cell counts in the lamina propria of their rectal biopsies. Although it was not possible to collect small bowel biopsies it would seem very unlikely that the very low levels of IgA in their WGL specimens was due to a mucosal IgA deficiency as has been described by our group (Sallam, 1995; Ferguson *et al* 1995). The child who had a second lavage for severe constipation later in the study had a WGLF total IgA concentration of 74, compared with 3 µg/ml in his first lavage.

When the diagnoses of the specimens supplied by Dr Keeling initially were revealed the two children with very low number of IgA secreting cells were also the only two that had Hirschsprung's. They were also the youngest aged 1 month and 3 days respectively. This is consistent with the findings of a study (Perkkiö & Savilahti, 1980) where IgA secreting cells are few or absent in the first 2 months after birth. We then requested more specimens from older children with Hirschsprung's to show that IgA deficiency was not a feature of this disease. As the table shows the three older children with Hirschsprung's all had normal numbers of IgA secreting cells.

These results indicate that the children with severe constipation have normal numbers of IgA plasma cells and thus there is no evidence for a mucosal IgA deficiency.

Table 7.1 IgA cell counts in rectal biopsies from children.

NAME	Diagnosis/ Findings at Colonoscopy	Age at biopsy	WGLF Total IgA µg/ml	IgA Plasma Cells /mm ²
DMcG	Constipation	12 yrs	5	487
JL	Constipation	10 mths	10	619
LM	Constipation	8 yrs	3	435
LM	Constipation (Second Lavage)	8 yrs	74	ND
LMcD	Constipation	9.8 yrs	6.5	ND
AH	? Crohn's	13 yrs	118	1079
CT	Normal	5 yrs	60	1094
AD	Juvenile Polyp	5 yrs	55	450
TI	Normal	8 yrs	63	862
SD	? Hirschsprungs Biopsy NAD	1.6 yrs	ND	668
VM	? Hirschsprungs Biopsy NAD	1.9 yrs	ND	686
DM	Hirschsprungs	1 month	ND	35
HMacF	Hirschsprungs	3 days	ND	0
HM	Hirschsprungs	6.3 yrs	ND	1395
HT	Hirschsprungs	6.5 yrs	ND	944
HMcM	Hirschsprungs	5.3 yrs	ND	974

ND = Not done.

Dilution of Intestinal Secretions by WGL Solution

The next possibility to consider was that secretions present within the GI tract were diluted by the WGL solution as it arrives in the small bowel/colon.

For this to be the case one would expect the concentration of the PEG in the resulting specimen to be less than in the solution being administered. It has previously been shown that effluent collected after WGL is 15 % gastrointestinal secretions and 85 % polyethylene-glycol lavage solution (O'Mahony *et al* 1990). However to get the very low levels of IgA in the solution one would expect the level of the PEG in the resulting effluent to be of the order of 1/10 of the original solution if dilution was the reason for the low total IgA. In view of this PEG levels in specimens collected from the children with low total IgA were measured. The concentrations measured were > 80% of the concentration of the fluid administered and are very similar to the concentrations found from lavages collected from adults previously (O'Mahony *et al* 1990). Thus dilution of intestinal fluid by Klean-Prep is not the cause for the low IgA levels.

Degradation Due to Contamination With Faecal Material

The rate of degradation of IgA in WGLF has been described in chapter 5. At two hours there is 80% of the original amount remaining, at 8 hours 44%. This rate of degradation is insufficient to explain the very low levels of total IgA in the WGLF of the four constipated children. For degradation to cause the breakdown of the IgA to 10% of the original the immunoglobulin would have had to been exposed to the enzymes for more than 24 hours. Whole gut lavage in these four children took a median of 9.5 hours (range 6-15), the exposure of IgA to the degradative enzymes would have been less than this figure as the actual transit time of a given bolus of fluid would be roughly 2-3 hours before being passed and processed immediately with anti-protease and anti-bacterial inhibitors.

Thus degradation within the GI tract is insufficient to explain these very low levels of total IgA.

Alternative Explanations

Binding of IgA to Bacteria in Faecal Material

Bacteria within the GI lumen are known to have IgA bound to the surface although whether this IgA is measurable by ELISA is not known. It would seem feasible that if the majority of IgA secreted into the lumen of the GI tract were bound to the surface of bacteria (which would be at highest numbers in faecal material) then the amount of IgA measured in faecal or unclear lavage specimens would be lower than in clear WGLF.

Measuring the concentration of IgA in corresponding unfiltered and filtered (filtering removes most bacteria) WGLF would not necessarily answer this question as if IgA bound to the bacterial wall were undetectable by ELISA then filtering out bacteria should not alter the measured concentration. Early in my work I compared unfiltered (UF/P) and filtered WGLF collected at the same time in 7 subjects. The filtered specimens were slightly lower but not to a significant degree.

Table 7.2: Concentration of IgA in filtered, processed (F/P) and unfiltered, processed (UF/P) whole gut lavage fluid collected simultaneously.

	Concentration of IgA (μ /ml)	
	Mean (n=7)	Median (n=7)
UF/P	124.3	55
F/P	101.1	39
Difference	23.1	16
p=	0.1	0.05
	(Two Sample T Test)	(Wilcoxon Signed Rank)

This possibility could be looked at further by spiking unfiltered and filtered WGLF with IgA where one would expect unfiltered specimen to have a lower measured

concentration of IgA as IgA would be bound to the bacteria still present in the unfiltered specimens. In this situation one would assume that the bacterial surface is not saturated with IgA. The ideal situation would be to use a broth of faecal flora known not to have IgA present (or WGLF from subjects known to be IgA deficient) and spike this with IgA.

Interference by Substances Present in Faecal Material

The possibility that substances present in faecal material interfere with the total IgA ELISA assay has not been studied in this thesis. The implication of this is that there would be a normal levels of IgA present which were not detected using ELISA. However this would be unlikely as studies that have analysed faecal extracts for total IgA by other techniques such as RIA or immunonephelometry have found similar or lower amounts of IgA. It would be very unlikely for there to be factors that interfere with all these different techniques.

More work needs to be done to confirm beyond doubt that interference is not affecting the ELISAs. By adding faecal extracts to standard IgA solutions one could establish if these are interfering with the IgA assay.

In this thesis the IgA ELISA starting dilution for WGLF and ileostomy/faecal extracts is 1/100. For ileostomy and faecal specimens these are previously extracted at 1/5 or 1/10. Thus the starting dilution from the original specimen is at least 1/500. Any possible factors that are present are likely to be sufficiently diluted out to not significantly interfere with the analyses.

Inhibition of Secretion of IgA from the GI Mucosa by Substances in High Concentrations in Faecal Material

One hypothesis is that there are one or more factors in faecal material that inhibit the transport of sIgA into the lumen of the GI tract. This is a very important possibility particularly with regard to the understanding of the immune response in diarrhoeal disease. I was not able to study this further as it would have been a major digression from the main aims of this thesis.

In the scenario where a subject develops diarrhoea due to an infectious agent or other aetiology, the inhibitory substances within the GI lumen could be significantly reduced in concentration. This in turn could lead to increased IgA secretion into the GI tract which seems a logical non-specific immunological defence mechanism to combat antigen exposure. This could also explain why, as I will show in chapters 10 and 11, daily IgA secretion in faeces is very much less than in whole gut lavage fluid. In effect WGL mimics the situation in diarrhoea where raised IgA secretion has also been found.

In the situation of these very constipated children, if the WGLF specimens collected were not clear of all faecal material, there may still be sufficient inhibitory factors within the GI tract present to keep the total IgA secretion to a minimum hence the very low total IgA results in their WGLF. The child who had a second lavage had a normal total IgA result. As I knew that his original specimen had a low total IgA and that his biopsy had normal number of IgA plasma cells, I was careful to collect a specimen once it was definitely clear of all faecal material revealing a normal total IgA.

Recycling of IgA within the GI tract

A final hypothesis is that IgA is recycled within the gastrointestinal tract. IgA secreted by the small bowel may be reabsorbed distally, probably in the colon. In the situation of whole gut lavage or diarrhoea the rapid transit through the bowel would limit reabsorption of the immunoglobulin. This is unlikely to explain the low IgA in these children as the transit time of the lavage fluid through the bowel would be similar whether or not it is completely clear of all faecal residues.

Discussion

In this chapter I have presented evidence that the low IgA concentration in the severely constipated children is not due to local IgA mucosal deficiency, dilution of the intestinal fluid or degradation of the IgA during transit through the GI tract.

A final possibility is that whole gut lavage, either by direct action or by reduction of inhibitory factors in the lumen, effectively stimulates the secretion of IgA. In these children with low IgA levels the specimen is collected early in this process prior to the steady state of secretion being attained.

Therefore I have not found an explanation for the very low concentrations of IgA found in these severely constipated children. The fact that the one child who had a repeat lavage later in the study had normal IgA concentration suggests that this was to do with the collection of the specimen, most likely due to the specimens not being entirely clear of all faecal material. The problems of interpreting IgA secretion rates into the GI tract with different conditions in the will be discussed further in the final discussion (chapter 12).

Chapter 8: Intestinal Mucosal Inflammation and Immune Activation in Cystic Fibrosis

Introduction and Background Review

Definition and Incidence of Cystic Fibrosis (CF)

Cystic fibrosis is the commonest, life threatening, inherited disease in the United Kingdom. The classical features of the illness include chronic broncho-pulmonary infection and pancreatic insufficiency, with high sweat chloride and sodium concentrations. The incidence is approximately 1/2000 live births, with a carrier rate in caucasians of 1/22. Inheritance is autosomal recessive and the gene has been located on the long arm of chromosome 7 (Rommens *et al* 1989; Riordan *et al* 1989; Kerem *et al* 1989). So far more than three hundred mutations have been described (Tsui, 1992). The protein encoded for by the gene is known as the cystic fibrosis transmembrane regulator (CFTR) which is a channel for chloride ions (Santis & Geddes, 1994). A functional defect in this protein decreases excretion of chloride from the mucosal surface and increases reabsorption of sodium into the epithelial cells. This leads to less water being excreted into the lumen and more viscous and tenacious secretions.

Ten to fifteen percent of cases present in the neonatal period with meconium ileus where a combination of thick meconium and hypoplasia of the ascending colon causes obstruction in the first two days of life. The remainder present later, most commonly with a combination of recurrent chest infections, chronic diarrhoea and failure to thrive. Diagnosis is confirmed by establishing the concentration of chloride and sodium in sweat. With the methods used in Edinburgh sweat chloride >70 mmol/l is abnormal in children under 12 and indicative of cystic fibrosis, 50-70 mmol/ml is considered equivocal (Laboratory handbook, Department of Clinical Biochemistry, R.H.S.C., Edinburgh (1992)). It is now possible to confirm the diagnosis using genetic probes. 70% of CF patients in the United Kingdom are homozygous for $\Delta F508$, a deletion of phenylalanine at position 508 in the amino acid sequence. 85 %

of carriers can be identified by testing for the four most common mutations (Santis & Geddes, 1994).

CF in the Royal Hospital for Sick Children, Edinburgh

In Edinburgh seventy five children are followed up at the Royal Hospital for Sick Children under the care of Dr Tom Marshall. The majority of these come from the Lothian and Borders regions, with some living in Fife.

Clinical Features

Cystic fibrosis is a multi-system disorder that typically affects the lungs with chronic infection, and the gastrointestinal tract with malabsorption and its consequences. These problems originate from the very thick mucosal secretions that result from the defective CFTR. In the lung this predisposes to repeated and chronic infections, ultimately leading to cor pulmonale, respiratory failure and death.

Gastrointestinal Complications

There are a number of GI conditions associated with cystic fibrosis, including cow's milk sensitive enteropathy (Hill *et al* 1989), pseudomembranous colitis (Winesett *et al* 1994) and coeliac disease (Valleta & Mastella, 1989). It has recently been shown that there is an increased risk of digestive tract tumours, with an overall odds ratio of 6.5 which increases to 20 in the 20-29 age group (Neglia *et al* 1995). Although serum IgE antibodies to food were not shown to be associated with CF (Ferguson *et al* 1986), higher levels of IgA and IgG antibodies have been found (Troncone *et al* 1994). The importance of this with regard to food allergy or intolerance has not been established.

Crohn's disease is said to be seventeen times more frequent in CF patients than in matched controls (Lloyd-Still, 1995). In reviewing previous case reports of cystic fibrosis with Crohn's the author found that 7/12 had ileo-colic disease. It is possible that a proportion of these children who were said to have Crohn's Disease had the new complication of strictures in the ascending colon that I will describe below.

Histology showing granulomata would be specific for Crohn's, however if

radiological and surgical evidence of an ileo-colic stricture were the findings, without granulomata found in pathological material then it would be important to re-evaluate these patient's diagnoses.

Intussusception, rectal prolapse, vomiting and gastro-oesophageal reflux are other GI complications of CF.

Meconium Ileus

10-15 % of children with cystic fibrosis present in the neonatal period with meconium ileus, i.e. small bowel obstruction due to blockage of the ileum by meconium. This meconium has decreased water content (CF 65% water compared with controls 75% water), increased viscosity, the presence of serum proteins such as albumin and increased levels of disaccharidases (Swachman and Kulezyeki, 1958). The latter two findings are thought to be due to the lack of pancreatic proteolytic enzymes (Eggermont, 1987).

Distal intestinal obstruction syndrome

Distal intestinal obstruction syndrome (DIOS) also known as meconium ileus equivalent, is characterised by episodes of complete or partial intestinal obstruction and affects between 10 and 47 % of CF patients (Littlewood, 1992). The problem is similar to that of meconium ileus in that the bowel is blocked by an inspissated mass of faeces in the ileo-caecal region. Severe cases present with pain, distension, constipation and bilious vomiting, milder cases can present with abdominal pain, reduction in frequency of stools and a palpable right iliac fossa mass. There are a variety of possible treatments including lactulose, gastrografin, enemas, n-acetyl cysteine and whole gut lavage (Koletzko *et al* 1989).

Pancreatic Failure and Malabsorption

85% of children with CF develop exocrine pancreatic enzyme insufficiency (Sokol, 1990) which causes the children to malabsorb, with associated diarrhoea, and failure to thrive. For several decades this has been treated by using pancreatic enzyme supplements that the patients have to take with any meals or snacks. These are in the

form of tablets or capsules which contain lipase, proteases and amylase. They have been shown to improve nutritional status, and reduce morbidity and mortality (Sokol, 1990).

Enzyme treatment and preparations

Until three years ago the children were required to swallow large numbers (up to 15-20) of capsules containing enzyme supplements before meals and snacks. This can be a major problem in the younger children and can affect the appetite of children already susceptible to poor weight gain.

In the UK in 1992 new preparations were introduced with two to three times the amount of enzymes in each capsule (see table 8.1) allowing a significant reduction in the number of capsules taken with each meal or snack. This regimen is preferred by most patients and does not produce any change in fat absorption (Morrison *et al* 1992).

The actual dosage of the capsules has been tailored depending on symptoms such as abdominal pain, stool type and the presence of a palpable faecal mass in the right iliac fossa (Lebenthal, 1994). It has been generally held that improving fat absorption is of paramount importance, regardless of the amount of enzymes being taken. However the available preparations differ in their bioavailability and activity of the enzymes themselves (Kraisinger *et al* 1994).

The Food and Drug Administration in the USA recommend a dose of 1500-2500 units of lipase/kg/meal (Taylor, 1995). In a recent editorial discussing the new complication, Lebenthal suggests a maximum of 3000 units/kg/dose of lipase (Lebenthal, 1994).

Table 8.1: Enzyme Preparations Used in Edinburgh.

(Low dose preparation shown first of each pair)

Preparation	Capsule Contents	Enzyme BP units/capsule		
		Lipase	Protease	Amylase
Creon	microspheres	8000	210	9000
Creon 25,000	microspheres	25000	467	18000
Pancrease	microspheres	5000	330	2900
Pancrease HL	minitablets	25000	1250	22500
Nutrizyme 10	microspheres	10000	500	9000
Nutrizyme 22	minitablets	22000	1100	19800

New Complication of CF: Fibrosing Colonopathy

In December 1993, the Committee on Safety of Medicines notified all doctors in the UK of 7 children with cystic fibrosis who had developed fibrotic strictures of the large bowel, possibly related to the high dose pancreatic enzyme supplements (Rawlins, 1993). They advised that patients should be changed to the low dose preparations unless there were special reasons not to. This complication has now been given the name fibrosing colonopathy (Smyth *et al* 1995). All of the high dose preparations listed in table 8.1 were reported as being related to at least one case of fibrosing colonopathy.

In January 1994, Smyth described five of these children in a paper published in the *Lancet* (Smyth *et al* 1994). These children had presented with symptoms of distal intestinal obstruction syndrome which failed to respond to medical management. At operation strictures of the ascending colon or caecum were found. Pathology of the specimens showed similar features in all the children suggestive of ischaemic denudation of the lining epithelium. They also had disrupted muscularis mucosa

which was being replaced by granulation tissue, and a mild chronic inflammatory infiltrate around blood vessels in the submucosa. The authors interpreted these features as post-ischaemic ulcerative repair due to either pressure induced ischaemia or direct local toxicity. The only recent change in the management of the children was that they had changed from low- to high-dose enzyme preparations 12-15 months prior to presentation. Four had been changed to Pancrease HL, one to Creon 25,000. Although these children had reduced the number of capsules that they had to take by about 1/3 they had increased the overall dose of the individual enzymes taken daily (for example daily lipase dosage increased more than two-fold). In the same issue of the Lancet there were two further reports of strictures with similar pathology to those described by Smyth (Oades *et al* 1994; Campbell *et al* 1994). Fourteen cases, confirmed histologically, have been reported in the UK (Smyth *et al* 1995) and the recommendations persist that high -potency pancreatins should not be used if possible (Committee on Safety of Medicines, 1994). Recent American figures report a total 35 cases of colonic strictures confirmed by histological findings at surgery (Borowitz *et al* 1995). In published American studies higher daily doses of enzymes have been found in the children with strictures (Freiman & Fitzsimmons, 1996; Prestridge *et al* 1996; Schwarzenberg *et al* 1995), they have also reported children who were taking low-dose enzyme preparations (Freiman & Fitzsimmons, 1996). A high proportion of the American patients present with bloody diarrhoea (Prestridge *et al* 1996; Schwarzenberg *et al* 1995) and are found to have strictures involving other areas or the entire colon as opposed to the ascending colon as described in the British children (Freiman & Fitzsimmons, 1996; Prestridge *et al* 1996; Schwarzenberg *et al* 1995). Finally a group have reported a four year old child who was “pre-stricture” with focal fibrosis, increased eosinophils, plasma cells and ulceration (Lloyd-Still & Powers, 1994).

Most studies have discussed the enzyme dose taken by the children as units of lipase. Unfortunately the reference point varies between units/kg/meal or units/kg /day. I will discuss all the doses as units/kg/meal, assuming where the units have been written per

day in the original reference that the children have 3.5 meals per day (i.e. divide the amount per day by 3.5), the original figure is included in brackets afterwards.

In the USA, of 14 reported cases, the mean dose of lipase was 16,000 units/kg/meal (Lloyd-Still & Powers, 1994). In Denmark, where cases exhibited a longer segment colitis than in the UK cases, the figure was 45,000 units/kg/meal (Taylor, 1995). In Germany, where high dose enzymes have been used since 1986, there have been no cases of strictures reported. This is thought to be due to more conservative use of enzymes with very few children taking more than 11,500 units/kg/meal (40,000 units of lipase/kg/day) (Taylor, 1995).

A recently published study, using ultrasound, has shown that there is evidence of colonic wall thickening in children with cystic fibrosis taking high dose enzyme supplements and a reduction in the thickening after the children changed back to low dose enzymes (Mac Sweeney *et al* 1995). An association between the thickening and the dose of protease taken was demonstrated by multi-variate analysis, however no association was found between the dose of lipase (or amylase). They found that 48% of children taking more than 265 units/kg of protease per meal, in the form of high dose supplements, had bowel wall thickness greater than 1.5 millimetres (control children were all less than 0.8 mm). The remaining children, either on LDES at any dose or on HDES but taking less than 265 units/kg/meal, only 12 % had evidence of bowel wall thickening. The median dose of lipase whilst taking the high dose enzyme preparations was 5700 units/kg/meal (20000 units/kg/day). A later analysis of this data presented at the Royal Society of Medicine, London 10/10/95 showed errors in the interpretation of protease concentration in the specimens and retracting the association with the dose of protease.

Theories of aetiology

A case control study by Smyth published in November 1995 confirmed that in histologically confirmed cases there was an association with high dose pancreatic enzyme usage (Smyth *et al* 1995). This was highest in boys aged 2-7 in whom 1.2% have developed this complication. The dose being taken of individual constituents

such as lipase, amylase and protease was also positively predictive. The cases reported in the UK took a mean daily dose of 13200 units/kg/meal (46200 units/kg/day). They also found that certain preparations had increased odds ratios with Nutrizym 22 having the highest odds ratio of 43.4 (95% CI 2.51-751), Pancrease HL the next at 8.4 (1.95-36.1) and Creon 25000 the lowest at 0.38 (0.1-1.42). Cases were also found to have been more likely to be taking laxatives and had increased usage of antibiotics and steroids.

Some theories for the pathogenesis of these lesions include direct toxicity of enzymes to the mucosa (Oades *et al* 1994), the same group suggesting in a later paper that protease is the most likely candidate (Mac Sweeney *et al* 1995), pressure induced ischaemia (Smyth *et al* 1994), ischaemia secondary to a vascular abnormality (Briars *et al* 1994), and fibrocyte stimulation (Knabe *et al* 1994).

van Velzen has also reported two children who developed fibrosing colonopathy who had been taking Nutrizym GR (a low dose preparation) (van Velzen, 1995). He postulates that a constituent of the coating of the Nutrizym and Pancrease enzyme preparations called eudragit, which is not present in Creon, could be leading to the complication.

Response in Edinburgh

Following notification of all doctors and pharmacists of this condition in December 1993 by the Committee on Safety of Medicines it was decided at the Royal Hospital for Sick Children, Edinburgh that all children taking high dose pancreatic enzymes would be changed back to the low dose preparations unless there were exceptional circumstances. This was arranged as the children and their parents attended the clinic.

Previous Investigation of Intestinal Mucosal Inflammation or Immunity in Cystic Fibrosis

The gastrointestinal complications of cystic fibrosis are both common and important. Despite this there have been relatively few studies of the intestinal mucosa.

Histological Studies

Generally light or electron microscopy of small intestinal tissue have not shown gross abnormalities of inflammatory cells in children with cystic fibrosis (Morin *et al* 1976; Freye *et al* 1964). However increases in the number of IgA secreting plasma cells (Falchuk & Taussig, 1973) and chronic inflammatory infiltration (Antonowicz *et al* 1978) have been described.

In 1963 a post mortem study revealed accumulation of acidophilic secretion in the lumen of intestinal glands in patients with cystic fibrosis who had died of meconium ileus (Thomaidis & Arey, 1963), the paper does not comment on the cellular aspects of these specimens. One year later a study reported twenty one children with cystic fibrosis who underwent peroral duodenal biopsies (Freye *et al* 1964). These showed no evidence of inflammatory exudate or abnormal mucosal architecture. There was an increase in the level of mucin on the surface of the epithelium.

Antonowicz, in a paper primarily looking at disaccharidase levels in small bowel biopsies of 63 patients with cystic fibrosis, describe 27 that had grade 1 (out of 4) atrophy, and 11 that had grade 2 atrophy (Antonowicz *et al* 1978). One aspect of this scoring system included the presence of chronic inflammatory infiltrate, however they did not present the results with regard to the four individual aspects of the scoring system. Thus it is not possible to establish the frequency of this inflammatory infiltrate from the paper. The article included a picture of one pathological specimen with inflammatory cells present.

Light and electron microscopy of rectal biopsies showed no evidence of mucosal inflammatory cells in children (Hage & Andersen, 1972). However lipid accumulation has been shown to be increased in surface epithelial cells from cultured rectal biopsies (Neutra & Trier, 1978).

IgA synthesis

A study of IgA synthesis in jejunal mucosal biopsies demonstrated an increase in children with cystic fibrosis compared to both patients with hereditary pancreatitis and controls (Falchuk & Taussig, 1973). This did not correlate with Swachman score,

severity of lung disease, bacteria in sputum cultures or serum IgA levels. IgA synthesis was highest in the CF group and lowest in the control group, the patients with hereditary pancreatitis coming in between. This suggested that IgA synthesis was inversely related to the degree of pancreatic insufficiency. As this was an in vitro study it also shows that this effect is due to increased local production and not decreased catabolism of IgA, a possibility in vivo.

Permeability and Uptake

In children with CF, intestinal permeability has been shown to be increased for cellobiose, a disaccharide (molecular weight 342) but not mannitol, a monosaccharide (Dalzell *et al* 1990). This is thought to be due to the different absorption mechanisms between the two molecules. The former is absorbed through the tight junctions between the enterocytes, the latter by transcellular mechanisms that are reduced in mucosal atrophy such as coeliac disease (Dalzell *et al* 1990). This dual sugar absorption test thus shows when the intestinal mucosa is damaged by demonstrating increased absorption of the disaccharide compared to the monosaccharide. This data has been confirmed more recently using lactulose as the disaccharidase (Sierra *et al* 1993; Flick & Perman, 1990). In the former study the increase in larger molecule absorption did not correlate with chymotryptic activity, Swachman score or faecal fat. The latter studied patients with cystic fibrosis and Swachman syndrome (inherited disorder with pancreatic dysfunction, skeletal and haematological abnormalities) who were divided by fat absorption tests into pancreatic sufficient and insufficient groups. There was also a healthy control group. They found that pancreatic insufficient patients had higher absorption of lactulose than pancreatic sufficient patients (from both CF and Swachman groups), controls had the lowest levels of all the groups. They also found that the lactulose absorption inversely correlated with duodenal trypsin output measured by CCK-secretin stimulation testing. This provided evidence for abnormal intestinal permeability being related to the presence of pancreatic insufficiency.

Faecal alpha-1-antitrypsin clearance is also increased in children with cystic fibrosis (Wulkan, 1995) which is suggestive of increased intestinal permeability.

Glucose absorption has been shown to be increased in CF, in a study using a jejunal perfusion technique (Frase *et al* 1985). For fructose and glycine, although absorption was increased, this was not to a significant level. D-xylose in this study did not show any increase in absorption. It should be remembered that unlike the other techniques this method only studies a short segment of the bowel and if mucosal abnormalities are patchy they may be missed.

In a study of uptake of amino-acids in children with CF this was shown to be reduced, the conclusion being that this defect was likely to be an intestinal mucosal problem (Morin *et al* 1976).

Small Bowel Transit Time

It is known that the small bowel transit time is prolonged in CF and other conditions with pancreatic insufficiency (e.g Swachman Syndrome) (Dalzell *et al* 1990; Durie, 1990). This has not been shown to affect the small bowel intestinal flora (Anderson & Langford, 1958).

Faecal Cytokines

In 1995 Briars reported that stool concentrations of IL-8 and TNF- α were increased in children with cystic fibrosis (Briars *et al* 1995). They found that the IL-8 (and not TNF- α) concentration negatively correlated with the Swachman score and enzyme dosage and was higher in five children colonised with pseudomonas compared to five without. With these results, and their comment that IL-8 is relatively resistant to acidity and proteases, they suggested that faecal IL-8 could be a measure of lung disease activity. However they previously described one child who had a moderately raised faecal IL-8 level who underwent treatment for a chest infection. The faecal IL-8 concentration did not change after one week of intravenous antibiotics. Four months later he presented with a stricture of the splenic flexure of the colon (Briars *et al* 1994).

Summary

In summary there a number of studies of gastro-intestinal mucosa which provide some evidence of abnormal mucosal immunity. These have found increased inflammatory cells in biopsies, mucosal IgA production, mucosal permeability, and faecal alpha-1-antitrypsin clearance. Whether raised faecal IL-8 and TNF- α originate from lung or gut inflammation is unresolved.

Whole gut lavage has never been used in the study of intestinal mucosal immunity in patients with cystic fibrosis.

Index Case

In July 1993, six months prior to public concerns about high dose pancreatic enzyme supplements, a child was treated at the RHSC for distal intestinal obstruction syndrome (DIOS) using whole gut lavage. His presentation is described below with two other children with distal intestinal obstruction who presented later in the study. The resulting WGL effluent was collected, stored and analysed over the next few months. This specimen showed very abnormal parameters, compared to both adult data and paediatric control specimens, with elevated albumin, A1AT, IgG, IL-1 β , IL-8, GE and ECP. Subsequently we planned to study patients with DIOS, treated with lavage, to assess the possible significance of the findings in this single case. However when the reports of strictures in children taking high dose pancreatic enzyme supplements were released it seemed possible that inflammation related to the use of high dose enzyme supplements (HDES) could be the cause of these abnormalities. Thus we organised a study to investigate the children on high dose pancreatic supplements prior to changing them back to the low dose enzymes.

Aims of study

To establish:

1. Whether children with cystic fibrosis, taking high dose pancreatic enzyme supplements, have abnormal WGLF parameters when compared with control children;

2. if so, do these recover when changed to low dose enzyme supplements.

The next section of this chapter describes and discusses the results of the lavage specimens related to aims 1 and 2.

Recruitment

The CF children were all recruited by Dr Marshall from the CF clinic or ward at the RHSC. Informed consent was obtained prior to the lavage from the parent/carer and assent from the child.

Patients

The patients were divided into 6 groups;

1. **Controls** (n=12)

- This group included four specimens from children with severe constipation and eight from the disease control group described in chapter 6.

2. **CF, HDES** - Cystic fibrosis children who were taking high dose pancreatic enzyme supplements, (n=12).

3. **CF, LDES** - CF taking low dose enzyme supplements (n=6)

- Five from group 2 as repeats after changing preparations
- One from the high dose section of group 6 after recovery of the DIOS.

4. **CF, PI** - CF, Pancreatic insufficient, prior to starting enzyme therapy, n=1.

5. **CF, PS** - CF, Pancreatic sufficient, n=1.

6. **CF, DIOS** - CF with distal intestinal obstruction syndrome (DIOS)

- on high dose enzyme supplements, (n=2)
- on low dose pancreatic enzyme supplements, (n=1).

Clinical Details

After consent had been confirmed on arrival in the ward, the children's height and weight were recorded. From this, anthropometric indices were calculated using Anthro, software available from the WHO (see table 8.2 below). There were no significant differences between the groups recorded below. Details of their current state of health and any medication was recorded. This included whether the children were taking antibiotics for chest infections. As an objective means for assessing the severity of chest infections these children were later divided into those on no antibiotics, those on oral antibiotics and those on intra-venous antibiotics.

Swachman score was estimated using the standard methods (Schwachman *et al*, 1975). This is a clinical scoring system designed for children with cystic fibrosis based on four parameters, the general activity, physical examination, nutrition and chest x-ray. The highest score possible is 100.

Table 8.2: Clinical details of CF and control patients.

Group of Patients	Number	Median Age months	Median WAZ	Median HAZ	Median Swachman Score	Antibiotics	
						Oral	IV
Control	12	101	-0.1	-0.8	Not applicable	0	0
CF, HDES	12	108	-0.6	-0.5	70	5/12	1/12
CF, LDES ²	6	97	-0.5	-0.1	83	3/6	1/6
CF, PI	1	75	-0.3	-0.6	65	No	Yes
CF,PS	1	135	1.26	0.9	90	No	No
CF, DIOS ¹	3	99.5	-0.5	-2.2	85	1/3	2/3

WAZ: weight for age Z score, HAZ: Height for age Z score

¹ Includes two taking high dose enzyme supplements, one on low dose supplements.

² Includes the repeat lavage of the index child with DIOS (DIOS1)

The preparation and number of enzymes taken per meal was recorded and the dose that the children was taking in units/kg/meal for each of lipase, protease and amylase was calculated. Table 8.3 includes the dosage for lipase and protease. Amylase correlates strongly with lipase in all the preparations whereas one preparation (Creon 25000) has much lower levels of protease in each capsule so calculations have been performed separately for lipase and protease.

Table 8.3: Dose of Enzymes taken.

Group of Patients	Number	Lipase u/kg/meal		Protease u/kg/meal	
		Median	Range	Median	Range
CF, HDES	12	6147	3472-10050	117	65-503
CF, LDES	6	3660	3488-8675	203	96-242
CF, DIOS	3	2750	2679-6957	177	138-348

Description of CF children with distal intestinal obstruction syndrome

Patient 1: DIOS1

This is the index case who is a boy who presented in July 1993, at 8 years of age, with a two day history of right iliac fossa pain, vomiting, less frequent hard stools, and a palpable right iliac fossa mass. He had had two previous episodes of DIOS treated with oral gastrografin. He was taking high dose pancreatic enzymes (Nutrizyme 22); 2750 units of lipase/kg/meal, 140 units protease/kg/meal. His DIOS was treated using whole gut lavage although afterwards there was still a 1 cm right iliac fossa mass palpable. Swachman Score was 85. At the time of the lavage he also required treatment for a chest infection with intravenous antibiotics.

In January 1994 he was changed to low dose enzyme supplements (Nutrizym GR), taking lipase 2750 units/kg/meal and protease 138 units/kg/meal.

In April 1994, he had a right iliac fossa mass palpable although without the symptoms of pain and vomiting. Whole gut lavage was repeated prior to a normal barium enema and the resulting effluent was collected for analysis.

Patient 2: DIOS2

In January 1994, whilst a long term in-patient with poor nutrition and lung disease, this 8 year old boy complained of recurrent episodes of abdominal pain with constipation, and on examination a right iliac fossa mass was palpable. Swachman score was 46. He was taking a high dose enzyme preparation (Nutrizyme 22); lipase 7000 units/kg/meal, protease 348 units/kg/meal. He was treated with whole gut lavage and his symptoms and right iliac fossa mass resolved. At the time of the lavage he was on intra-venous antibiotics for a chest infection. A barium enema in March 1994 was normal.

Patient 3: DIOS3

A boy presented to the surgeons at the age of 12, with abdominal pain, infrequent bowel motions and a tender right iliac fossa. Appendicitis was ruled out after observation and DIOS was diagnosed. At the time his Swachman score was 90 and he was taking a low dose enzyme preparation (Pancrease LD); lipase 2700 units/kg/meal, protease 177 units/kg/meal. He was initially treated with gastrografin orally but the mass persisted and he was treated with whole gut lavage with resolution of his symptoms and mass. No barium enema has been performed.

Methods

Lavage Specimen Collection

Whole gut lavage was performed as previously described, the children being admitted as day patients unless they were already in the ward for other reasons.

The parents or carers were asked to continue with the child's usual physiotherapy in the morning prior to the lavage. The children were asked to expectorate any sputum during the lavage.

Whole gut lavage fluid was collected once clear of all faecal material, processed and stored for analysis later. Details of the time taken, rate and volume required, route (orally/naso-gastric), and side effects were all documented.

Analyses Performed

Methods as detailed in chapter 4.

- Mucosal inflammation/plasma leakage albumin; Total IgG, A1AT, albumin.
- GI bleeding; Hb.
- Cellular mediators; Eosinophil cationic protein (ECP), granulocyte elastase (GE).
- Cytokines; Interleukin-1 (IL-1), interleukin-8 (IL-8).
- Enzymes; Chymotrypsin.
- Secretory immunoglobulins; Total IgA, total IgM (results discussed in chapter 11).

Results

Recruitment

Twelve children taking high dose pancreatic enzyme supplements were recruited by Dr Marshall from the CF clinic. It is not possible to ascertain how many of the 75 children being followed up were approached to see if they would consider being involved in the study. This was for two reasons; 1) a proportion had already changed to the low dose supplements prior to the ethical approval, and 2) Dr Marshall does not see all the patients at each clinic attendance.

Five of the original 12 CF children taking high dose enzyme supplements repeated the lavage. Of the remainder, one was considered to be too ill to undertake the repeat lavage, in one case it was not possible to contact the parents, the remaining five were not prepared to repeat the procedure. When told originally about the study, the children

and the parents were told that we planned to repeat the lavage if the child had any abnormal immune parameters. At that stage they were happy to be involved as the change to the low dose enzyme supplements was considered a step backwards. However by the time of the repeat lavages the children had got used to taking the low-dose preparations and some did not want to undergo the repeat procedure. I had, when gaining their consent, made it clear that any decision to return to taking high-dose pancreatic enzymes could not be made solely on the basis of results of the lavage performed, although any information gained would be helpful in determining if these preparations could be safe. The length and tedium of the lavage were cited as the most common reasons for not wanting to repeat the procedure.

Results of first lavages

CF on HDES

All the children with cystic fibrosis taking high dose pancreatic enzyme supplements had one or more of the parameters above the range recorded in the control children and outwith the adult normal range. Figures 8.1-8.6 demonstrate that the children with cystic fibrosis taking high dose enzyme supplements had significantly higher levels of albumin, total IgG, IL-1, IL-8 and ECP. Granulocyte elastase, although absent in all except two of the control children, was not significantly increased when comparing medians using the Mann-Whitney test. When comparing the presence or absence of GE using the chi-squared test there was a significant increase ($p=0.045$) in the cystic fibrosis group. There was no evidence of blood loss in any of these children. Alpha-1-antitrypsin was not altered.

CF, pancreatic insufficient, prior to starting enzyme supplements

This child's range of results were within the range of the children taking high-dose pancreatic enzyme supplements despite the lavage being performed prior to starting enzyme supplements. This would suggest that the abnormalities detected are associated with pancreatic insufficiency, but are not necessarily a consequence of the enzyme supplements.

CF, Pancreatic Sufficient

The one child who had WGL who is pancreatic sufficient had results within the range of the control children except for a raised IL-1.

CF, DIOS

Two of the three children with DIOS were taking high dose enzyme supplements, and at the time of lavage these two also required intra-venous antibiotics. They both had raised concentrations of total IgG and albumin, IL-1, IL-8, and particularly ECP and GE. These results are strongly suggestive of intestinal inflammation and immune activation with both neutrophil and eosinophil activity. In the child that had a repeat lavage after recovering from DIOS and changing to low dose enzyme supplements (prior to a barium enema) these concentrations dramatically reduced as demonstrated in figures 8.7-8.12.

The child with DIOS taking low dose enzyme supplements (DIOS3) had lower levels of the parameters than DIOS1&2 although they were at the higher end of the CF, HDES group.

It is possible that the very high levels of the children DIOS 1 and 2 were in part due to their chest infections (both were requiring intra-venous antibiotics at the time of the lavage). It was at this stage that because of the potential of swallowed sputum affecting the lavage results that I planned to collect sputum at the time of the lavage in order to investigate its potential influence. Figures 8.13-8.15 show that of five children who were on IV antibiotics at the time of the lavage the two with DIOS described above had very much higher levels of the WGL parameters than the remaining three.

Daily Dose of enzymes in the CF children

None of the abnormalities detected showed any relationship with the dose of lipase, protease or amylase in units/kg/meal. This included the data from both the first and the repeat lavages.

Figures 8.1-6: Concentrations of substance in whole gut lavage fluid versus group of patients. Horizontal bars indicate the median. Mann-Whitney test was used to compare the medians of the control and CF, HDES groups.

The three children with DIOS are labelled 1,2,3 as in the clinical description above.

Number of subjects (unless otherwise indicated); Controls = 12; CF,HDES = 12; CF,DIOS = 3; CF,PI = 1; CF,PS = 1

Figure 8.1: Albumin

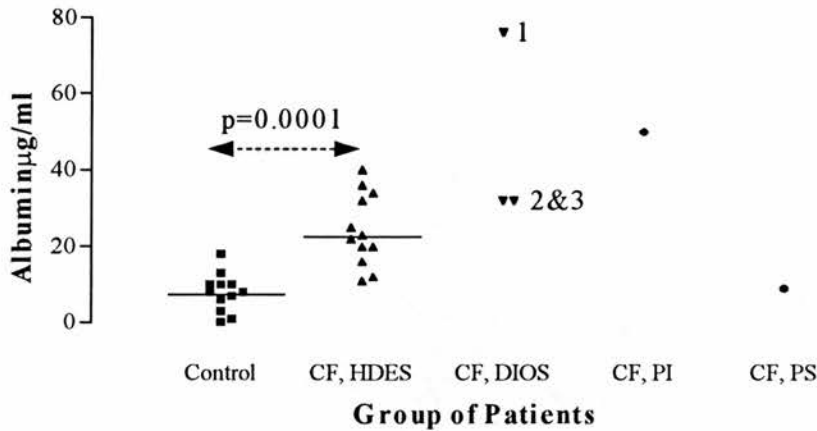


Figure 8.2: Immunoglobulin G

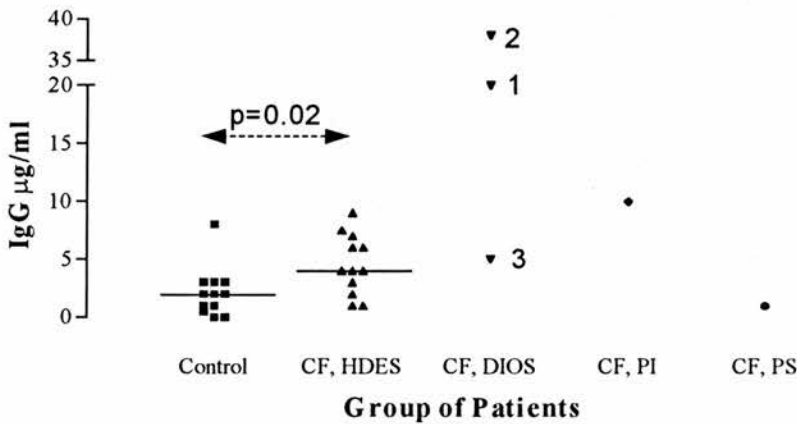
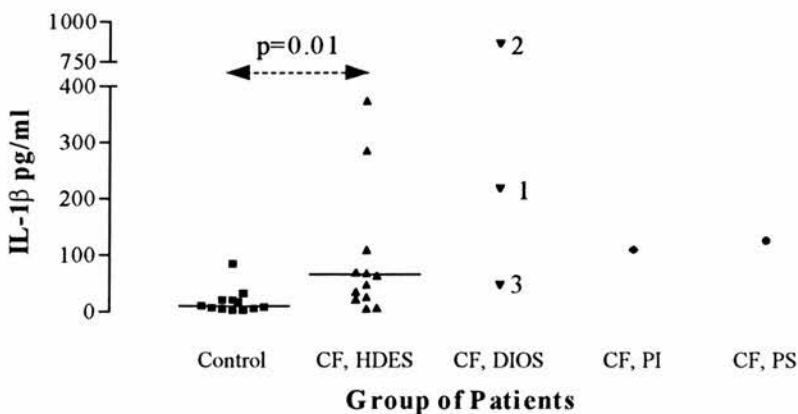


Figure 8.3: Interleukin-1β



Role of Clinical Score (Swachman)

Swachman score showed no relationship with any of the abnormalities detected.

Role of chymotrypsin in lavage

Chymotrypsin measured in the lavage showed no relationships other than a weak negative correlation with IL-8. This may be because chymotrypsin, as a protease, degrades a proportion of the IL-8 present.

Role of enzyme preparation

The case control study that suggested that Nutrizym 22 was strongly associated with the development of fibrosing colonopathy (Smyth *et al* 1995) led us to look at the results with regard to the enzyme preparation.

This showed that the two children with intestinal inflammation, who had DIOS whilst on a high dose enzyme preparation, were also the only two in this study taking Nutrizym 22. The remaining children were all taking either Creon or Pancrease supplements.

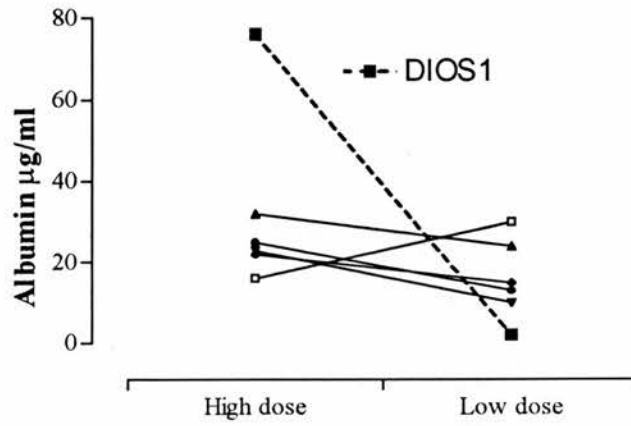
Results of repeat lavages

The results of the repeat lavages are shown in figures 8.7-8.12. The five children with CF on HDES who had repeat lavages showed no significant changes in the rate of the lavage or level of the abnormal factors measured in the two lavages. The median dose of lipase being taken at the time of the lavage showed a reduction from a mean of 8300 to 4731 units of lipase/kg/meal ($p=0.04$). Protease dosage also showed a non-significant reduction from 279 to 181 units/kg/meal ($p=0.3$). Patient 1 of the DIOS group (DIOS1) showed an impressive reduction in all the lavage parameters except for ECP (see figures 8.7-8.12 below). This suggested recovery of the inflammation present at the time of the first lavage.

Figures 8.7-8.12: Concentrations of substance in WGLF from individual patients on high- then low-dose pancreatic supplements.

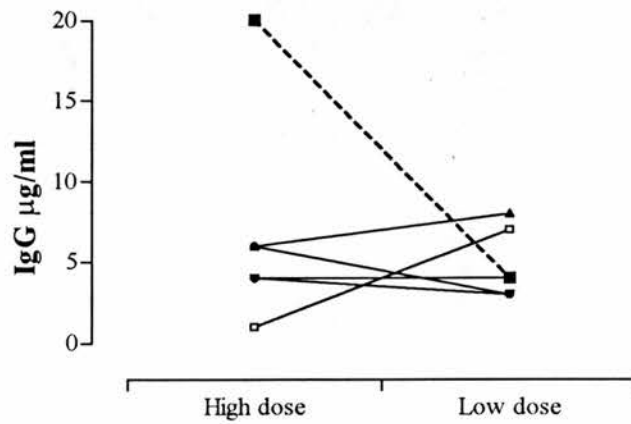
The one child with DIOS whilst on high dose enzymes (DIOS1) who had a second lavage whilst on low dose enzymes is indicated by a dotted line.

Figure 8.7: Albumin



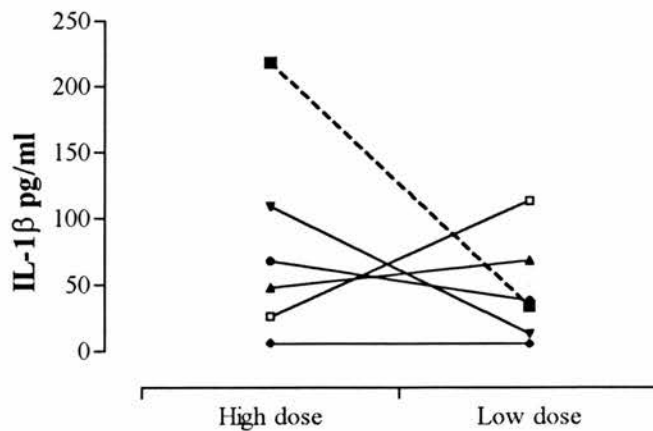
Enzyme Preparation

Figure 8.8: IgG



Enzyme preparation

Figure 8.9: Interleukin-1β



Enzyme Preparation

Figure 8.10: Interleukin-8

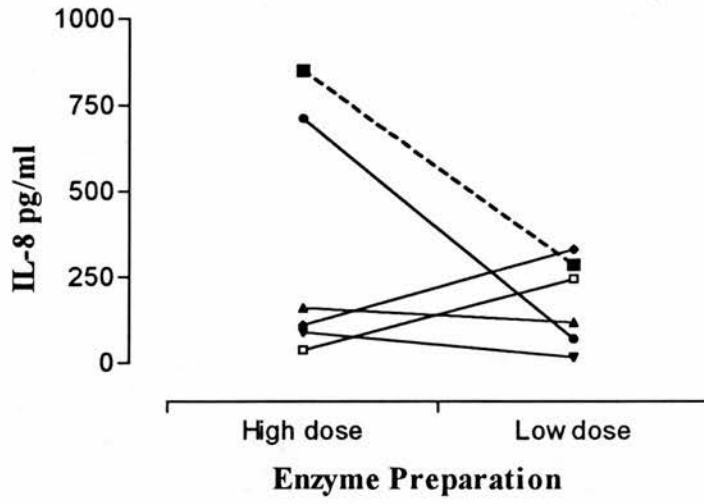


Figure 8.11: Eosinophil Cationic Protein

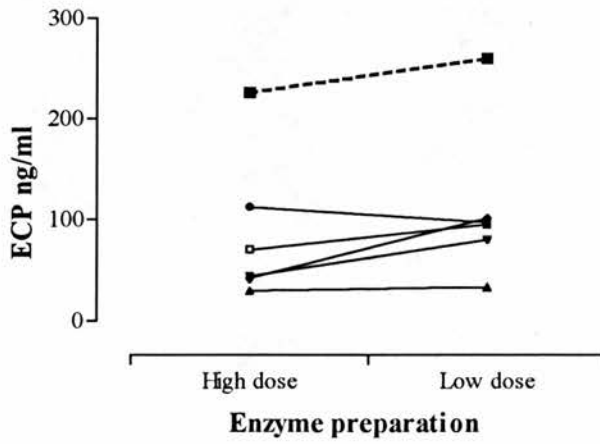
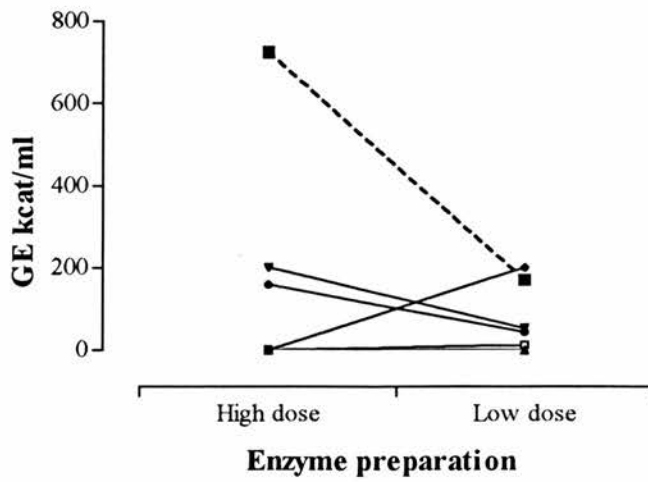


Figure 8.12: Granulocyte Elastase



Discussion

These results show that, as a group, children with cystic fibrosis taking high dose pancreatic enzymes have significantly higher concentrations of albumin, IgG, IL-1 β , IL-8 and ECP in whole gut lavage fluid when compared to controls. There is also evidence to suggest gross intestinal inflammation and immune activity in two children taking Nutrizym 22 who were being treated for distal intestinal obstruction syndrome.

Explanations for these differences are:

1. Direct immune activation of the intestinal mucosa due to;
 - toxicity of pancreatic enzymes and/or other drugs,
 - stimulation of the intestinal mucosa due to small bowel bacterial overgrowth in CF patients (secondary to abnormal intestinal motility).
2. Secondary immune activation of the intestinal mucosa;
 - generalised up-regulation of mucosa associated lymphoid tissue originating from chronic lung infection.
3. decreased breakdown of normally secreted substances due to pancreatic insufficiency.
4. Swallowed mucous secretions from the respiratory tract.

Direct toxicity of high dose pancreatic enzyme supplements has not been confirmed as the five children who underwent repeat lavages did not show recovery of the abnormalities once on the low dose preparations. In this sub-group the median levels of substances assayed, although higher than the controls, were lower than initial group of 12. Thus it is possible that the children who had two lavages, had less severe inflammation initially, and were less likely to benefit from the change to the lower dose preparations. This group of five patients were taking a higher median dose of lipase and protease than the seven children who had a single lavage (and the combination of these two groups). If the dose of enzymes were responsible one would

expect these children to have higher levels of lavage parameters to fit with their higher levels of enzymes.

Whether enzyme preparations could have caused abnormalities in the CF group at all is less likely when one sees the abnormal WGLF concentrations detected in the pancreatic insufficient child who had not started enzyme supplementation.

Chronic stimulation from abnormal intestinal flora is not the cause of these abnormalities as CF patients have been shown to have normal intestinal flora (Anderson & Langford, 1958).

Generalised immune up-regulation of all mucosal surfaces in these children is another possibility. Chronic bronchopulmonary infection leads to continuous immune stimulation. This could in turn increase concentrations of chemokines and numbers of migrating B and T cells in the serum travelling to and activating the gastrointestinal mucosa. For this to be the case one would expect the Swachman score to negatively correlate with the abnormalities found as the more severe, chronic lung infection leads to lower Swachman score and should also lead to increased inflammation in the GI tract. There was no relationship between the Swachman score and the abnormalities found. Checking serum levels of cytokines or migrating cells would not answer this question as high levels could originate from the gut mucosa as well as the lung. Studying this further in humans would require collection of lavage specimens from patients with bronchiectasis or other chronic lung infection without pancreatic insufficiency.

There is only one study that has correlated endoscopic findings in the large bowel with WGLF parameters. They found a mean IL-8 WGLF concentration of 36.4 pg/ml in patients with ulcerative colitis compared with a level of <4 pg/ml in control patients (Hommes *et al* 1995b), this was found to correlate significantly with a visual endoscopic score of inflammation. In these children with CF the median level of WGLF IL-8 was 158 pg/ml.

The possibility that these abnormalities are a consequence of decreased breakdown within the GI tract is unlikely for two reasons. First the method of lavage minimises

transit time of substances through the GI tract and immediate processing inhibits any proteolytic activity. Thus exposure to proteolysis is minimised as much as is possible without using a balloon perfusion system. Secondly IL-8 and ECP, the two substances which showed the biggest increases in the CF children, have been reported as being relatively stable in the GI tract (Briars *et al* 1995; Berstad *et al* 1993).

I was able to identify two adult patients from the Western General Hospital who had pancreatic insufficiency who had previously had whole gut lavage for clinical reasons.

Patient 1 has idiopathic chronic pancreatitis and requires 12-15 tablets of Creon per day (he had not started them at the time of the lavage). Patient 2 has alcohol related pancreatitis with an abnormal pancreolauryl test confirming pancreatic insufficiency, at the time of the lavage he was not taking any enzyme supplements.

Table 8.4: WGLF Parameters of Two Adults with Pancreatic Insufficiency.

Patient	Enzymes	IgG	Albumin	A1AT	Hb	IL-1	IL-8	GE
		µg/ml	µg/ml	µg/ml	µg/ml	pg/ml	pg/ml	kcat/ml
1	Creon	1	8	3	1	ND	ND	ND
2	None	3	0	5	2	15.4	ND	ND

ND = None detected

Neither of these two patients show any of the abnormal parameters identified in pancreatic insufficient children with cystic fibrosis. Again this suggests that pancreatic insufficiency is not the only cause for the abnormal parameters found.

DIOS

The grossly abnormal immune parameters found in two of the children with DIOS, both of whom were taking Nutrizym 22, show that these children had gastro-intestinal inflammation and immune activation. The marked difference between these two

children and the third with DIOS on low dose enzymes could be due to the different preparations.

A combination of high dose-preparations and the presence of inspissated faeces in the right iliac fossa and increased delivery of active ingredients to this area could lead to mucosal ulceration. In a study of 12 children with cystic fibrosis and DIOS, two had evidence of mucosal ulceration on their barium enema (Matseshe *et al* 1977).

Two of the 12 children taking high dose preparations without DIOS had palpable right iliac fossa masses without further symptoms to suggest DIOS. The analyses performed on these two children were not any higher than found in the remaining 10 children. Thus the presence of a right iliac fossa faecal mass cannot be the only cause for the abnormalities found.

The possibility that, in the two children with DIOS, the concurrent chest infection is responsible is impossible to exclude definitively retrospectively, however seems unlikely when one looks at the figures 8.13-8.15. These graphs demonstrate that the two DIOS patients, who were on IV antibiotics at the time of the lavage, had grossly elevated parameters, whereas the three other children also investigated whilst on intravenous antibiotics for chest infection did not come close to these levels.

It will be of great importance to study this group of patients further by collecting specimens from more patients. As WGL is used as a treatment for DIOS this should not involve major ethical problems as specimens of WGLF can be collected in the course of treatment.

Figures 8.13-8.15: Whole gut lavage fluid concentrations in cystic fibrosis children divided into groups by method of administration of antibiotics for chest infections. DIOS1,2,3 are the three children with DIOS described above.

Figure 8.13: Total IgG

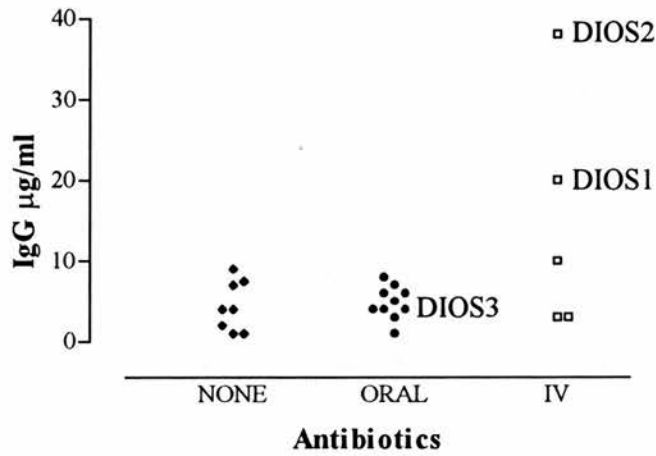


Figure 8.14: Interleukin-8

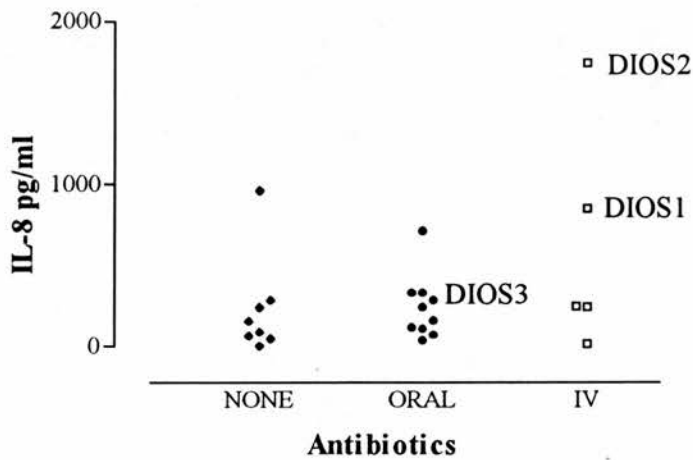
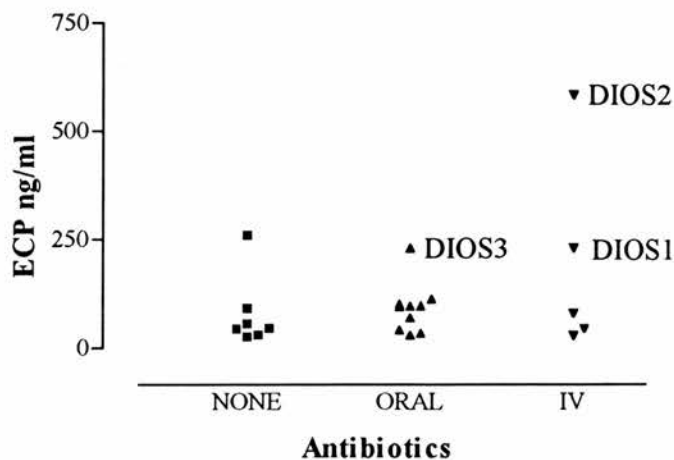


Figure 8.15: Eosinophil Cationic Protein



Summary

In this chapter I have shown that children with cystic fibrosis have raised concentrations of albumin, IgG, IL-1, IL-8 and ECP in WGLF compared to control children. This is most likely to originate from the gastrointestinal mucosa. These abnormalities appear to be related to the presence of pancreatic insufficiency and in the group of patients who had two lavages performed there was no evidence that the high-dose as opposed to low-dose enzyme supplements caused the increased concentrations.

Two children who had DIOS and were taking Nutrizym 22 showed very abnormal parameters of intestinal inflammation. In one of these children parameters improved after recovery from the DIOS and changing to a low dose preparation. The cause of their inflammation has yet to be identified, further studies involving collaboration with other centres are underway to elucidate whether the changes were a feature of the medication or DIOS or both.

None of the children in this study has so far developed fibrosing colonopathy and so it is impossible to relate any of these results to the development of this condition. If any do develop this condition it will be important to look back at the results of their lavage specimens.

Chapter 9: Potential Influence of Respiratory Tract Secretions in the

CF Study

Sputum in CF

When attempting to demonstrate intestinal mucosal secretory abnormalities, one must bear in mind that the children would continue swallowing some respiratory tract secretions. This is important in children with cystic fibrosis as they regularly require treatment for chest infections with one of the indications for antibiotics being increased sputum production. The potential effect of sputum has never been considered in published studies of immunity in the intestine of patients with CF.

To minimise the potential effect of sputum in the subjects the parents were asked to ensure that the children have their usual physiotherapy prior to coming up to the hospital and asking the children to expectorate any sputum during the lavage. The majority of the children in the study did not have a productive cough at the time of the lavage.

Production of Sputum

In adults, more than 1.5 g sputum per hour is considered excessive (Falk *et al* 1984). Two studies have looked at the amount of expectorated sputum during physiotherapy in adults with cystic fibrosis who had concurrent chest infections (Falk *et al* 1984; Pryor *et al* 1979). One demonstrated mean sputum production of 55 g/day (0.04 g/kg/hr) (Pryor *et al* 1979). The other reported levels of 163.2 g/day (0.13 g/kg/hr) (Falk *et al* 1984). No studies have quantified the amount of sputum that is produced within the respiratory tract that is swallowed as opposed to expectorated.

Immunological analysis of sputum

A wide variety of immunological analyses have been performed in the sputum of patients with cystic fibrosis including immunoglobulins (Reynolds, 1987; Konstan *et al* 1995), antibodies (Pedersen *et al* 1992), cytokines including IL-8 (Assadulahi *et al* 1992), IL-1, IL-6, IL-1 receptor antagonist (IRAP), TNF-alpha (Kronborg *et al*

1993), and cellular mediators such as eosinophil cationic protein (ECP)(Virchow, Jr. *et al* 1992; Reimert *et al* 1995; Koller *et al* 1994) and neutrophil elastase (Konstan *et al* 1995). All these factors are present at high concentrations in the sputum of patients with cystic fibrosis, however most of them can also be found in the GI tract of adults with inflammatory bowel disease.

Aims of Study

To estimate amounts of sputum that would have to be swallowed per hour to cause the differences found in the lavage parameters in the children.

Patients and Methods.

Sputum collection was attempted at the time of the lavage in seven of the 12 lavages of the children taking high dose pancreatic enzyme supplements and all the five children who underwent a second lavage. It was only possible to collect any in three of the first seven children as the other four were unable to expectorate any sputum.

Sputum Collection

The child was asked to expectorate into a universal container which was transported on ice and stored in a -70°C freezer. Specimens that were saliva without sputum were discarded. Sputum was then prepared as described in the methods chapter.

Sputum Analyses performed

In attempting to establish the potential influence of sputum in the lavage I chose ECP and IL-8 as marker molecules. Both of these substances are thought to be relatively stable in GI tract specimens (Briars *et al* 1995; Berstad *et al* 1993) and were significantly increased in the lavage fluid of the children on high dose pancreatic enzyme supplements. By knowing the rate at which the lavage fluid was administered and the concentration of the substance in lavage effluent and sputum it is possible to estimate how much sputum would have to be swallowed per hour in order to cause the levels found in the lavage fluid, using the following equation.

$$\text{Sputum Swallowed (g/hour)} = \frac{\text{Rate of Lavage (ml/hour)} \times [\text{Substance}]_{\text{lavage}} \text{ (ng/ml)}}{[\text{Substance}]_{\text{sputum}} \text{ (ng/g)}}$$

[Substance] is the concentration of the substance in the lavage effluent or sputum.

For IL-8 the median level in control lavages was 0 pg/ml so no subtraction as a 'background' concentration is necessary. There is no figure to base an estimate for control levels of IL-8 in sputum of controls as these children were not expectorating any. Omitting any 'background' concentration in sputum swallowed would, if anything, lead to an underestimate of the volume swallowed to cause the differences in the WGLF.

For ECP the lavage background level for controls is 14.5 ng/ml, again there is no available figure for control sputum. I have assumed that the influence of the background level in sputum will be similar to that of lavage and have not made any subtraction.

For the purposes of this study I have assumed that there is zero breakdown of IL-8 and ECP during passage through the GI tract.

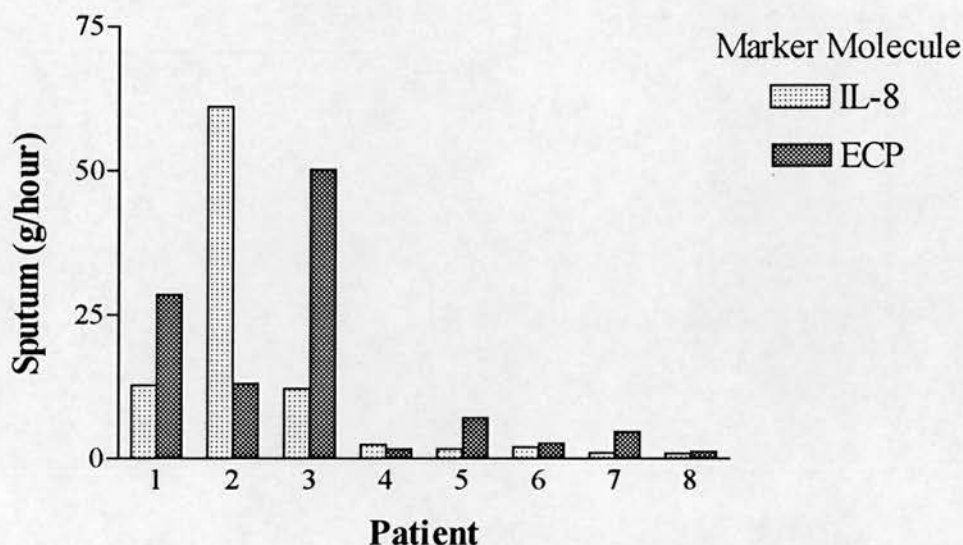
Results

The median (range) estimated weight of sputum swallowed using ECP as the marker molecule was 5 (1-57) g/hour and using IL-8 was 2.3 (0.9-61) g/hour or, with the median weight of 26.5 kg in this group, 0.19 g/kg/hour and 0.09 g/kg/hour respectively. These weights equate to 120 and 55 g/day respectively which would be considered very large amounts for a child to be expectorating.

Figure 9.1 shows the weight of sputum which would have to be ingested per hour to account for WGLF concentrations of the substance measured for each child who had sputum collected. The first three children, who had estimates of between 10 and 60 g/hour, are very unlikely to be producing this amount of sputum. It is also important to note that of these three children, two were not on any antibiotics for a chest

infection and had considerably higher volumes of sputum which would need to be swallowed per hour to produce the lavage concentrations, than the remaining five children who were taking antibiotics (4 oral, 1 IV). This is a very unlikely scenario if sputum was responsible for the abnormalities.

Figure 9.1: Weight of sputum required to be swallowed to account for WGLF concentrations of marker molecules



The ideal situation to clarify this problem would be to identify a substance in WGLF that is not present in simultaneously collected sputum. One possibility is a chemokine called RANTES. This is an eosinophil chemoattractant and activator (Kameyoshi *et al* 1992; Rot *et al* 1992) and has been found both in bronchoalveolar lavage fluid and in vitro cultures of colonic epithelial cell lines (Oda *et al* 1995; Yang *et al* 1995). No publications have reported the presence of RANTES in vivo from the human GI tract. Using the same techniques as in the IL-8 assay I found that RANTES was measurable in 2/8 WGLF specimens (and detectable in 6/8 at low levels). None of the sputum specimens had any detectable levels of RANTES. The absence of significant amounts of RANTES in the sputum, when it was present in corresponding WGLF, confirms

that this was being secreted in the GI tract. This is further evidence to suggest that contamination by sputum is not the cause of the immune abnormalities in WGLF of children with cystic fibrosis.

Discussion

From this data I can exclude the possibility that sputum is causing the abnormal parameters in 3/8 of the children from whom I was able to collect sputum because of the excessive estimated amounts required to be swallowed.

The only way to study the intestinal secretory immune system in vivo to exclude both sputum and pancreatic secretions is with balloon perfusion systems. Double balloon systems would not be suitable as they only investigate a short segment of the gut. However it could be feasible, in adult volunteers, to use a single balloon, positioned distal to the pancreatic/cystic duct, and to perfuse the rest of the GI tract with a PEG based solution, collecting the specimens passed per rectum as in whole gut lavage. This would allow the GI tract to be perfused without interference from bile, pancreatic enzymes or sputum. This is the only way to prove whether ingested sputum could have caused any of the above abnormalities. It is invasive and would require careful ethical consideration with regard to the risks and benefits for the subject.

*SECTION IV: Faecal Material Compared with Whole Gut
Lavage*

Chapter 10: Comparison of Faeces and Whole Gut Lavage for Assessment of Gastrointestinal Mucosal Immunity and Inflammation

Introduction

In previous chapters evidence has been presented for the argument that whole gut lavage can be an ethical and useful method for investigating gastro-intestinal mucosal immunity and inflammation in children. However in certain groups of patients this method would not be feasible. The most common and important example of this is the situation of acute gastroenteritis where children will often have associated vomiting making whole gut lavage both ethically and practically impossible.

Striking differences have been reported in the faecal concentrations of molecules of interest, when diseased cases (normally, diarrhoeal diseases) and controls are compared. These data would be sound only if there were a standard rate of recovery of such molecules in faeces, irrespective of the presence or absence of diarrhoea, intestinal inflammation, infection or allergic reaction.

Relatively few studies have attempted to compare immune analysis of faeces with alternative specimens such as serum, small bowel aspirates, and saliva. Those that have mostly looked at the presence or absence of specific antibodies (Hjelt *et al* 1986; Benhamou *et al* 1995; McLean *et al* 1980; Coulson *et al* 1990). Questions remain whether faecal material can represent the true secretory immune status of the gastrointestinal tract and whether faeces can be an alternative to other established and often more invasive methods of investigating intestinal mucosal immunity. It is equally unclear whether perfusion systems truly represent the physiological secretion rates of immune substances in the GI tract.

Chapter 10 part I describes a study comparing inflammatory and immunological substances in faecal specimens with whole gut lavage, in chapter 10 part II I go on to study the output of immune substances in ileostomy specimens, a similar situation to diarrhoeal disease.

Literature Review

Immunological studies in faecal material

Numerous studies have been performed looking at measures of immunity or inflammation in faecal specimens including total immunoglobulins (Haneberg & Aarskog, 1975; Koutras & Vigorita, 1989; Haneberg, 1974; Schanler *et al* 1986; Kapel *et al* 1990; Meillet *et al* 1987; Sasai *et al* 1992), antibodies (Ogra *et al* 1977; Benhamou *et al* 1995; Siddons & Chapman, 1995; Coulson *et al* 1990; Yoshizawa *et al* 1980; Onorato *et al* 1991), A1AT (Haneberg & Tonder, 1973; Bernier *et al* 1978; Sullivan *et al* 1992; Davidson & Lonnerdal, 1990), ECP (Berstad *et al* 1993), granulocyte elastase (Adeyemi & Hodgson, 1992; Saitoh *et al* 1981; Bohe, 1987), and cytokines (Nicholls *et al* 1993; Briars *et al* 1995).

Studies in diarrhoeal material

Total immunoglobulins

In one study faecal total, monomeric and polymeric IgA in healthy adults and adults with chronic diarrhoea was measured (Meillet *et al* 1987). The daily output of these substances were significantly increased in the patient group. Although protease inhibitors for the preservation of faeces were used, this was after the specimen had been collected and the time of exposure to enzymes within the bowel would have been very much longer in the healthy group than the disease group. This problem of a lack of a suitable diarrhoeal control group has been present in other studies, including those looking at total immunoglobulins (Saha *et al* 1990; Blecka, 1978; Prentice *et al* 1989), and antibodies (Coulson *et al* 1990; Kletter *et al* 1971).

Some studies have used groups of patients with diarrhoea as controls for their studies (Benhamou *et al* 1995; Reed & Williams, Jr. 1971). The problem in this situation is the definition of diarrhoea as well as the severity. A patient with severe cholera will tend to pass high volume clear watery stools whereas with parasitic infestation the diarrhoea is more likely to be loose but still regarded as diarrhoea by the subject.

IgG has been detected more commonly in faecal specimens of patients with diarrhoea (Haneberg & Aarskog, 1975), in the same study IgM was not found to differ between the groups studied. Assuming IgG to be primarily serum derived, measurement of IgG in intestinal secretions will depend on the degree of inflammation of the mucosa which depends upon the pathogenesis of the diarrhoea.

Antibodies

Since Davis et al found evidence for specific gut immunity to dysentery (Davies, 1922), assays for antibodies in diarrhoeal specimens have been extensively used. These studies have either looked for the presence or absence of the antibody (Kletter *et al* 1971; Yoshizawa *et al* 1980) or have actually quantified the amount of antibody in the stool specimen and compared it with the control groups or follow up specimens (Reed & Williams, Jr. 1971; Grimwood *et al* 1988). The possibility that in the same patient, diarrhoea may be more likely to contain measurable antibodies than normal faeces has never been studied.

A1AT

Measurement of faecal A1AT has been extensively investigated as a method for measuring enteric protein loss (Bernier *et al* 1978; Florent *et al* 1981; Moran *et al* 1995) and applied to a number of different studies (Kapel *et al* 1992; Bhan *et al* 1989; Davidson & Lonnerdal, 1990; Sullivan *et al* 1992).

Cytokines

In one study of cytokines in stool (Nicholls *et al* 1993), a control group of children with diarrhoea was included. This showed that TNF- α was increased whereas IL-6 showed no differences between the three groups.

Diarrhoea and the Immune Response in Children

In 1975 Haneberg found that patients recently recovered from acute infectious diarrhoea had higher concentrations of faecal IgA than controls (Haneberg & Aarskog, 1975). IgM was slightly higher but not to a significant degree. By collecting

faeces from recently recovered patients he did make some effort to use comparable specimens. He also found that children with ulcerative colitis had high levels of IgG.

In 1990 Saha also found that total IgA was increased in the stool and serum of undernourished children with chronic diarrhoea compared with controls (calculated per gram of dry faeces) (Saha *et al* 1990). In this study the controls were normal healthy children passing normal faeces.

In adults and children with shigella positive diarrhoeal stools higher concentrations of IgA were measured than in non-shigella diarrhoeal stools (Reed & Williams, Jr. 1971). IgG and IgM were not significantly different in the two groups.

IL-6 has been shown to be rapidly degraded over a period of 2 hours making this an inappropriate assay when assessing GI immune responses in faeces (Nicholls *et al* 1993). TNF α however was more stable when spiked into stool supernatant. This was found to be significantly higher in children with active inflammatory bowel disease compared with healthy controls (Nicholls *et al* 1993). They also found in three children with *shigella flexneri* infection, very high levels of TNF- α and IL-6.

Alpha-1-antitrypsin has been found to be increased in diarrhoeal stool of children with protracted diarrhoea when compared to children with normal stools (Bhan *et al* 1989). The patients with pathogens isolated had higher concentration than both controls and children with diarrhoea due to carbohydrate intolerance, with the concentration tending to reduce after treatment. However a more recent study only showed that 5/29 children with persistent diarrhoea had high faecal A1AT (Sullivan *et al* 1992), the group with strongyloides stercoralis being the only ones to have significantly higher concentrations of A1AT, in this study the patients with giardia did not have significantly higher levels of A1AT as a group.

Using ⁵¹Chromium labelled EDTA another group showed increased permeability of the gastrointestinal tract in seven children with acute gastroenteritis and eight with infantile eczema (Forget *et al* 1985).

Dual sugar permeability tests in Gambian children have shown increased permeability in malnourished children, especially those with marasmus, acute and chronic diarrhoea and those with measles (Behrens *et al* 1987).

Comparison of faecal and diarrhoeal material in the same patient

As a small part of a larger study Haneberg gave his three children laxatives and measured the IgA concentration in their diarrhoeal stools and compared the result with specimens collected prior to starting the laxatives (Haneberg & Aarskog, 1975). He found that it was 'largely unchanged'. There are no other publications that have investigated the effect of inducing diarrhoeal stools on the measured output of immune factors in faecal material.

Strygler in 1990 estimated the faecal clearance of A1AT in volunteers and patients (Strygler *et al* 1990). In normal subjects daily clearance of A1AT was 13.6 ml/day. They then induced diarrhoea in the normal subjects with a range of agents (lactulose, sorbitol, sodium sulphate and phenolphthalein). They found that the clearance of A1AT was significantly increased to 27.8 ml/day, $p < 0.05$. Thus in the situation of experimentally induced diarrhoea the daily measured excretion of alpha-1-antitrypsin in faeces was increased by a factor of two. It seemed very unlikely that this was due to toxic effects of the drugs on the mucosa. A1AT is known to be resistant to proteolysis so reduction in degradation would seem unlikely, the other alternative is that alteration in flow through the GI tract alters protein leakage or exudation. They then used the results for the subjects with experimentally induced diarrhoea as the controls for the disease groups who had diarrhoea.

The same group have gone on to investigate this subject further by looking at the effect of changing the flow rate in a jejunal perfusion system on altering intestinal permeability (Fine *et al* 1995). They found that faster flow rates reduce the average pore size for intercellular aqueous diffusion from 13 Å to 8 Å. In the discussion they postulate that due to increased exposure to the intervillus space, with higher flow rates, of fluid in the lumen, that the cells lining the sides of the intervillus space may be less permeable than the cells at the villus tips. This apparent decrease in mean pore

size with increasing flow rate at first sight does not seem to fit with their previous finding of increased output of A1AT in diarrhoeal stools. However increased exposure of the mucosal surface to faster flowing fluids is likely to allow increased diffusion of the molecule by exposing more pores to the fluid.

Lavage compared with perfusion systems

The methods generally accepted as the most representative for studying secretory immunity in humans in vivo are balloon perfusion techniques. These involve perfusing a segment (about 30-40 cm) of the small or large bowel with a non-absorbable fluid and assay substances in this fluid including total IgA, total IgM, albumin, A1AT, eosinophil cationic protein. In chapter 11 I will compare the secretion rates of immune substances measured in WGLF with previously published data in small and large bowel perfusion systems.

Diarrhoea, Transit Time and Faecal Water Content

The possibility of using diarrhoea as a more reliable means of assessing the intestinal immune response than normal faecal specimens is based partly upon the theory that the transit time and therefore the exposure to degradative enzymes is minimised.

The reduction in whole gut transit time in both patients and volunteers with experimentally induced diarrhoea is well established (O'Donnell *et al* 1990; Steadman & Kerlin, 1994). One of these studies (O'Donnell *et al* 1990) used a subjective, semi-quantitative assessment of stool form and related this to the whole gut transit time as measured using radio-opaque markers. The scoring for the stools ranged from 1: 'separate hard lumps like nuts' to 7: 'watery, no solid pieces'. They demonstrated a significant negative correlation between the transit time and the stool form score i.e. the looser looking the stool, the shorter the transit time.

A recent study has shown that a similar subjective assessment of the consistency of diarrhoeal specimens is related to the water content (Wenzl *et al* 1995). I have summarized their results in table 10.1 for patients without steatorrhoea.

Table 10.1: Subjective assessment of stool consistency compared with the measured water content (Wenzl *et al* 1995).

<u>Stool Consistency</u>	<u>Water Content (%)</u>
Normal	72.0 ± 1.2
Semi-formed	81.7 ± 1.3
Soft	86.3 ± 0.9
Semiliquid	88.5 ± 0.6
Runny	91.2 ± 0.6

Thus the most severe or 'runny' stools have faecal water content of approximately 91.2% whereas normal stools have water content of 72%.

Combining the results of these two studies would suggest that measurement of the faecal water content can be crude method of assessing the transit time of the whole gut, i.e. high water content has a shorter whole gut transit time. To confirm this would require a study measuring transit time in subjects with and without diarrhoeal disease and measure the water content of faecal material passed.

Summary of why this research needs to be done

1. No one knows how estimation of the daily output of immune (e.g. IgA and IgM) and other factors in faeces compare with WGL or more invasive methods of investigating the secretory immunity of the GI tract.
2. Antibodies may not always be detectable in faecal specimens. It is not known whether this is a reliable means to confirm the absence of antibodies.

How This Chapter Will Proceed.

The first part will describe a study performed in collaboration with Professor Anne Ferguson and Kenneth Humphreys looking at the daily output of various factors in faecal specimens compared to whole gut lavage. I was involved from the outset in the design of the study and some of the analyses performed with Ken Humphreys.

Professor Ferguson performed most of the calculations and writing up of the paper

which has been published (Ferguson *et al*, 1995). As a consequence of results of this first study I went on to study ileostomy patients.

Part I: Faecal Study

Aims

1. to compare daily output of total IgA, total IgM, total IgG, albumin and A1AT in faecal material and WGLF from the same patient.
2. to compare the detection of antibodies in faeces and WGLF.

Patients and Methods

Patients

Specimens were collected from 20 adult in-patients who were having WGL as bowel preparation for colonic investigations. Stools were collected and weighed for the previous 3 days. Matched specimens of faeces and WGLF were collected from all the subjects.

Ten had inflammatory bowel disease (IBD) (five men, five women; mean age 40.1 years, SD 17.5; six Crohn's disease, four ulcerative colitis). In all of these, their IBD was clinically active assessed subjectively by the clinician arranging the investigations and confirmed by the objective measure of WGLF IgG concentration $>10 \mu\text{g/ml}$ (Choudari *et al* 1993a).

Ten patients were disease controls (four men and six women, mean age 53.4 years, SD 24.4) who had other conditions: two irritable bowel syndrome, two dietary iron deficiency anaemia, two benign colonic polyps, and one each of diverticular disease, pancreatic insufficiency, drug-induced diarrhoea and non-steroidal enteropathy.

Specimen Collection

Under the supervision of an experienced nurse, patients drank polyethylene glycol-based lavage fluid (Klean-Prep, Norgine, UK), at a rate of 250 ml every 10-15 minutes.

Part of the first stool passed by the patient on the morning of the test was collected and frozen at -70°C , and an aliquot of the first completely clear specimen of WGLF, was collected, processed and stored for analysis.

The faecal specimens were extracted as described in the methods section. Specimens were stored both with and without processing agents added to examine the effect of adding processing agents on these extracts.

Assays performed

The following assays were performed on the stool extracts and WGLF: Hb, IgG, albumin, alpha-1-antitrypsin (A1AT), IgA, IgM. Results were expressed as $\mu\text{g/ml}$ WGLF or $\mu\text{g/g}$ faeces.

Isotype-specific antibodies to ovalbumin (OVA), gliadin (GLI) and beta-lactoglobulin (BLG) were assayed in a semi-quantitative ELISA procedure (O'Mahony *et al* 1991a). Results were read as optical density (OD) values, and expressed as percentages of the reference standard OD reading, with values $>10\%$ representing detectable antibody.

For absolute titres of IgA antibody to ovalbumin standard, ELISA was performed with a range of dilutions of test specimens and of high-titre standard sera, the latter being arbitrarily designated as containing 1000 units IgA class antibody per ml (O'Mahony *et al* 1991a).

Calculations

Faeces

Output of substance (μg or units/day) = conc./g of faeces x daily faecal weight (g)

Lavage

Output of substance (μg or units/day) = conc./ml of WGLF x rate of lavage (ml/day)

Results

Daily faecal weight

The daily faecal weight in the IBD patients (251 ± 122 g/day, range 120-510) was significantly greater ($p < 0.01$) than in the disease control patients (124 ± 39 g/day, range 57-203).

Concentrations of immunoglobulins and plasma-derived proteins

There were similar concentrations of Hb, IgA and IgM in WGLF from IBD and non-IBD patients, whereas concentrations of IgG, albumin and A1AT were higher in the IBD patients, in keeping with presence of active disease (Table 10.2).

Table 10.2: Concentrations of substances in whole gut lavage fluid (WGLF) of patients with inflammatory bowel disease (IBD) and with other GI diseases (mean \pm s.d.).

Substance assayed in WGLF	Concentration ($\mu\text{g}/\text{ml}$)		Significance of difference between groups
	Active IBD (n=10)	Other GI diseases (n=10)	
IgA	177.1 ± 58.6	166 ± 112.8	NS
IgM	15.4 ± 10.8	7.7 ± 10.4	NS
IgG	93.3 ± 93.5	4.3 ± 5.4	$P < 0.02$
Albumin	101.1 ± 72.2	30.9 ± 61.2	$P < 0.05$
A1AT	35 ± 56.6	8.0 ± 7.1	NS
Hb	5.4 ± 2.8	3.5 ± 3.9	NS

Relative recoveries of immunoglobulins and plasma-derived proteins

These concentrations were then expressed as estimated daily output, and with the amount in WGLF taken as 100% using the following equation the relative (%) recovery of the various substances could be calculated.

$$\% \text{ Recovery} = 100 \times \frac{\text{Estimated daily output in faecal material}}{\text{Estimated daily output in lavage fluid}}$$

The proportions of the various substances which could still be detected in faeces varied greatly: <0.1%-30.8% for IgA, <0.1%-35.5% for IgM, <0.1%-15.4% for IgG, <0.1%-25.5% for albumin and 1.2%->100% for A1AT.

There were clear differences in the relative amounts recovered from faeces in the IBD and non-IBD groups. Differences between control and IBD groups in median percentage recovery of IgA (control = 0.4 % vs IBD = 5.5 %, p=0.001), IgM (1.6 % vs 8.5 %, p=0.03), IgG (0 % vs 2 %, p=0.001), albumin (0 % vs 9.2, p=0.001) demonstrated higher rates of recovery in the IBD group. This data is shown in graphic form in figures 10.1 and 10.2 in the next section discussing ileostomy patients.

Effects of adding protease inhibitors to faecal extracts

There were no differences between processed and unprocessed faecal extracts for IgA, IgM, IgG, and A1AT concentrations. However specimens treated with protease inhibitors had significantly higher concentrations of albumin, with more than double the values obtained with unprocessed specimens (p<0.05) and, as described below, the frequency of detection of specific antibodies was greater.

Detection and relative recoveries of antibodies

The three semi-quantitative assays for isotype-specific antibodies were performed in processed and unprocessed WGLF and in processed and unprocessed faecal extracts from four IBD and six non-IBD patients. Results are summarised in Table 10.3, which shows frequencies of detectable antibodies of IgA, IgM and IgG classes.

Frequencies in WGLF were similar for the IBD and non-IBD groups, (apart from IgG, probably reflecting plasma leak). Apart from a single IBD patient, results for faeces showed positive results only in processed samples, and although antibodies were known to be present in both IBD and non-IBD patients on the basis of WGLF data, detection rates of antibodies were higher in faecal extracts from IBD patients.

For example IgA antibodies were detectable in 9/12 processed faecal extracts and 11/12 processed WGLF from IBD patients but only 2/18 processed faecal extracts and 15/18 processed WGLF from non-IBD patients.

Table 10.3. Frequencies of detection of isotype specific antibodies to three food antigens in processed and unprocessed specimens of whole gut lavage fluid (WGLF) and faeces from 10 patients (4 IBD, 6 disease controls).

Isotype	Diagnostic Group	Number of Assays	Number of specimens with detectable antibody in:			
			Processed WGLF	Unprocessed WGLF	Processed faeces	Unprocessed faeces
IgA	IBD	12	11	5	9	0
	Other	18	15	11	2	0
IgM	IBD	12	2	0	1	0
	Other	18	5	0	0	0
IgG	IBD	12	5	3	3	1
	Other	18	2	0	0	0

The quantitative ELISAs showed that IgA antibodies to OVA were present in WGLF from 19 of the 20 patients, with similar concentrations in IBD and non-IBD groups, whether expressed per ml WGLF, per mg IgA or per mg Hb (Table 10.4).

This was matched by the presence of antibody in processed faecal extracts in eight of the ten IBD cases, antibody at low titre was detectable in processed faecal extracts of two out of ten non-IBD patients, with diverticular disease and NSAID enteropathy.

Discussion

This data demonstrates that there are disease influences on the recovery from faeces (when compared with gut lavage fluid) of immunoglobulins, plasma-derived proteins and antibodies.

Table 10.4: Concentrations of IgA anti-ovalbumin (OVA) antibody in whole gut lavage fluid (WGLF) from patients with inflammatory bowel disease (IBD) (n=10) and with other diseases (n=9).

Diagnosis	Reference Measure	Concentration of IgA anti-OVA in WGLF (arbitrary units)		
		Mean	s.d.	
IBD	per ml WGLF	7.2	4.1	NS
Other		6.8	3.3	
IBD	per day (x 10 ⁶)	42.9	28.4	NS
Other		44.8	33.5	
IBD	per mg IgA	23.7	20.4	NS
Other		18.6	11	
IBD	per mg Hb	1620	1765	NS
Other		2054	1280	

NS, Not significant, Hb, haemoglobin

In patients with active IBD, faecal IgA recovery was up to 30.8% of the amount present in WGLF, whereas the maximum proportion detected in the faeces of non-IBD cases was 1.7%.

The loss of specific IgA antibody activity in faeces is even greater than loss of IgA content. In the case of IgA anti-OVA, some antibody activity could be detected in faecal extracts of 8 of the 10 IBD cases, and 2 of the 10 non-IBD cases, all of whom had measurable antibody in WGLF. Results of faecal antibody tests, if considered alone, would have suggested that there is abnormal mucosal immunity to this dietary protein in active IBD, whereas the use of gut perfusate specimens shows that most adults have intestinal IgA antibody to ovalbumin in similar concentrations.

Summary

In faecal specimens from disease controls the estimated daily output of IgA is only up to 1.7 % of that measured in WGLF and that specific IgA antibodies such as anti-

OVA IgA are not always detectable in faecal extracts when they are detectable in corresponding WGLF.

In contrast, adults with active inflammatory bowel disease, who are likely to have diarrhoea (in part shown by their significantly increased daily output of faecal material), the estimated daily faecal output is up to 30.8% of that measured in WGLF and antibodies are more frequently recovered from the faecal extracts.

The detection of IgA antibodies in both the WGLF and the faecal extracts is improved if the specimen is processed.

Other substances measured (IgM, IgG, albumin) also show increased recovery in the IBD group.

Thus there seems to be a difference in these two groups of patients when studying matched faecal and WGLF specimens, possibly related to the presence or absence of diarrhoea.

Part II: Ileostomy study

Rationale

Working on the theory that diarrhoeal illness improves the recovery of substances in faecal material when using whole gut lavage as the standard I then planned an experiment using volunteers with ileostomies as the subjects.

Ileostomy fluid is similar to diarrhoeal fluid in that it has a short transit time through the gut and has a high water content. Recovery in ileostomy fluid will thus give some indication of how good the recovery of substances in diarrhoeal specimens can be.

The suggestion that water content could be related to transit time led me to also examine the relationship between the water content and the recovery of substances in ileostomy faecal material.

Aims

1. to compare daily output of total immunoglobulins (A, M, and G), specific antibodies, albumin and A1AT in ileostomy output and matched WGLF.
2. to determine if there is a relationship between the water content of the ileostomy specimen and the recovery of immune substances in faecal material.

Patients

Adult patients and volunteers with ileostomies were recruited from the outpatient clinic and ward at the Western General Hospital.

Fourteen subjects with ileostomies were recruited, all had surgery as a complication of inflammatory bowel disease (9 Crohn's disease, 5 ulcerative colitis). There were 9 males and 5 females with a mean age of 42 years (range 18-65 years).

Methods

Specimen Collection

The subjects collected their 24 hour ileostomy output in a pre-weighed container prior to attending the GI unit. They emptied their ileostomy bag just prior to leaving home to attend the hospital.

On arrival an aliquot of ileostomy output (less than 1 hour old) was collected from their bag and was immediately mixed 1/5 (weight by volume) in PBS at 4⁰C , and centrifuged at 3000g, at 4⁰C. The resulting supernatant was then filtered and processed as in WGLF specimens and stored at -70⁰C.

Whole gut lavage was then started at a rate of approximately 250 ml every 15 minutes, the exact rate was recorded by the attending research nurse. The first ileostomy specimen passed after starting the lavage was collected and processed as above. Once the effluent ran clear the lavage specimen was collected and processed in the standard manner.

Assays

Total immunoglobulin A, M and G, albumin, and A1AT were assayed as described previously. Specimens from individual patients were run on the same plate in order to minimise inter-assay variations.

A range of quantitative antibody assays were also run including anti-ova IgA and IgM, anti-gliadin IgA and anti-cholera toxin (Anti-CT) IgA and IgM. The presence of cholera toxin antibodies in WGLF from subjects not exposed to cholera disease or vaccine has been described previously and is felt to be due to cross reactive antigens originating from other gram negative organisms (Ferguson *et al*, 1995a). Units for these are arbitrary, based upon the serum standards used. IgG antibodies were not assayed as these were frequently not detectable in WGLF and faecal material in the previous section.

Calculations

Estimated percentage recovery of substances in ileostomy fluid daily outputs were calculated as in the previous experiment.

$$\% \text{ Recovery} = 100 \times \frac{\text{Estimated daily output in ileostomy fluid}}{\text{Estimated daily output in lavage fluid}}$$

Results

Figures 10.1 and 10.2 demonstrate the different recoveries of substances in the three groups of patients which include the disease control and active IBD patients from the previous section of this chapter. Ileostomy patients have clearly higher rates of recovery than the other two groups.

Figure 10.1: Recovery of total IgA and M in different groups of subjects.

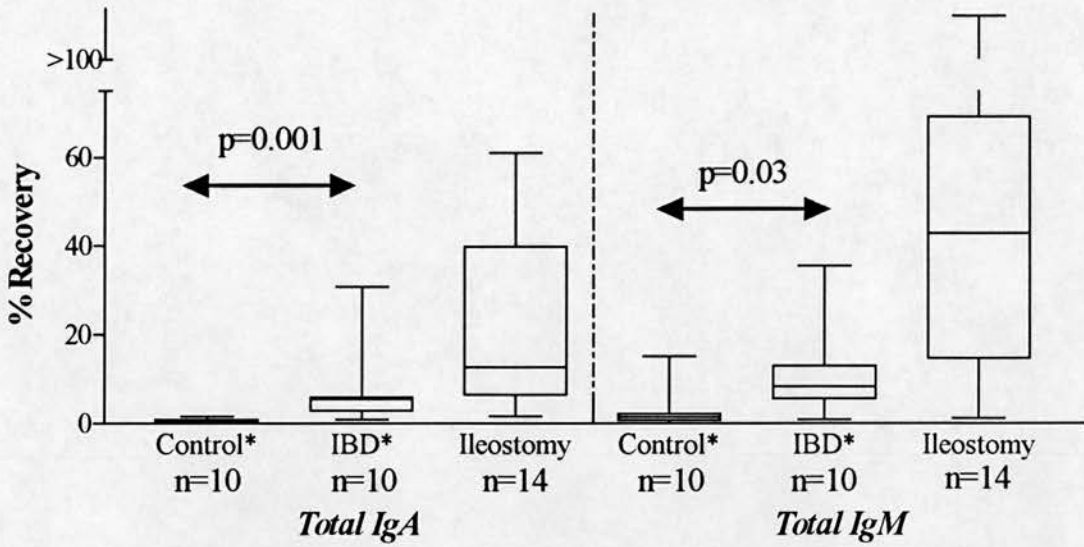
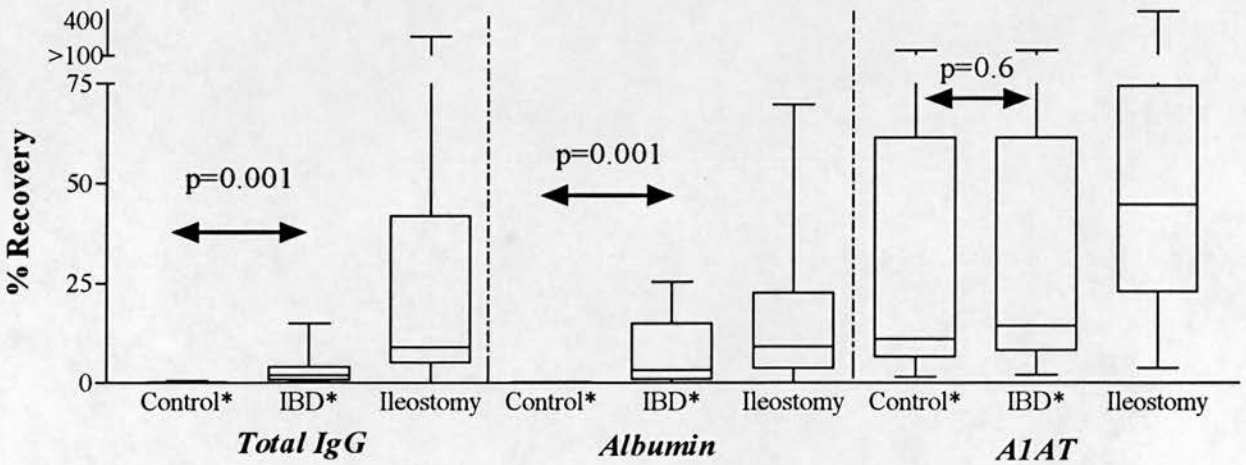


Figure 10.2: Recovery of Plasma Derived Proteins in Different Groups of Subjects.



* Results from chapter 10 part I. Box and Whisker plot: Median is the horizontal line within the box, the upper and lower limit of the box indicates the interquartile range, the whiskers indicate the full range.

Detection of antibodies

When assessing the presence or absence of antibody the top optic density of the doubling dilutions of the specimens had to come within the standard curve. The table

below show that the rate of detection of the presence of antibody in ileostomy effluent is similar to WGLF. For the results described in the previous section, the criteria for detection of antibody were slightly different and so I have included in brackets the results using those criteria (optic density of the top concentration of the specimen > 10% of the OD of the top standard).

Table 10.5: Detection of isotype specific antibodies in WGLF and ileostomy effluent.

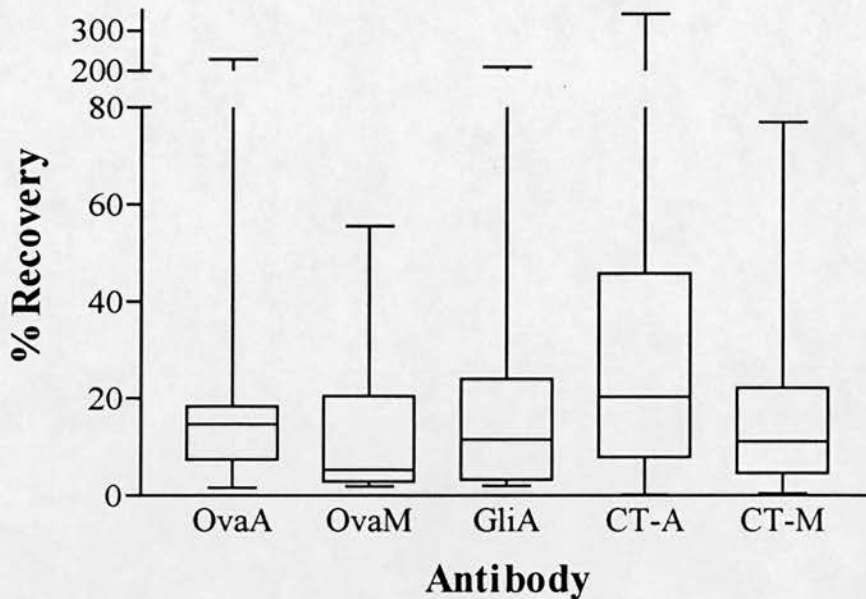
<u>Antibody</u>	<u>WGLF</u> N=14	<u>ILEOSTOMY</u> <u>EFFLUENT</u> N=14
OVA-A	14 (14)	14 (14)
OVA-M	14 (11)	12 (9)
GLIADIN-A	14 (14)	14 (12)
CHOLERA TOXIN-A	14	14
CHOLERA TOXIN-M	14	14

Recovery of antibodies from ileostomy effluent compared with lavage

Table 10.5 shows whether or not antibodies can be detected in the ileostomy fluid. I then went on to examine the estimated rates of recovery of antibodies in ileostomy effluent compared to WGLF.

These show medians of 12.3 %, 10.1 % and 19% for the IgA antibodies Ova, Gli and CT respectively. The IgM antibodies Ova and CT had recoveries of 9.3 and 11.1 respectively. The IgA antibodies thus have very similar median recoveries to those for total IgA (12.75%).

Figure 10.3: Recovery of antibodies in ileostomy fluid



'Fresher Specimens' of Ileostomy Effluent

In 8 of the subjects two ileostomy specimens were collected. The first was from the bag on arrival (Specimen 1) and the second was a fresher specimen (Specimen 2) which was the first output from the ileostomy after commencing the lavage, but before the clear specimen was collected. Analyses were performed for total IgA and IgM, Ova-A, Ova-M, Gli-A, CT-A, CT-M. Percent recovery was then estimated as described above for both the specimens.

I have compared these two sets of specimens in table 10.6 using the following equation: % Difference = % Recovery in specimen 2 ('fresher specimen') - % Recovery in Specimen 1.

Statistics were calculated using the Wilcoxon Signed Rank Test.

All differences were positive thus the estimated output in Specimen 2 (the 'fresher' of the two) was higher than in Specimen 1. This was only significant for total IgA and Gli-A antibody.

Table 10.6: Difference between the first and second ileostomy specimen.

	% Difference (Median)	p=
IgA	+ 6.3	0.014
IgM	+ 1.2	0.441
Ova-A	+ 9.5	0.107
Ova-M	+4.7	0.059
Gli-A	+ 7.8	0.022
CT-A	+ 7.2	0.441
CT-M	+ 4.2	0.141

Relationship of recovery and % water content

Figures 10.4 to 10.7 plot the % recovery versus the % water content. For total IgA and IgM, and Ova-A and Ova-M there is a trend to increasing recovery with increasing percentage water. None of the other substances (IgG, albumin, A1AT, and remaining antibodies) showed any relationship with the percentage water content.

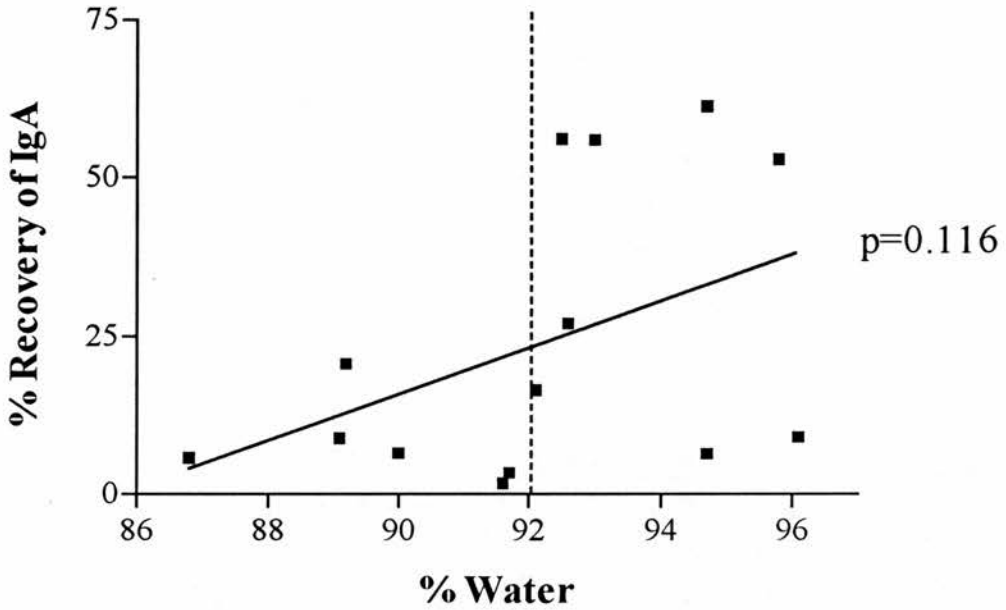
By dividing the ileostomy specimens into two groups, those with water content above 92 % water and those below 92% water as marked on figures 10.4 and 10.5 it is possible to compare these as distinct groups. Table 10.7 shows the results of comparing the recoveries of the medians for the specimens <92% with those ≥92% water.

Table 10.7: Recovery of substances in ileostomy extracts related to water content.

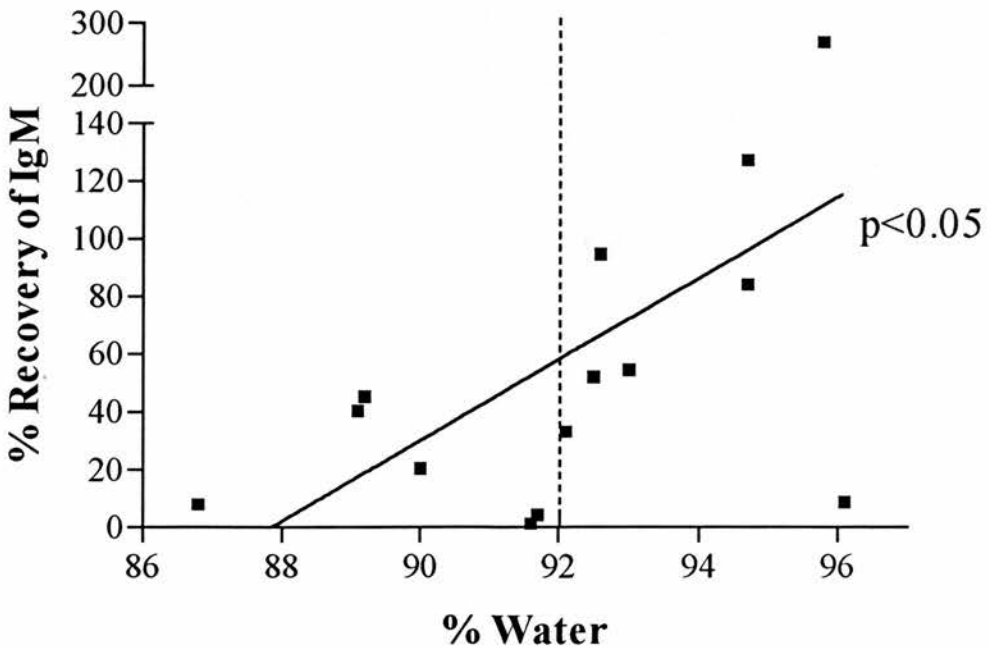
Substance	Median Recovery		Mann Whitney p=
	< 92 % Water n=6	≥ 92% Water n=8	
Total IgA	6.1	39.9	0.02
Total IgG	5.3	21.3	0.22
Total IgM	14.2	69.3	0.02
Albumin	3.7	13.4	0.14
A1AT	26.5	82.1	0.21
Ova-A	7.3	18.3	0.03

Figures 10.4 and 10.5: Relationship of recovery of total IgA and IgM and % water content of ileostomy effluent. Linear regression is plotted with the p values for that line.

% IgA Recovery vs % Water

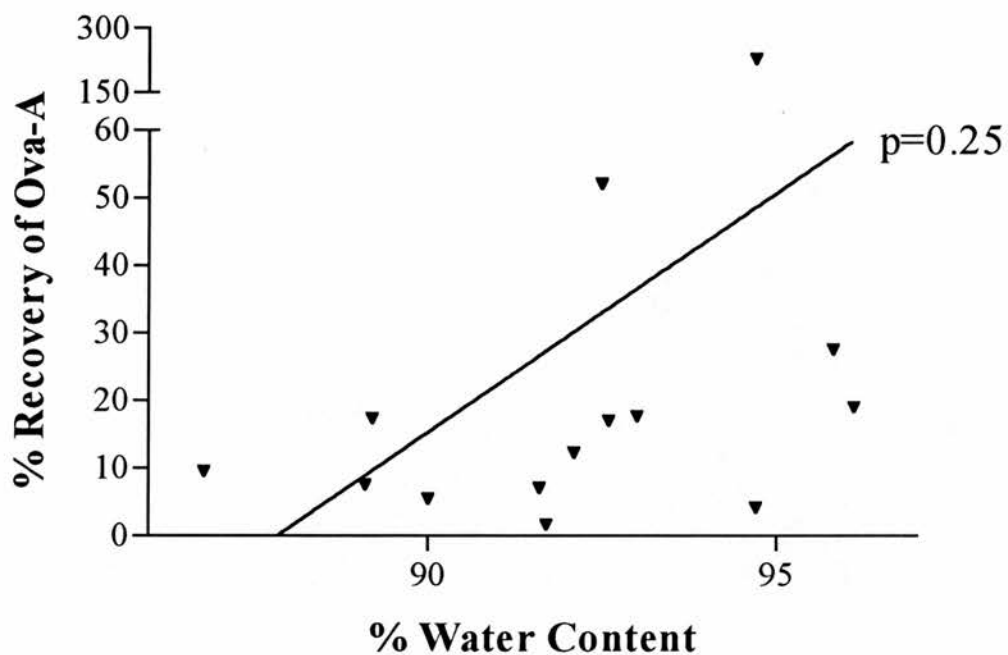


% IgM Recovery vs % Water

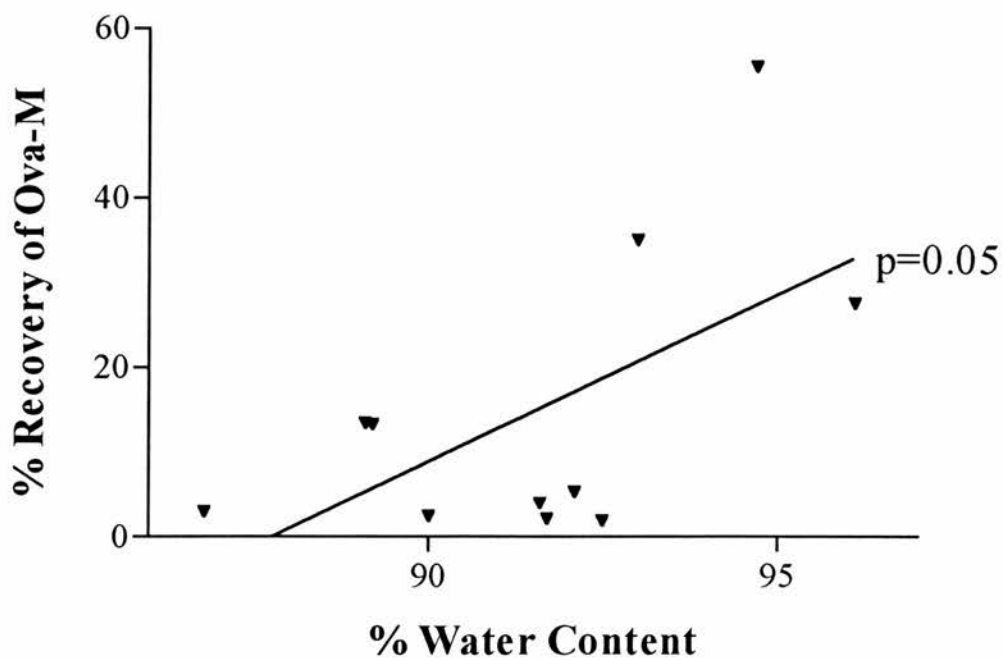


Figures 10.6 and 10.7: Relationship of recovery of Ova A and M and % water content of ileostomy effluent. Linear regression is plotted with the p values for that line.

% Ova-A Recovery vs % Water Content



% Ova-M Recovery vs % Water Content



Discussion

In this chapter I have shown that measurement of daily outputs of immunoglobulins, antibodies and plasma derived substances in faecal material is very different to outputs recorded in a whole gut perfusion system (WGLF) and that these differences depend upon the disease group of the patient, probably related to the presence or absence of diarrhoea.

Table 10.8 shows the percent recoveries of the various assays performed in ileostomy patients. Individual results show recoveries of greater than 100% in A1AT, IgG and IgM. These figures are due to very low concentrations found in WGLF. Total IgA and albumin which tend to always be present at readily measurable levels in WGLF show ranges up to 70%.

Table 10.8: Summary of data recovery of immune and plasma derived substances in patients with ileostomies.

Substance Assayed	Median n=14	Mean n=14	Range	95% CI
IgA	12.7	23.7	1.6-61	11-37
IgG	9	38.6	0-268	-2-79
IgM	42.8	60.2	1.2-269	19-101
Albumin	9.2	19.2	0-70	5-33
A1AT	44.7	99.5	3.8-476	18-181
Ova-A	14.6	30.2	1.7-225	-2.9-63.5
% Water	92.3	92.1	86.8-96.1	90.6-93.7

In ileostomy patients the median recoveries varied widely from 9.0 % for total IgG to 44.7 % for A1AT.

When collecting the ileostomy specimens two of the subjects had very low 24 hour outputs of ileostomy material when compared with the other patients. As all the patients were clinically well it seems possible that they had failed to collect all the output for that day. This would affect their results by reducing the percentage recoveries of the various factors. The two patients with the lowest outputs were the youngest, one of whom was still at school.

For consistency all the patients had their ileostomy specimen collected first thing in the morning after fasting overnight. However whether the concentration of total IgA and IgM measured in this sample is representative of the remainder of the day has not been looked at. Four fresh specimens plus one from the 24 hour container were collected and processed from a single in-patient with an ileostomy throughout a 24 hour period. This data is shown below, expressed per gram of faeces (wet weight). A 2.7 fold difference between the highest and the lowest concentration was found. This patient was not fasted during this period and the differences may be due to the different food and fluid intakes during the day. Studies in animals and humans suggest that nutrients such as amino-acids may stimulate immunoglobulin secretion (Freier *et al*, 1987; Colombel *et al*, 1992a). To clarify this detail further more patients need to be studied whilst fasting. The time 0 specimen, which was the equivalent one collected by all the remaining subjects, had the lowest concentration. If this finding were reproducible in more subjects the estimated recoveries would be 2-3 fold higher.

Table 10.9: Concentration of IgA and IgM in a single patient with an ileostomy throughout a 24 hour period.

TIME (hours from start of collection)	CONC. IgA ($\mu\text{g/g}$)	CONC. IgM ($\mu\text{g/g}$)
0 (fasting)	435	111
2.5	724	345
6	1165	715
24	546	374
24 hour collection	591	263

Extracts from patients with ileostomies had the highest recovery for all substances (as shown in figures 10.1 and 10.2). This shows that ileostomy fluid is more representative of the secretion of immune and other substances into gut perfusion fluid than faeces from control patients. However the specimens from patients with ileostomies still had a wide range of recoveries such as total IgA ranging from 1.6-61 % (median 12.7 %) whereas recovery of total IgA in all 10 control patients in part 1 was less than 2 %.

For total IgA and IgM this increase appears to be associated with an increase in the % water content of the ileostomy fluid above 92 %.

A number of reasons for the differences in results obtained with faecal material and gut perfusate can be suggested.

1. Breakdown of substances exposed to degradative enzymes is increased by increased time of exposure as I have shown in the degradation assays for total IgA and IgM in WGLF in chapter 5. In this study I showed that over 24 hours at 37⁰C, total IgA in WGLF reduced to 22 % of the original concentration. This could be an underestimate of the rate of degradation in faecal material, as the concentration of degradative enzymes is likely to be higher in the faecal material and these enzymes are being continually secreted into the gastro-intestinal tract. Ideally one would spike faecal material with total IgA to measure the breakdown, however this is not feasible for a solid material and the degradative enzymes themselves may be inactivated in faeces.
2. Secretion or translation of substances is increased by the perfusion technique and the diarrhoeal state when compared with the normal state.
3. There may be mechanical or biological interference with assays by substances present in faeces to a variable extent (reduced in diarrhoea) whose effect in WGLF is minimised by dilution, this could include factors originating in bile.

Summary

These experiments have shown that the output of immune substances measured in faecal or diarrhoeal material is considerably less than in a whole gut perfusion system, and that the amounts recovered vary, relating in part to the presence or absence of diarrhoea.

At least part of this effect for total and specific IgA and total IgM is likely to be due to the breakdown of the substances by degradative enzymes as shown by the reduction in concentration in WGLF stored for up to 24 hours at 37°C.

Previous studies of immune substances in diarrhoea using normal faecal specimens as control material are thus flawed as they have assumed that recovery of the substance measured is the same in both the normal and diarrhoeal state.

If attempting to compare diarrhoeal specimens from control patients with the group being studied researchers should attempt to show that the diarrhoeal material collected for both groups is comparable. One method for this could be to measure the percent water content in the control and study groups. In this chapter I have found that in ileostomy patients a faecal water content of > 92% is associated with an increased recovery of immune and inflammatory markers.

The problems of being able to collect suitable control material in studies of immune or other substances in faeces or diarrhoea mean that ideally a gut perfusion technique should be used, which allows a similar intra-luminal physiological state to be attained between all the groups. This is not feasible for all studies of gastrointestinal secretory immune system in children.

The data from this chapter, showing the percentage recoveries of various substances in patients with ileostomies, allow comparisons to be made between the outputs measured in diarrhoea (assuming that the recoveries in ileostomy specimens are similar to those found in the diarrhoeal state), and control specimens from children who have had WGL for other reasons. I will discuss this further in the next chapter on daily outputs of IgA in various groups of children.

Future Studies

There are two studies that would clarify some aspects of this complex area, particularly with regard to the analysis of faecal material for immune substances.

1. In order to confirm that the measurement of faecal water content can relate to the whole gut transit time (WGTT) patients with diarrhoeal disease should have their WGTT measured simultaneously with the faecal water content of stool passed. The control group can be patients or volunteers without diarrhoeal disease. My hypothesis is that the shorter the transit time the higher the water content of the diarrhoeal/faecal material.
2. To prove that the presence of diarrhoea alters the measurement of immune factors in the GI tract would involve a similar study to that performed by Fordtran's group (Strygler *et al* 1990). This would mean collecting a group of volunteers without diarrhoeal disease and measuring the daily output of immune substances in their normal faecal specimens. Diarrhoea would then be induced using a set dose of a PEG based diarrhoeal agent and again measure their output of immune substances. The subjects would finally undergo WGL to establish the output in the perfused state. It would be possible in a large study to also measure the WGTT in these patients in these three situations (normal, diarrhoeal and perfused) while measuring the % water content of specimens collected. This study would show any differences between the measurement of the output of immune substances in the normal, diarrhoeal and perfused state and relate them to the water content of the material passed.

Chapter 11: Daily Output of IgA and Other Substances in WGLF and Acute Watery Diarrhoea in Children

Introduction

In the first part of this chapter previous studies that allow estimation of the rate of secretion of various immune and other substances into the GI tract are reviewed. I will then calculate the daily output of these substances in the groups of children that have been studied in this thesis i.e. control children, children with cystic fibrosis, healthy African children and African children with acute, watery diarrhoeal disease. The substances that I will be looking at are total IgA, total IgM, total IgG, Albumin, A1AT and ECP.

Literature Review

I have previously discussed the problems of different methods of collecting, processing, analysing and interpreting different types of specimens from the gastrointestinal tract. Many studies describe the concentration per unit of wet or dry weight faecal material. The latter avoids the problems of water content in the faecal material but does not allow for variable intake of food between the subjects.

Another method for comparing results between different groups is to calculate the daily output of the substance of interest, thus the results are expressed as mg/day or mg/kg/day. The latter method is preferable if comparing outputs between adults and children.

For plasma derived proteins a common way to express results is the clearance per day (ml/day) as this allows for the concentration of the substance in the serum from which the protein (albumin or alpha-1-antitrypsin) originates (Bernier *et al* 1978; Strygler *et al* 1990; Kapel *et al* 1992).

In order to estimate the daily output one needs to know both the concentration of the substance and the volume per day of the material which you are studying. For the

gastrointestinal tract the types of specimens that can be used to calculate daily outputs are faeces/diarrhoea, balloon perfusion fluid and whole gut lavage fluid. Faeces has the disadvantage of prolonged transit time with exposure to degradative enzymes in the gastro-intestinal tract. Balloon perfusion techniques are able to exclude degradative enzymes, however they only study a small area of the gastro-intestinal tract and for daily outputs from the whole gut approximations of the length of the bowel and the secretion rates in the different areas of the bowel have to be made. Whole gut lavage, like faeces, studies the whole gastrointestinal tract and degradative enzymes are present in the lumen, even though the time of exposure to them is minimised. I will now review previous studies from which it is possible to estimate the daily secretion rates of various substances. All these calculated outputs and the results of this study are included in tables 11.1 and 11.2.

Assumptions

So that realistic comparisons can be made with children, the daily output will be expressed as mg (or μg) of the substance/kg/day. In papers including adult data, where the figures have been expressed as mg/day I have assumed that the weight of the adults is 65 kg.

In perfusion studies where data has been expressed as mg/minute/unit length of bowel, like Conley and Delacroix I will assume that the length of the bowel is 3.6 m (Conley & Delacroix, 1987) and multiply the results accordingly. The actual length of the small bowel can vary, in one study from 160-430 cm with median of 291 (Fanucci *et al* 1988). Other *in vivo* estimates of the length of the small bowel are 220 cm (Prigent-Delecourt *et al* 1995), and 261 cm (quoted in (Fanucci *et al* 1988)).

Some papers report the clearance of alpha-1-antitrypsin and other plasma derived proteins. Clearance is calculated using the following equations:

$$\text{Clearance (ml/day)} = \frac{\text{Daily output (mg/day)}}{\text{Concentration in serum (mg/ml)}}$$

$$\text{Daily output (mg/day)} = \text{Concentration in faeces/fluid (mg/ml)} \times \text{Volume of faeces/fluid per day (ml)}.$$

Where papers have only included the clearance I have calculated the daily excretion assuming that the normal serum A1AT is 3.5 g/l. The daily output can thus be calculated by multiplying the clearance by the serum concentration.

Faecal Studies

In adults the total IgA output in faeces, measured by electro-immunodiffusion showed a daily output of 0.9 mg/kg/day in controls, a group of patients with chronic diarrhoea secreting 4.1 mg/kg/day (Meillet *et al* 1987).

A more recent study estimated daily faecal polymeric IgA output in adult patients and controls (Kapel *et al* 1992). Polymeric IgA is known to be 73% of the total IgA measured in faeces from healthy subjects (Meillet *et al* 1987) so the estimated total IgA would be approximately 30% higher. This works out as a total IgA secretion of 0.04 mg/kg/day in controls, 0.22 in inactive Crohn's disease, 3.0 in active Crohn's disease and 2.9 in active ulcerative colitis (Kapel *et al* 1992). IgM output was 0.02 mg/kg/day in controls, 0.03 mg/kg/day in inactive Crohn's disease, 0.05 mg/kg/day in active Crohn's and 0.05 mg/kg/day in active ulcerative colitis (Kapel *et al* 1992). This group also quote figures for the daily clearance of IgG, in order to convert these to daily outputs I have multiplied the clearance by the mean serum level of IgG of 9.4 mg/ml. The estimated IgG clearance was 0.02 mg/kg/day in controls, 0.04 in inactive Crohn's, 0.10 in active Crohn's and 0.14 in active UC.

In a study of diarrhoea from cholera patients the daily output of IgA was estimated to be 1.55 g/day (or 23.8 mg/kg/day) (Northrup *et al* 1970). For IgG, of the 5 (out of 15) patients who had detectable levels, the average was 2.5 mg/kg/day. Assuming that the 10 who had undetectable amounts had zero IgG would mean an average of 0.8 mg/kg/day. IgM was detectable in 8/25 with a mean of 2.6 mg/kg/day for the whole group.

A study of IgA output in faeces in children ranging from 1 day to 12 years estimated a median of 85 mg/day, ranging from 15-700 mg/day (Haneberg & Aarskog, 1975). This data is extracted from a log graph in the paper so is only approximate. Unfortunately it is not possible to calculate the rate of secretion per kg from this data as the ages of the children who had outputs measured are not available.

A1AT clearance in faeces has been estimated at 10.5 ml/day (approximately 0.57 mg/kg/day) (Kapel *et al* 1992), 3.07 ml/day (0.16 mg/kg/day) (Bernier *et al* 1978), and 13.6 ml/day (0.73 mg/kg/day) (Strygler *et al* 1990) in control adults. Inducing diarrhoea in the latter study led to a daily clearance of 27.8 ml/day (1.50 mg/kg/day) (Strygler *et al* 1990). Patients with active inflammatory bowel disease had higher outputs of 3.08 mg/kg/day (Kapel *et al* 1992) and 4.94 mg/kg/day (Bernier *et al* 1978).

Owing to the survival of A1AT in the GI tract and high levels of A1AT in breast milk, faecal A1AT is not felt to be a useful investigation in breast feeding infants (Davidson & Lonnerdal, 1990).

Perfusion Studies

There are two main groups who have developed segmental jejunal perfusion techniques:

Rimbaud described a single balloon technique where a tube is passed into the small bowel (Rimbaud *et al* 1981). A single proximal balloon is inflated to prevent degradative secretions contaminating the perfusate which is infused at a rate of 10 ml/minute immediately below the balloon. Forty centimetres distal is the entrance to the lumen which collects the perfusate for analysis. Thus the length perfused is 40 cm. They have recently described a similar method to perfuse the whole colon at 15 ml/minute using an obstructive balloon in the distal ileum (Prigent-Delecourt *et al* 1995).

Knutson described a two balloon technique where fluid is perfused between two balloons which are 10 centimetres apart, sited in the small bowel (Knutson *et al* 1989). The rate at which they perfuse the segment is 3 ml/minute.

Single Balloon Perfusion Studies

Taking data for colonic (Prigent-Delecourt *et al* 1995) and small bowel (Jonard *et al* 1984) perfusion techniques from the same group separately and assuming that the small bowel is 220 cm long (Prigent-Delecourt *et al* 1995) and the large bowel is 140 cm it is possible to estimate the IgA secretion into the whole gut. Into the small bowel estimated daily IgA secretion is 28.3 mg/kg/day. This plus the colonic IgA secretion of 3.58 mg/kg/day leads to a total IgA secretion rate of 31.9 mg/kg/day.

Using the same calculations albumin secretion is 18.6 mg/kg/day (16.1 small bowel and 2.5 large bowel), IgG secretion is 5 mg/kg/day (4.26 small bowel, 0.74 colon), and IgM secretion is 3.06 mg/kg/day (2.68 small bowel, 0.38 colon). This group have gone on to study patients with coeliac disease and have found a five fold higher rate of secretion of IgM and two fold increase of total IgA (Colombel *et al* 1990). IgG and albumin secretion showed no significant differences.

Hällgren *et al*, in a study using the same single balloon perfusion technique (Hällgren *et al* 1989) estimated the secretion of albumin to be 28.2 mg/kg/day in controls, 24.4 mg/kg/day in patients with active Crohn's disease (mean Crohn's disease activity index of 211 and normal mucosa at endoscopy) and 32.2 mg/kg/day in patients with active coeliac disease. These results for control patients are similar to those estimated by Rambaud above. The surprising result is that the patients with active Crohn's disease had a normal albumin secretion rate, as this has previously been shown to be increased in whole gut lavage fluid from adults with active IBD (Choudari *et al* 1993a). The probable explanation, also one of the disadvantages of segmental perfusion, is that Crohn's Disease is a disease of a patchy nature and the area perfused was endoscopically normal and thus plasma leakage was not increased in the region perfused. In the same study secretion rates of eosinophil cationic protein were also estimated as being 15.4 µg/kg/day in controls, 32.3 µg/kg/day in Crohn's and 132.8

$\mu\text{g}/\text{kg}/\text{day}$ in the patients with active coeliac disease. The last figure being a significant increase ($p < 0.01$) on the previous two, consistent with the presence of activated eosinophils in coeliac disease (Talley *et al* 1992). Both the Crohn's and the coeliac groups also had increased levels of myeloperoxidase secretion compared to controls, suggestive of neutrophil activation.

Double Balloon Perfusion Studies

Using the double balloon perfusion technique the rate of leakage of albumin into the whole bowel has been estimated as 5520 mg/day or 84.9 mg/kg/day (Knutson *et al* 1989), a later study looking at ethanol challenge in the same system estimated a similar rate of 79.8 mg/kg/day in control subjects (Lavö *et al* 1992a). The reason for this very much higher output compared to the rates calculated using single balloon perfusion is not known but could relate to the different techniques, possibly the different flow rates in the two groups.

An estimation of the ECP secretion rate in the same study was 4.3 $\mu\text{g}/\text{kg}/\text{day}$ (Lavö *et al* 1992a). This lower rate to that in the study above of 15.4 $\mu\text{g}/\text{kg}/\text{day}$ (Hällgren *et al* 1989) may be due to the fact that the higher outputs were from disease controls as opposed to healthy volunteers in the latter study. A more recent study reported secretion of ECP in control patients as 11.3 $\mu\text{g}/\text{kg}/\text{day}$, with albumin being secreted at 66.3 mg/kg/day (Knutson *et al* 1993).

IgA secretion in healthy volunteers, using the same technique, can be estimated as 40.3 mg/kg/day with IgM secretion of 17.1 mg/kg/day (Lavö *et al* 1992b). In the same study patients with coeliac disease had IgA secretion of 70.9 mg/kg/day and IgM secretion of 30.5 mg/kg/day.

Whole Gut Lavage

IgA secretion of 32.5 mg/kg/day in whole gut lavage fluid has been reported by Dr Jamal Sallam in adult healthy volunteers (Sallam, 1995), he also albumin secretion was estimated to be 2.2 mg/kg/day. The advantage of WGL for calculation of the rate of secretion of substances is that no assumptions are made with regard to the length

of the bowel or the uniformity of the numbers of the IgA or other plasma cells throughout the bowel. It is thus notable that the estimated output of IgA daily is so close to that calculated in the balloon perfusion systems. The lower rate of albumin secretion may be due to the differing rate at which the perfusion fluid is administered. Alternatively the presence of pancreatic enzymes in WGLF may lead to some breakdown of albumin prior to collection and treatment with protease inhibitors.

Conclusions of Previous Studies

There is no data estimating secretion rates per kilogramme of these substances in children. I will now go on to compare the rates of secretion of these substances in children that I have studied. These include WGLF from control children in the UK, children with cystic fibrosis and African children (with specimens collected by Dr Mary Hodges).

In the chapter 10 the recovery of immune substances in ileostomy output, when compared with WGLF, was found to be more representative than normal faecal material, and the recovery may improve with increasing water content, particularly above 92%. In Freetown I was able to arrange to collect acute watery diarrhoeal specimens from children attending the National Rehydration Training Centre run by the Sierra Leone Red Cross. The specimens collected from children, who were being rehydrated, were watery specimens similar in form to WGLF. I had planned to collect similar specimens from children attending the infectious diseases ward at the City Hospital, Edinburgh. However children referred to this hospital were almost always recovering from their illness and did not have watery diarrhoea. In order to compare secretion of immune substances in watery diarrhoea to WGLF specimens it is essential to compare the daily outputs per kilogramme as it would be meaningless to simply compare the absolute concentrations.

Aims

To compare the daily rate of gastro-intestinal secretion of total IgA, IgM, IgG, albumin, A1AT and ECP in different groups of children.

Patients

Groups 1-3 have been described in more detail in chapter 3.

1. Twelve controls consisting of the the eight disease controls plus four who underwent whole gut lavage as treatment for constipation. The four children treated for severe constipation were excluded from the IgA calculations as they were found to have very low levels of total IgA as discussed in chapters 6 and 7. Their remaining results were within the range of the remaining control children and so have been included.
2. Seventeen children with cystic fibrosis (see chapter 8 for description). In this chapter I have included the first lavages from all 17 children and excluded any repeat lavages.
3. A total of 40 Sierra Leonean Children who completed WGL as part of study into their gastro-intestinal mucosal immunity and inflammation. Twenty five of these children have had some of their results published (Hodges *et al* 1994).
4. Sierra Leonean children with acute watery diarrhoeal disease:

These children were recruited from the National Rehydration Training Centre, Freetown, Sierra Leone in collaboration with Dr L H Kabba. This unit works as a casualty for all children in Freetown, although most come from the local area. The majority of the children attending have acute diarrhoea. The patients that I recruited had acute watery diarrhoea, bloody diarrhoea was excluded because of the problems of interpreting results due to the presence of blood. Ethical permission was approved by the Sierra Leone National Ethical Committee.

Details Recorded

The child was weighed and the length measured. From these figures the weight-age Z score, height-age Z score and the weight-for-height Z score were calculated using Anthro, an anthropometric software package (World Health Organisation, 1992).

In addition to the anthropometric data the children were examined by myself and details of the length of the diarrhoea prior to presentation were recorded.

Specimen Collection

Specimens were collected while the mother was rehydrating the infant. The child was settled in the mothers lap, over a receptacle for the collection of the acute watery diarrhoea. If the child urinated in the receptacle, then that specimen was discarded. As all the children had frequent watery diarrhoea the time taken to collect a specimen was usually less than one hour.

Once collected the diarrhoeal specimens had processing agents added immediately as in whole gut lavage specimens, but prior to filtering. Specimens were then transported across the centre of Freetown on ice, to the St Andrew's Clinic for Children, where they were centrifuged, filtered and aliquotted. Unfiltered, unprocessed (UF/UP) specimens were also collected for haemoglobin estimation and microbiological investigations. One aliquot had formalin added to allow microscopy for ova, cysts and parasites.

Diarrhoeal Output

The measurement of diarrhoeal output was performed using pre-weighed disposable nappies. Once a specimen had been collected a nappy was placed on the child and urine was collected separately by placing a second piece of nappy over the urethra of the child.

The time that the nappy was put on and removed was recorded and the nappy was reweighed after removal. The increase in weight was the weight of the diarrhoea. As all the specimens did not include any solid matter and were pure fluid I have taken 1 gramme of this weight to be equal to 1 ml of diarrhoea for calculations of outputs. One attempt at recording the output failed because the aunt of the child kindly washed the nappy immediately after removing it.

Methods

Total IgA, IgG, IgM, A1AT, Albumin and ECP (filtered/processed) were analysed in these specimens.

The specimens were tested for rotavirus antigen by staff at the Regional Virus Laboratory, City Hospital, Edinburgh under the guidance of Dr Burns, consultant virologist. Culture and microscopy of the stools were performed at the microbiology laboratories at the Western General Hospital, Edinburgh with the assistance of Dr Masterton, consultant microbiologist and his staff.

One aliquot was freeze dried to establish the water content of the fluid.

Results

On my first trip to Freetown six single specimens, without outputs, were collected from children to establish the feasibility of the collections. On my second trip a further 14 specimens were collected including the outputs.

Children

The African children with diarrhoea had a median age of 12 months. Their median weight was 6.7 kg and the WAZ -2.0, HAZ -1.4 and Ht/Wt Z -2.0. These are all lower than in the other groups of children (see table 3.2). One child was marasmic, one had kwashiorkor on examination.

Diarrhoeal Output

The median duration of diarrhoea prior to presentation was 2.5 days, the maximum being 12 days. Acute diarrhoea was defined as < 14 days.

The median length of time over which the diarrhoeal output was measured was 3.4 hours with an interquartile range of 2.6-4.2 hours. Most of the children arrived in the morning and returned home later the same day after rehydration.

The median estimated output of diarrhoea was 995 ml/day or 154 ml/kg/day.

Microbiology

In only two specimens were pathogens confirmed, both had rotavirus. The remainder had no evidence of parasites or bacteria. The bacterial cultures performed in Edinburgh do not specifically identify enteropathogenic E Coli, a common cause for diarrhoeal disease in children in tropical countries. There is no laboratory in Sierra

Leone currently culturing faecal specimens for the diagnosis of bacterial infections and the specimens taken back to Edinburgh for culture had been frozen. Thus it is not possible to make any conclusions about the pathogenic organisms except that rotavirus was present in two of the children. Any future studies looking at the immune response to particular organisms would need to clarify in more detail the causative organisms.

Water Content

The median water content was 95.6%, ranging from 89.6-98.8%.

Concentrations of Immune Substances in Watery Diarrhoea

The absolute concentration of substances in the diarrhoeal fluid shows a wide range in all the parameters as demonstrated in figures 11.1-11.6. IgG, A1AT and albumin have both high and low levels suggesting that some of the children have significant plasma leakage and protein loss. One child had no IgA or IgM in her stool. The children with rotavirus, marasmus and kwashiorkor did not show any distinctive results when compared with the group as a whole.

Results: Daily Output of Substances

Tables 11.1, 11.2 and figures 11.7-11.12 show the daily secretion rates of the substances in the four groups of children that I have described above plus the data from previously published studies in faeces and balloon perfusion systems as discussed previously.

The labelling of the figures are CF = Cystic Fibrosis Group, SL Lav. = Healthy African children (who had WGL), SL Dia. = Sierra Leone Children with diarrhoea.

The graphs (figures 11.7-11.12) are box and whisker plots showing the median as the line within the box, the box showing the interquartile range and the whiskers demonstrating the full range.

Figures 11.1-11.6: Concentration of Substances in Acute Watery Diarrhoea.

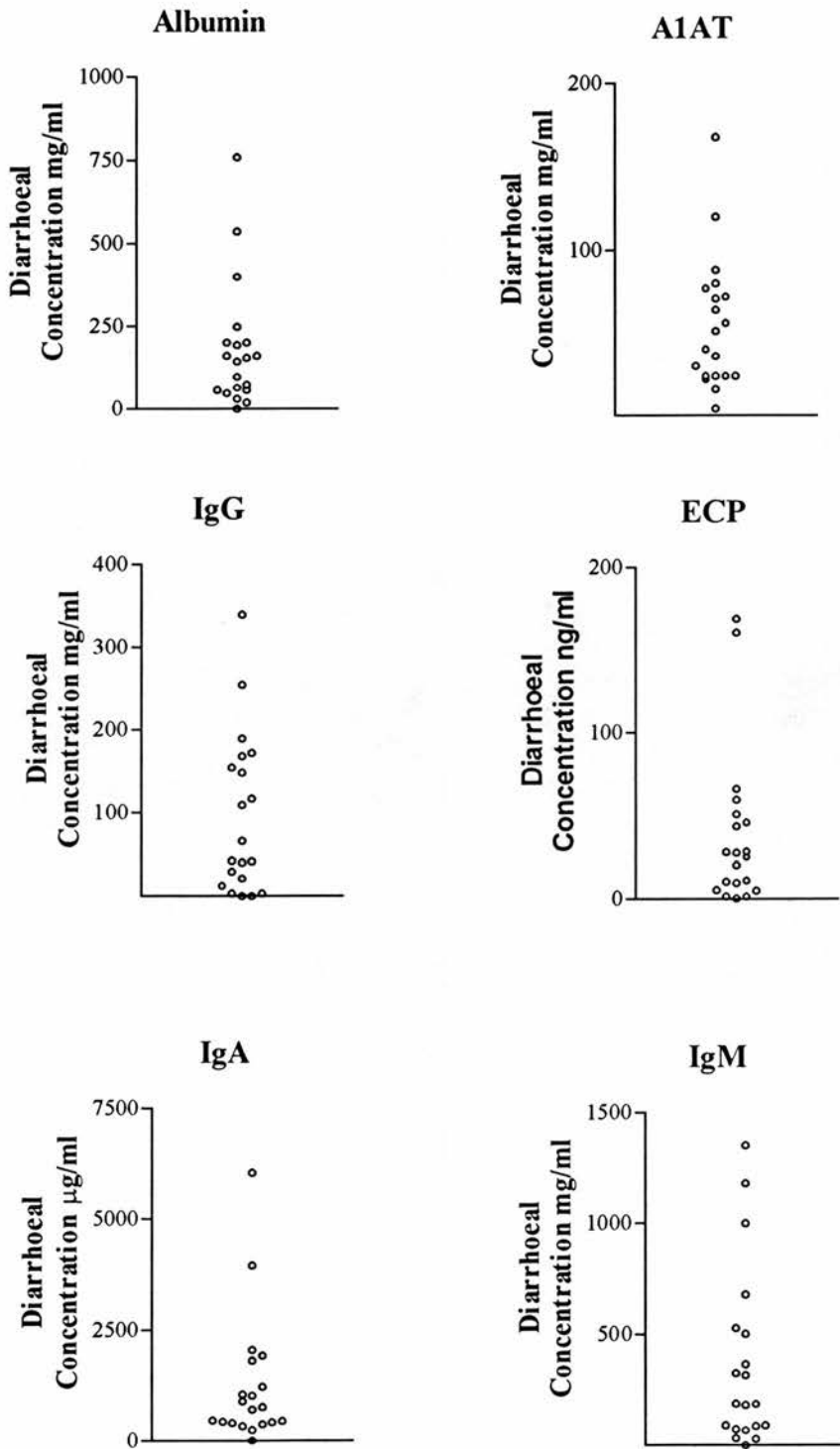


Table 11.1: Studies from which estimations of daily intestinal output of total IgA, IgM, and IgG have been made. Individual studies are described above

TYPE OF SPECIMEN	STUDY	TYPE OF PATIENT	Output per day		
			IgA mg/kg	IgM mg/kg	IgG mg/kg
Whole Gut Lavage	(Sallam, 1995)	Healthy Volunteers	32.5		
	Croft Thesis 1996	Control + Constipated (Children)	35.4*	0.9	0.65
		CF Children	58.0	2.1	1.9
		African Children (lavages)	45.9	2.1	1.0
Watery Diarrhoea	Croft Thesis 1996	African Children (watery diarrhoea)	80.7	21.7	6.5
Faecal	(Meillet <i>et al</i> 1987)	Control	0.9		
		Chronic diarrhoea	4.1		
	(Kapel <i>et al</i> 1992)	Control	0.04	0.02	0.02
		Inactive Crohn's	0.22	0.03	0.04
		Active Crohn's	3.0	0.05	0.10
	(Northrup <i>et al</i> 1970)	Active UC	2.9	0.05	0.14
Cholera		23.8	2.6 (17/25 ND)	0.8 (5/15 ND)	
Single Balloon Perfusion	(Jonard <i>et al</i> 1984) plus (Prigent-Delecourt <i>et al</i> 1995)	Control	31.9	3.1	5
Double Balloon Perfusion	(Lavö <i>et al</i> 1992b)	Control	40.3	17.1	
		Coeliac	70.9	30.5	

* Excludes four constipated children who had very low IgA results.

ND = Not detected

Table 11.2: Studies from which estimations of daily output of A1AT, Albumin, and ECP have been made. Individual studies are described above.

TYPE OF SPECIMEN	STUDY	TYPE OF PATIENT	Output per day		
			A1AT mg/kg	Alb mg/kg	ECP µg/kg
Whole Gut Lavage	(Sallam, 1995)	Healthy Volunteers		2.2	
	Croft Thesis 1996	Control + Constipated Children	2.6	3.0	8.3 (UF) 1.5 (Fi)
		CF Children	2.8	10.2	22.3 (UF) 3.9 (Fi)
		African Children	2.0	3.5	2.8 (Fi)
Watery Diarrhoea	Croft Thesis 1996	African Children	6.1	24.9	3.0 (Fi)
Faecal	(Kapel <i>et al</i> 1992)	Control	0.57		
		Inactive Crohn's	2.78		
		Active Crohn's	4.91		
		Active UC	3.22		
	(Bernier <i>et al</i> 1978)	Control	0.16		
		Active IBD	4.94		
(Strygler <i>et al</i> 1990)	Control	0.73			
	Induced Diarrhoea	1.50			
Single Balloon Perfusion	(Jonard <i>et al</i> 1984) plus (Prigent-Delecourt <i>et al</i> 1995)	Control		18.6	
	(Hallgren <i>et al</i> 1989)	Control		28.2	15.4
		Crohn's (Mean CDAI: 211, endoscopy: normal mucosa)		24.4	32.3
		Active Coeliac Disease		32.2	132.8
Double Balloon Perfusion	(Knutson <i>et al</i> 1989)	Control		84.9	
	(Lavö <i>et al</i> 1992a)	Control		79.8	4.3
	(Knutson <i>et al</i> 1993)	Control		66.3	11.3

UF = Unfiltered specimens, Fi = Filtered specimens.

Immunoglobulin A Secretion (figure 11.7)

These results are the first to estimate daily intestinal secretion rates of IgA in children.

The control children have a very similar total IgA daily secretion rate (35 mg/kg/day) to those estimated by both whole gut lavage and balloon perfusion systems in adult healthy volunteers/controls.

The daily secretion rates in WGLF were significantly lower in control children than those with cystic fibrosis ($p < 0.05$) but not compared with healthy African children ($p = 0.26$). The secretion rates were also lower than in African children with acute watery diarrhoea ($p = 0.01$).

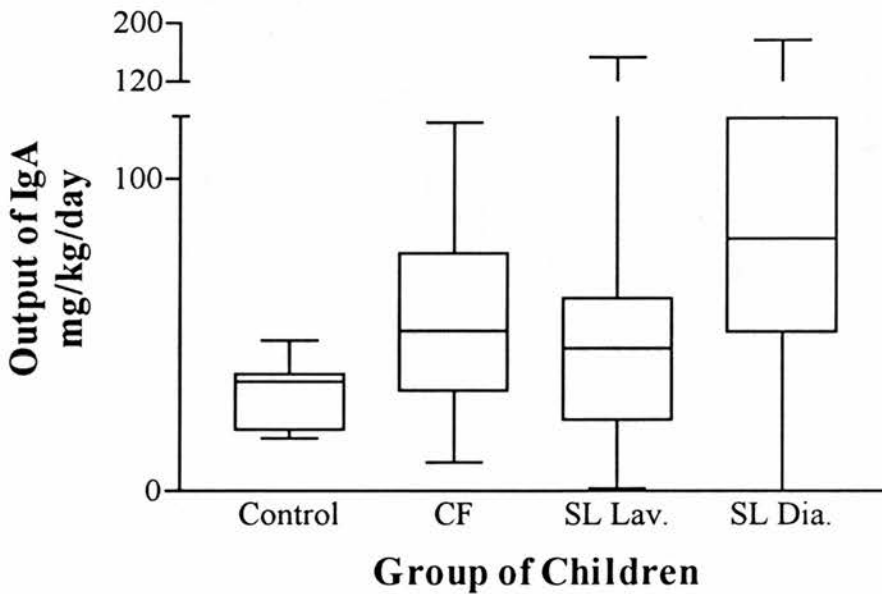


Figure 11.7: Daily output of total IgA in children

There was no significant difference between the output of IgA in the CF children or healthy African children.

The output measured in acute watery diarrhoea was significantly higher than the healthy African children ($p = 0.03$) as well as the UK control children, but not the children with CF ($p = 0.15$).

Immunoglobulin M secretion (figure 11.8)

IgM secretion in control children was estimated at 0.9 mg/kg/day. This was not significantly different to WGLF from CF children or African children. However IgM secretion was significantly increased in African children with watery diarrhoea at 21.7 mg/kg/day compared with all three WGLF groups.

Compared with previous studies, as shown in table 11.1, the children who underwent WGL had higher levels than had been measured in faeces and similar levels to that found in the single balloon perfusion system. However the double balloon perfusion system seemed to estimate much higher levels of daily IgM secretion. As this is only over a 10 centimetre distance it is possible that the area perfused has higher numbers of IgM plasma cells than in the rest of the GI tract, leading to an overestimate of the secretion rate into the whole gut.

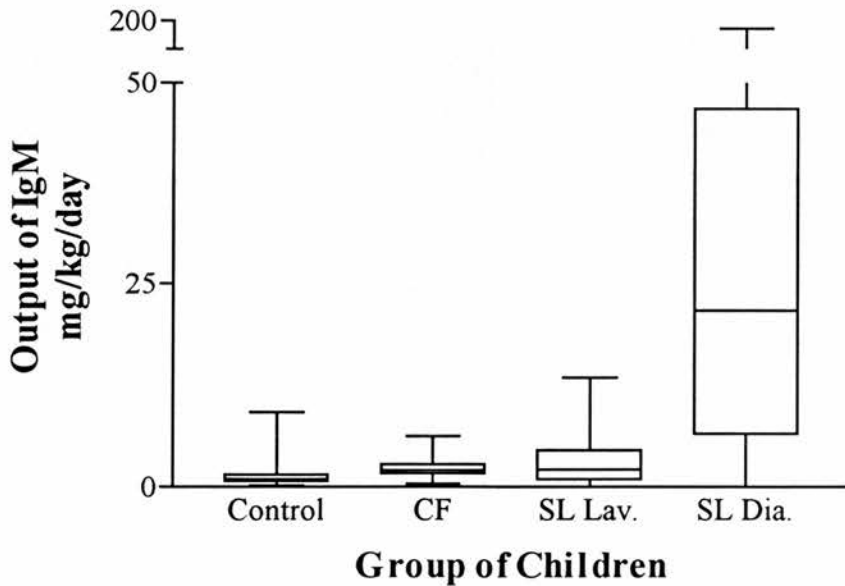


Figure 11.8: Daily output of IgM in children

Immunoglobulin G (figure 11.9)

Daily secretion of IgG in control children was 0.65 mg/kg/day, lower than that found in the CF children ($p < 0.05$) but not significantly different to WGLF from African children. Watery diarrhoea showed significantly increased levels of IgG over the control, CF and African lavage specimens suggestive of increased plasma leakage.

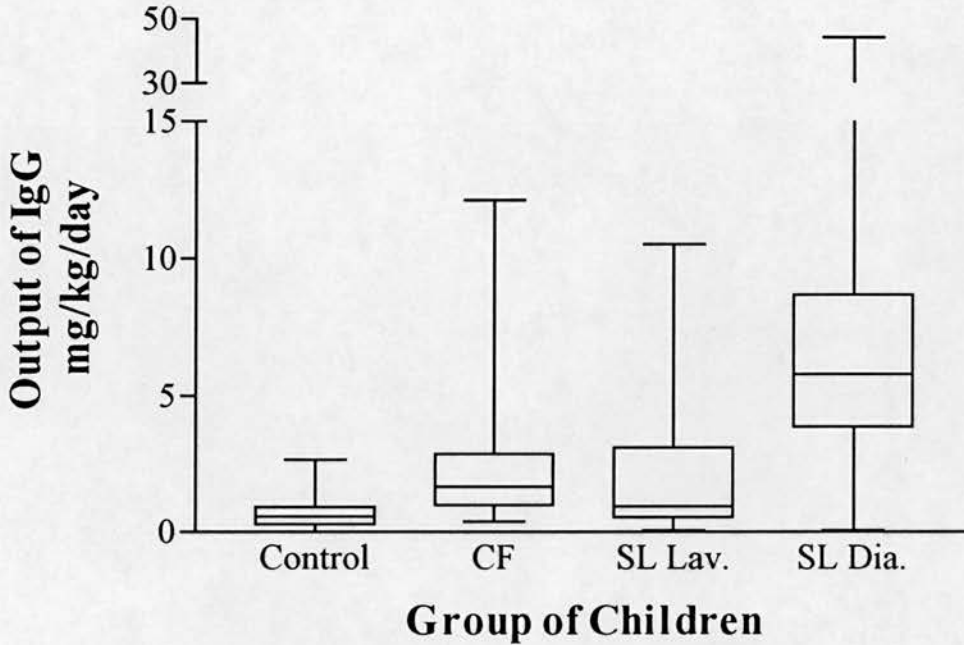


Figure 11.9: Daily output of IgG in children

Albumin (figure 11.10)

The daily output of albumin in control children was similar to that found in adults who underwent WGL. However the rates when compared with balloon perfusion systems, were considerably lower. This cannot be solely explained by the presence or absence of degradative enzymes, as the cystic children who are pancreatic deficient, also had lower levels than the perfusion systems, although higher than the control children. Albumin output was significantly increased in the children with acute watery diarrhoea.

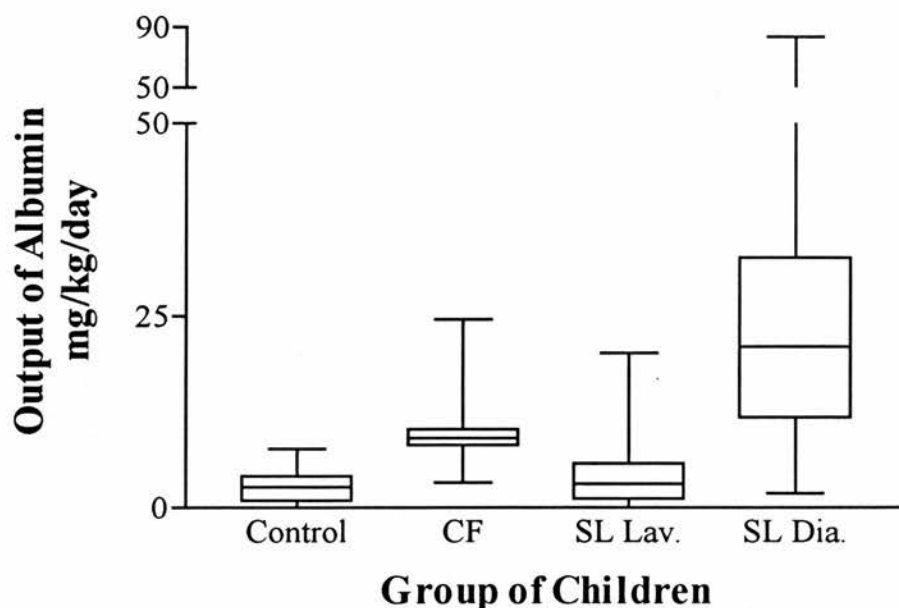


Figure 11.10: Daily intestinal output of albumin in children

A1AT (figure 11.11)

A1AT secretion in children gave very similar results to those found in previous studies estimating A1AT output in faeces. This would be consistent with A1AT being resistant to degradation in the GI tract.

As is the case for albumin the daily output was increased in the SL diarrhoea groups over all the other three groups. There were no other significant differences between the three groups.

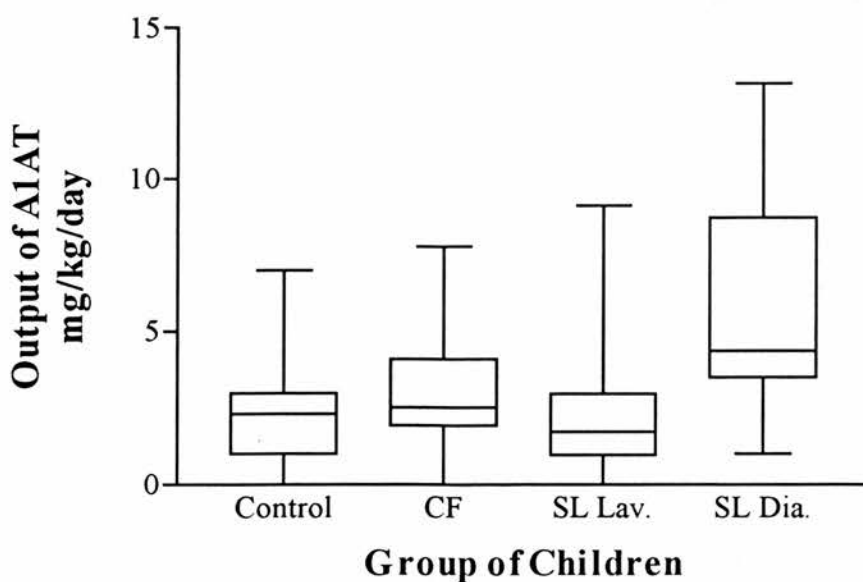


Figure 11.11: Daily output of A1AT in children

ECP

To compare the results in control children with the data from previously published studies it is necessary to use unfiltered specimens as filtering reduces the measured ECP. As shown in table 11.2 previous estimates in adult controls are 15.4, 4.3, 11.3 $\mu\text{g}/\text{kg}/\text{day}$, a similar range to the 8.3 $\mu\text{g}/\text{kg}/\text{day}$ that I have estimated in unfiltered WGLF from control children. The CF children show significantly higher levels of ECP output than control children for both unfiltered (22.3 $\mu\text{g}/\text{kg}/\text{day}$) and filtered (3.9 $\mu\text{g}/\text{kg}/\text{day}$) specimens.

The African children who underwent whole gut lavage only had filtered specimens collected and so for comparison of these children I have calculated the rate of output of filtered ECP for all the children both for statistical analyses and figure 11.12 below.

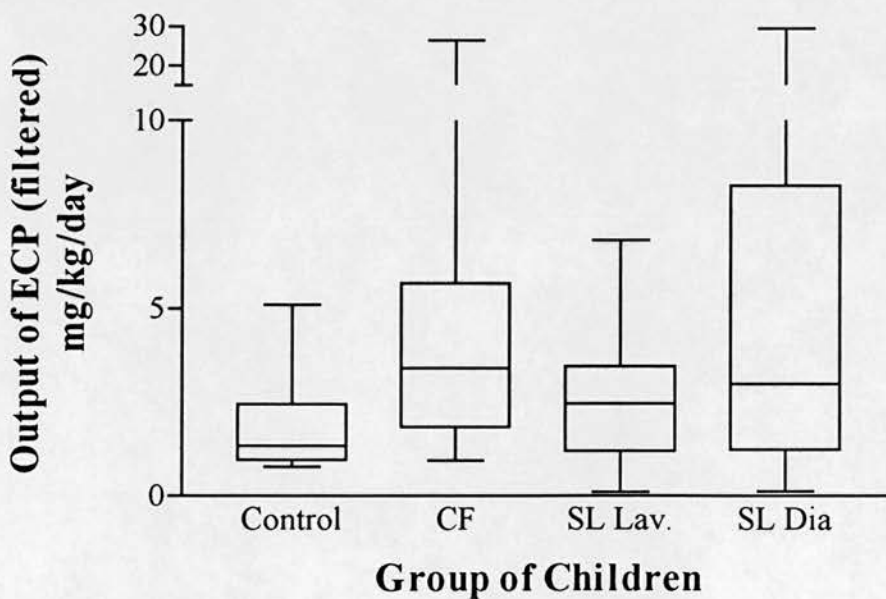


Figure 11.12: The daily output of ECP (filtered or 'free') in children.

The only significant difference was between the control children and the children with cystic fibrosis. Other than this there were no statistically significant differences found between these groups.

Discussion

These are the first estimates of the daily intestinal output of immune factors from children and has shown that the output of IgA expressed per kilogramme in control children is similar to that found in whole gut lavage and perfusion systems in adults.

Whole Gut Lavage Output in Children

IgA secretion into WGLF in UK control children is significantly lower than children with cystic fibrosis but not children from Sierra Leone.

This could be explained by a generalised up-regulation of mucosal IgA secretion in children with CF. It is also possible that the CF children have reduced breakdown of total IgA due to their pancreatic insufficiency which could lead to the higher estimated output of IgA compared to pancreatic sufficient, control children. However the fact that the adults and children who underwent whole gut lavage had very similar outputs of total IgA to studies using balloon perfusion systems (which exclude pancreatic secretions) would be suggestive that the presence or absence of pancreatic enzymes do not significantly affect the measured outputs.

I had expected the African children to have had a higher output of total IgA than UK control children, due to their constant exposure to intestinal pathogens, however this has not been confirmed. The rate of output was higher in the Sierra Leone specimens but not to a significant degree. There were no other significant differences between the UK control and African children who underwent WGL.

Compared with CF children, controls have lower ECP, albumin, and IgG which have been discussed in chapter 8.

Children with cystic fibrosis also show a significantly increased level of albumin over Sierra Leonean children. This could be due either to the lack of pancreatic enzymes or the presence of intestinal inflammation in the CF children. Alternatively if African children had significantly lower serum albumin than the UK controls due to dietary differences then one could expect the albumin output in these children to be reduced.

As is shown in table 3.2 these African children have lower weight for age and weight for height Z scores suggestive of worse nutritional status.

Output in Watery Diarrhoea in African Children

The Sierra Leone diarrhoeal patients show an increase in all the parameters except IgA and ECP over the other three groups. IgA output was significantly increased compared with the UK control children and the SL lavage children.

The median % water in these diarrhoeal specimens was 95.6%. The figures from ileostomy extracts consisting of >92% water in chapter 10 suggests that the recovery of IgA in diarrhoeal fluid compared with what would be expected in WGLF would be 39.9%. Thus if WGL had been performed in these children the IgA secretion rate may be up to 2.5 times the outputs measured here. Equivalent figures for other parameters were IgM 69.3%, albumin 13.4%, IgG 21.3%, and A1AT 82.1%. Thus all these differences are, if anything, minimised by using diarrhoea (as opposed to WGLF) as the means of assessing output of immune substances. What is clear is that total IgG, IgM, albumin, A1AT, and IgA have increased outputs in this group of children with acute watery diarrhoea. IgA is also increased over UK control children but has not been proven to be increased when compared with CF children who underwent WGL. The ideal situation in the children with acute diarrhoea would have been to collect whole gut lavage fluid, however this would not be ethically or practically possible as half of the children had concurrent symptoms of vomiting.

It was disappointing not to collect watery diarrhoeal specimens from children in the UK. This could be feasible in a community based study seeing the children early on in their illness, as most of these children are managed at home with oral rehydration.

Conclusions

In this chapter I have shown significant differences in the daily secretion rates of substances between UK control children and children with cystic fibrosis. Surprisingly there were no significant differences between UK control children and the Sierra Leone children who underwent WGL, effectively Sierra Leone control patients.

The increases shown in African children with acute watery diarrhoea over both Sierra Leone children and UK control children are almost certainly minimised by not being able to use WGLF as a technique to investigate them in the acute phase. Increases in IgG, albumin and A1AT suggests an increase in intestinal mucosal inflammation and plasma leakage in acute watery diarrhoea. The markedly raised IgM output in these children is likely to be part of the immune response to the pathogens causing the symptoms.

Recording output of substances over a fixed period in materials from the gut allows direct comparison between different types of specimens. It is essential to be able to discuss differences knowing how the different intra-luminal states can affect secretion or degradation rates of the substances being assayed.

Chapter 12: General Discussion and Conclusions

Introduction

In this thesis the major aim was to study the use of whole gut lavage for research into gastrointestinal mucosal immunity and inflammation in children. In the first section evidence is presented to support an argument that this technique is ethical and can be used for this purpose. The study on the children with cystic fibrosis has shown interesting results, suggestive of intestinal mucosal inflammation, which is now under further investigation. As whole gut lavage is used for clinical reasons, collection of specimens for research into specific illnesses such as inflammatory bowel disease is feasible. However the use of whole gut lavage for research purposes should only be done in centres with appropriate expertise. As with any research in children these studies should only be performed if they cannot be undertaken in adults and if the problem being addressed is an important problem for children.

As my experience of whole gut lavage in children increased I was aware that this could not be used to study certain illnesses, in particular acute gastroenteritis, where the mucosal immune system is of fundamental importance. The hypothesis that diarrhoea may be more representative of what can be measured in WGL than normal faecal material led to the work described in the second part of the thesis where the output of substances in faecal material was compared with whole gut lavage.

Practicalities of WGL

The most important aspect regarding practical details of WGL in children was that the lavages were prolonged and the children participating became bored and hungry. As the study progressed, the time taken for the lavage to be completed tended to decrease, almost certainly because of initial caution with the rate of administration of the fluid to minimise side effects. As both I and nursing staff on the wards in the RHSC, Edinburgh were relatively inexperienced with the use of WGL in children this confirmed how important familiarity with the use of this technique is. Based on my experience of WGL I have written a sheet of instructions for use on wards.

It was of great interest to see the reduced duration of WGL in children from Sierra Leone, thus making the technique acceptable and feasible for studies in similar countries in the developing world. It was clear that these children took less fluid (per kg) to acquire clear specimens than the UK children, which is likely to be due to the different dietary and bowel habits in West Africa. As the relationship between nutrition, mucosal immunity and diarrhoeal disease is far from established but is highly relevant to the understanding of the problems of diarrhoeal disease this could prove to be a very important technique. A paediatrician from Bangladesh based in the GI laboratory at the Western General Hospital in Edinburgh is currently setting up studies of mucosal immunity in the International Centre for Diarrhoeal Diseases Research Institute in Dhaka.

The proposed study comparing whole gut lavage with conventional treatment for inpatient treatment of severe constipation did not succeed as the consultants in charge of the children requested that they received WGL as the preferred treatment. This was not unreasonable as there is published evidence that WGL is a successful and practicable treatment. The question as to whether WGL is a better treatment than the currently accepted methods remains unanswered. However this method succeeded in all the children, the oldest of which preferred WGL to previous treatments which included enemas. A proportion of these children had excellent long term results, having previously failed with conventional therapy.

Analyses of WGLF in Control Children

At the outset specimens were collected from children undergoing WGL for clinical reasons, mostly prior to colonoscopy, who did not have diagnoses made at the time of the colonoscopy and were intended as controls. Of the pre-colonoscopy specimens three children had results that were outwith both the adult normal range and the range of the control children as a whole. One of these three children has since been confirmed as having ulcerative colitis, one has problems strongly suggestive of Crohn's disease and the last was an infant with failure to thrive and a large cystic hygroma with a percutaneous gastrostomy in situ. Thus these disease controls,

although the mucosa was grossly normal at colonoscopy, may have had abnormalities elsewhere in the GI tract leading to high WGLF parameters. The remaining children presented with rectal bleeding and either had rectal polyp or nothing abnormal detected. This latter group's WGLF parameters were similar to adult healthy volunteer data. Without collecting specimens from healthy, normal children these would be the most appropriate children to collect specimens from to use as 'normal' data in future studies.

Five specimens were collected from four children with severe constipation who had very low total IgA levels in their initial WGLF. This was an unexpected finding, most likely to be as a consequence of the specimens collected not being entirely clear of all faecal material. One of these children had a repeat lavage later in the study which showed a normal concentration of total immunoglobulin. The remainder of their WGLF results were within the range of the control children and adult data. The possible reasons for faecally contaminated specimens having low total IgA levels remains unknown, this will be discussed further later in this section.

Children with Cystic Fibrosis

In the children with cystic fibrosis the original hypothesis was that children taking high-dose pancreatic enzyme supplements would have raised WGLF parameters suggestive of mucosal inflammation and immune up-regulation, which would return to normal when the children changed to the low-dose enzyme supplements. Although the CF children (as a group) had significantly raised total IgG, albumin, ECP, IL-1 and IL-8 there was no reduction in the five children that had a repeat lavage after changing to the LDES, and thus HDES was not shown to be the cause for these abnormalities. These five children had lower concentrations as a group whilst taking HDES than the group of 12 on HDES and so it is possible that the change to LDES was less likely to reduce these parameters.

The pancreatic insufficient child who was not on any enzymes had similar parameters to the 12 who were on HDES, and the pancreatic sufficient child with CF had essentially normal parameters (although he did have a mildly raised IL-1). The data

from these two suggests that abnormalities in the original group of 12 were associated with the presence of pancreatic insufficiency but not necessarily with enzyme supplement usage.

Of the three children with distal intestinal obstruction syndrome two had abnormalities consistent with intestinal mucosal inflammation similar to that found in active inflammatory bowel disease. These two children were also the only two who were taking Nutrizyme 22, which has recently been implicated as the enzyme preparation with the highest risk ratio for developing fibrosing colonopathy. Whether this inflammation was secondary to DIOS, or the enzyme preparation, or a combination of the two is impossible to say. Further studies are needed to look for evidence of intestinal inflammation in children with DIOS. In centres who use whole gut lavage as a treatment for DIOS the collection of further samples from patients should be encouraged.

Why the concentrations of the parameters were raised in the original 12 children with CF is not known. Although abnormalities of the intestinal mucosa are the most likely there are two factors that complicate the interpretation of these results.

First is that swallowed respiratory secretions could have influenced the concentration of parameters measured in the WGLF. In order to minimise any sputum ingested during the WGL the parents were asked to perform the usual chest physiotherapy in the morning prior to attending the ward. The children were also asked to expectorate any sputum during the lavage. Briars *et al* studied faecal cytokines, particularly IL-8, in patients with cystic fibrosis, concluding that measuring IL-8 in faeces could be an indirect means of assessing cytokines in the lung (Briars *et al* 1995). Unlike Briars, in this thesis, there was no correlation between WGLF IL-8 concentration and Swachman scores, pancreatic enzyme dosage or pseudomonas colonisation. There was also no relationship between concentration of parameters in WGLF and X ray scores (looking at the severity of the lung lesions) or the usage of antibiotics (whether none, oral or intravenous). Of the five children receiving intra-venous antibiotics at the time of the lavage three were within the range of the group of twelve children, the

other two had very high parameters. These two were also the two children with DIOS taking Nutrizyme 22. All these findings would be against respiratory secretions being responsible for the abnormal parameters in WGLF. In 8 of the children (unfortunately not the two with DIOS) I collected sputum at the time of the lavage and measured the concentration of IL-8 and ECP. Four other children were unable to expectorate when requested. Using these figures the weight of sputum that would have to be swallowed per hour to cause the levels detected in WGLF varied between 0.9 and 61 g/hour. The median amount per day was between 55 and 120 g/day which are excessive volumes for children to be producing.

Patients with chronic lung disease present a specific problem when attempting to directly assess secretion from the intestinal mucosa. The ideal situation is to exclude all respiratory secretions by the use of balloon perfusion systems. An alternative is to identify a substance that is measurable in the WGLF but is not present in corresponding sputum. Preliminary results in this thesis measuring RANTES in the sputum of these children has found none detectable in sputum but detectable levels were found in corresponding WGLF. Using a perfusion system such as WGLF allows rational assessment of the potential influence of these secretions, which cannot easily be made in faecal material or aspirates. Future studies where respiratory tract secretions may influence the assessment of intestinal secretory immunity (such as in specimens of faecal material, jejunal aspirates and WGL) should make provision for this by minimising the amount of secretions present and measuring the concentration of immune substances in secretions to allow estimation of the weight required to be swallowed to cause the measured concentrations in intestinal specimens.

The second complicating issue in these children is the absence of pancreatic enzymes. The mild increases in albumin and IgG in the group of children could be as a result of this deficiency, whereas raised ECP, which is stable in faecal material is unlikely to be explained by this lack of enzymes.

In conclusion these children have shown increased parameters suggesting intestinal mucosal inflammation and immune activation. Neither swallowed respiratory

secretions or reduced degradation due to pancreatic insufficiency is likely to be the cause. To confirm this further studies, excluding both pancreatic and respiratory secretions in subjects and controls, would have to be performed.

Faeces versus Whole Gut Lavage

Assessing IgA secretion in the GI tract

Four results in this thesis need to be linked together to discuss further what measuring total IgA outputs in faeces and whole gut lavage might really mean.

1. Comparing secretion rates as estimated in previously published studies of jejunal perfusion systems in adult control patients, generally accepted as the best method for directly investigating intestinal mucosal immunity, reveal very similar outputs to those measured in WGLF in control children and data in adult healthy volunteers.
2. In severely constipated children very low levels of total IgA in WGLF were thought to be related to the specimens collected not being clear of all faecal material. This finding in unclear WGLF specimens has previously been shown by O'Mahony et al in adult subjects.
3. The daily output of IgA measured in faecal material was < 1.5% of that measured in WGLF in adults without active inflammatory bowel disease, this rose to a median of 12.5 % (maximum 60 %) in adults with ileostomies. Thus the daily output of IgA measured in WGLF is of the order of 100 times that measured in non-diarrhoeal faeces and 8 times that measured in ileostomy material.
4. In ileostomy patients there was a trend suggesting that the higher the water content of the faecal material passed from the ileostomy, the higher the recovery of the immunoglobulin A when compared with whole gut lavage.

In this study a number possibilities have been examined or discussed that could explain the difference between the measured daily output of IgA in faecal and WGL material (taking WGL as 100%). Most of these could have some effect but none

explain the very large differences seen in the results comparing faecal and WGLF material.

Degradation of IgA in WGLF at 37°C leads to 80% of the original concentration at two hours, 44% at 8 hours and 23% at 24 hours. This cannot explain a recovery of 1% in faecal material if one assumes in these adults the transit time through the gut is approximately 24 hours. It also cannot explain the recovery of IgA being 12.5 % in adults with ileostomies who had a transit time of less than two hours where one would expect at least 80% of the IgA to still be present. Thus degradation is not the sole cause for IgA output in faecal material being so much less than in WGL.

Dilution of intestinal secretions by WGLF was proposed as an explanation for the low IgA results in very constipated children. The concentration of PEG in the resulting WGLF specimens was as predicted from previous studies in WGLF. If dilution had been responsible then one would have found very low PEG concentration in the resulting WGLF.

Hypotheses

My first hypothesis is that whole gut lavage (and other perfusion systems) actually stimulate the secretion of immunoglobulin A. Thus in unclear specimens the IgA measured is simply that already present in the bowel lumen. Subsequent specimens (including those clear of all faecal material) are collected at the time that secretion of IgA from the mucosa into the WGLF is maximal.

Why the secretion is stimulated is not known but may be associated with excess fluid in the lumen of the GI tract. This could explain the higher recovery rates found in specimens from patients with ileostomies compared with control adults, as well as the increased recovery found in ileostomy specimens with higher water content.

It would be important to know if in balloon perfusion studies whether during the first few minutes of the perfusion whether IgA secretion is maximal or if the secretion rate increases with time to a peak. If this were the case it would suggest that the IgA secretion is being increased due to the perfusion state.

An interesting study using single balloon perfusion systems showed that secretion of polymeric and monomeric IgA, IgM, albumin, IgG and secretory component was increased when using an elemental diet as the perfusate (consisting of amino-acids, glucose, maltose and oligosaccharides) compared with a control solution (NaCl, KCl and mannitol) (Colombel *et al* 1992a). The pH and osmolality were equal in both solutions. The suggestion was that nutrients stimulate immunoglobulin secretion and this has been shown in perfused isolated loops of bowel in rats (Freier *et al* 1987). This demonstrates that it is likely to not just be rates of flow of fluid or water content that alter immunoglobulin secretion rates.

Alternatively there may be factors in the lumen that in the normal state inhibit the rate of IgA secretion but when reduced in concentration allow increased secretion of IgA. This would make sense in diarrhoeal disease as once the high water content of acute diarrhoea is present in the GI lumen, the concentration of the inhibitory factors is reduced and IgA 'floods' in and can suppress the pathogenic organisms whether viral or bacterial. This would be a non-specific response which would also be found in the situation of diarrhoea of non-infective origin.

Using Ussing chambers to measure IgA secretion with different physiological situations on the luminal side would allow study of some of these possibilities *in vitro*. There are no methods described that can truly recreate the physiological secretion of substances from the intestinal mucosa *in vivo* and allow collection of specimens for estimation of secretion rates. How to recreate the *in-vivo* physiological state of the intestinal lumen and be able to collect intestinal fluid secreted from a fixed area but excluding pancreatic enzymes is a problem that I cannot see being resolved. Isolating a loop of bowel from a mammal and collecting all secretions is a possibility, however the process of isolation and the absence of secretions from higher up the gastrointestinal tract would make this non-physiological.

An alternative might be the intra-venous administration of labelled dimeric IgA (including J chain). Although the majority of IgA is thought to be manufactured locally, if a fixed proportion of secreted IgA were to originate from serum, then after

intra-venous administration of labelled IgA the measurement of the secretion rate of the label in faeces, intestinal juice, perfusion fluid or whole gut lavage, and the ratio of this labelled IgA to non-labelled IgA, could allow alternative estimates of the secretion rate. A study by Jonard has shown that after administering intra-venous radio-labelled dimeric IgA (without secretory component), a proportion (< 2 %) of IgA in jejunal perfusion material (from non-inflamed gut) originates from serum (Jonard *et al* 1984). If the differences in the measured daily output of IgA in faeces and WGL were not due to increased secretion during WGL (but were due to degradation), then output of the labelled IgA ought to be the same in both systems. By using a label that is stable then the problems of degradation of IgA are eliminated as it would be the label that is measured rather than the IgA molecule. When doing this it would be essential to confirm that the GI tract was not inflamed as the presence of radio-labelled IgA passing through inflamed areas directly from the serum would invalidate this method.

A second hypothesis is that IgA is recycled within the large bowel. In this situation during both WGL and the diarrhoeal state, reduced amounts of IgA are reabsorbed from the lumen of the large bowel and thus the output in faecal or other material increases. The study where the colon was perfused with a proximal balloon demonstrating IgA secretion would make this unlikely as one would expect the vast majority of the IgA to be reabsorbed within the lumen. Using the balloon perfusion system in the large bowel and labelled IgA (in the perfusion fluid) one could study this by looking for the appearance of labelled IgA in the serum of the subjects. Ideally one would perform the perfusion both in the small and large bowel and would expect to find labelled IgA in the serum from the large bowel perfusion but not from the small bowel study.

Antibody secretion in the GI Tract

From the point of view of interpreting previous studies of faecal material this thesis shows the error of comparing concentrations of IgA in diarrhoeal and non-diarrhoeal

stools. Of particular importance is the finding that specific IgA antibodies can be undetectable in faeces from adults without diarrhoea whereas they are present in the same patients in whole gut lavage. Interpretation of results based upon the absence of antibodies in this type of specimen is based upon incorrect assumptions.

On the other hand the fact that antibodies were always detectable in ileostomy fluid when also detectable in WGLF suggests that, in certain cases, diarrhoeal stool may be a suitable means to look for the presence or absence of antibodies. When essential to look for the presence of faecal antibodies in subjects without diarrhoea it would be possible to stimulate diarrhoea in a non-invasive way in order to collect suitable specimens for analysis.

Output of IgM, Albumin, A1AT, and IgG in the GI Tract

Immunoglobulin A is the major secretory substance in the GI tract, in this thesis the difficulties of interpreting results measuring IgA and IgA antibodies in intestinal specimens has been looked at in some detail. Other substances (IgM, albumin, A1AT, IgG) measured both in faecal material and WGL also revealed much higher outputs in the latter. In the children with severe constipation, who had very low total IgA levels, the remaining parameters such as albumin, A1AT, haemoglobin, IgM, IgG were all within the adult normal range (as well as the remaining control children). Further work to establish the reasons for these differences needs to be performed.

Measurement of Substances in Diarrhoeal Specimens

I have used adult volunteers with ileostomies as a model to assess the recovery of immune substances in diarrhoeal specimens when compared with WGL. The short transit time within the gut lumen and high water content in ileostomy effluent make these two situations similar.

Chapter 10 discusses evidence that when comparing output (over a fixed period of time) of IgA, IgM, IgG, albumin, A1AT, and IgA antibodies in faeces/ileostomy effluent the output measured is lower than in the situation of whole gut lavage (see table 10.8). It is unlikely that subjects with diarrhoea would have a greater recovery of

substances (compared with WGL) than adults with ileostomies, if anything the recovery will be lower. Thus it is reasonable to assume that if diarrhoeal material records a higher output of one of these substances (e.g. IgA) when compared to WGL this is a true finding and not simply a technical difference between the type of specimen. Conversely if WGL has higher outputs of these substances than ileostomy, normal faecal or diarrhoeal material then no conclusions can be made.

Acute Watery Diarrhoea in Sierra Leonean Children

The very raised output of IgM, plus increases in the output of IgG, albumin, A1AT in acute watery diarrhoea suggest a combination of immune activation and increase in plasma leakage as a group. Further studies are now required to look in more detail at the pathogens involved so that the pathophysiology of the diarrhoeal illnesses can be better understood.

Eosinophil Cationic Protein

In the children with cystic fibrosis the raised levels of ECP suggest an increase in intestinal mucosal eosinophil activation which accords with the previous findings of eosinophils in pathological specimens. The importance of this in the setting of enzyme treatment is not known.

There was no evidence of eosinophil activation in African children with acute watery diarrhoea, suggesting that eosinophils are not of pathophysiological importance in these patients.

Final Conclusions

The first part of this thesis shows that whole gut lavage can be a useful means for studying intestinal secretory immunity and inflammation in children, however using this technique would not be feasible in all gastrointestinal diseases where the mucosal immune system is of fundamental relevance.

Interpretation of immunological analyses of faecal material needs to be done cautiously with every effort being made to ensure that control specimens are truly

comparable with the specimens under investigation. In certain situations, such as looking for the presence or absence of specific antibodies, diarrhoeal specimens may be a useful means to investigate the intestinal mucosa.

As an alternative to faecal material, whole gut lavage has the advantage that the same intra-luminal state can be achieved in all patients. Thus direct comparison of results between the patients is feasible. This is not the case when comparing faecal outputs of immune and inflammatory substances between subjects with and without diarrhoea.

Appendix 1: Lavage for severely constipated children: Information for ward staff

Lavage is a widely used safe procedure for bowel preparation in both adults and children. A number of children's hospitals already use lavage in the treatment of constipation but no-one has shown it to be better or worse than conventional treatment including enemas etc. The solutions used, either Klean-prep or Movie-Col are essentially the same except for differing flavours. They do not cause any significant alterations to the patients biochemistry or cardiovascular system. The main potential problems are nausea, bloating and occasionally vomiting. These settle with slowing the rate of administration.

Details of Admission:

- **PLACE:** If possible the patient (especially the older ones) should be admitted to a cubicle or easily screened off area with a bedpan/commode available once they start passing stools. During the lavage it is best to encourage the child to be as mobile as is feasible (partly to keep them from getting too bored).
- **TIME:** As the lavage in severely constipated children takes a long time (in one study a mean of 11 hours) it is best to start in the early hours of the morning. Thus admit the patient in the evening prior to the lavage. Pass an naso-gastric tube and start the lavage at say 0500. The children usually do not pass any stool for at least three hours after starting the lavage, depending on how fast it is given.
- **MEALS:** They should have a light evening meal and no further solids until the lavage is completed.
- **ORAL FLUIDS:** Small sips of fluid before and during the lavage are permissible. It is not necessary to push fluids during the procedure as they are not being dehydrated.
- **DRUGS:** All should be continued as normal except on the day of the lavage. Any given on the day are likely to have their absorption significantly affected (most likely reduced). Thus unless essential it is probably best to omit them. Oral metoclopramide can help adults tolerate the procedure (given either before or during) and may be given.
- **ROUTE:** Even in the most motivated children the amounts to drink are too large and we have found that **ng tube** is necessary.
- **METHOD OF ADMINISTRATION:** The best method in adults seems to be giving the fluid as half hourly boluses, however given continuously via a Kangaroo pump/IVAC is more feasible on a busy ward. The problem with this is that the

required rate of administration is sometimes faster than the pump can cope with and extra should be given.

- **RATE:** The fluid should be given as fast as the child can tolerate it. This is because the faster it is given the quicker the lavage will be completed. However if the fluid is given too fast in the early stages the child will vomit thus slowing the end result. We tend to start at about 10-15 ml/kg/hr for the first 1-2 hours then increase up to 20 ml/kg/hr (in younger children with an upper limit of 500 ml/hour).
- **OBSERVATIONS:** The most important problems to be aware of are those of nausea, abdominal distension or vomiting. Numerous studies have shown no significant effect on biochemistry although we do tend to check these before and after the procedure. We have had one child aged 10 months who developed asymptomatic hypoglycaemia towards the end of a prolonged lavage.

1 hourly:	abdominal girth symptoms
2 hourly:	pulse rate BP BM stix (under 18/12)

Abdominal girth seems to be a reasonable way to assess the possibility of vomiting. All the children over the first few hours tend to gradually increase their girth and the abdomen becomes quite firm to palpation. They then start to pass stool and once they do start it is worth increasing the rate to try to complete the lavage as soon as is possible. However if they have not opened their bowels we would generally slow (or stop) the infusion for up to 1 hour if the girth increases by more than 15-20 % and the belly is very taut.

- **TIME TAKEN:** Severely constipated children take a long time to complete the lavage, a great deal depends on the **end point** aimed for (see below). Generally a time of 8-12 hours would not be unusual, however this may be reduced by increasing the rate of administration more aggressively once the child starts to pass stools. In some cases it is preferable to stop the infusion in the evening and then restart in the early hours of the next morning.
- **END POINT:** Once the effluent runs clear of all faecal material (clear yellow colour) you can be sure of completely clearing the child of all stool. Because of the length of time taken to achieve this, continuing the lavage until all palpable faecal masses have gone is an alternative (but less satisfactory).

Appendix 2: Reagents and Instrumentation

1. Reagents

WGLF Processing Reagents

- **SBTI** - Soya bean trypsin inhibitor. 0.1 g/100 ml PBS. SBTI (BDH Cat. No. 39043 SX).
- **PBS** - Phosphate buffered saline pH 7.2. 2 tablets (Sigma P4417) dissolved in 400 ml distilled water, pH adjusted to 7.2.
- **EDTA** - 0.3 M disodium ethylene diamine tetraacetic acid (BDH Cat. No. 10093) pH 8.0. 11.0 g/100 ml distilled water.
- **PMSF** - 0.1 M Phenylmethylsulphonylfluoride (Sigma Cat No P-7626). 1.74 g/100 ml alcohol.
- **Na Azide** - sodium azide. 2 g/100 ml distilled water.
- **NBCS** - Newborn calf serum. Sigma (Cat No N 4762 - heat inactivated).

In House ELISA Reagents

Plates

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

Coating antibodies

- **Goat antihuman IgG**: Fc specific, Sigma Chemical Co. (Cat No I2136)
- **Goat antihuman IgM**: μ chain specific, Sigma Chemical Co. (Cat No I2386)
- **Goat antihuman IgA**: α chain specific, Sigma Chemical Co. (Cat No I2261)
- **Gliadin**: gliadin extract (REKTOR) 1990
- **Ovalbumin**: (Sigma Cat No A5503)
- **Beta-Lactoglobulin**: (Sigma Cat No L0130)
- **Monosialoganglioside (GM₁)**: (Sigma) 1 mg/ml in 2:1 chloroform: methanol
- **Cholera Toxin**: (List Biologicals)

Conjugated antibodies - alkaline phosphatase conjugated

All made up in the appropriate diluent.

Goat antihuman IgM, Sigma Chemical Co. (Cat No A3437) 1/10000

Goat antihuman IgA, Sigma Chemical Co. (Cat No A3036) 1/10000

Goat antihuman IgG, Sigma Chemical Co. (Cat No A9544) 1/5000

Other Reagents

• **Sterile Water**

1 litre sterile water containers (Baxter Healthcare Code F7114).

• **Coating buffer**

Carbonate-bicarbonate coating buffer, 0.05M, pH 9.6 at 25°C. Dissolve the contents of 10 capsules (Sigma Chemical Co. Cat No C-3041) in 1 litre sterile 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 Cat No P-1379)

• **Adult Bovine Serum**

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

• **Diluent**

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

• **CT and *Salmonella Typhi* Diluent**

0.05% Tween 20 + 0.9% saline + 0.05% (w/v) bovine serum albumin

• **Diethanolamine (DEA) substrate**

To make 5 litres of DEA substrate:

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

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

1.0 g sodium azide (NaN_3)

4 l sterile water

2. Adjust the pH to 9.8 with 6N 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.

Albumin/AIAT Reagents

All reagents are obtained from Merck (BDH) unless otherwise stated.

- **PEG Reagent:** 40g polyethylene glycol 6000 (biochemical grade), 6g tris, 2g Tween 20 and 1g sodium azide dissolved in 800 ml distilled water, adjusted to pH 7.0 with dilute hydrochloric acid and made up to 1 litre volume with distilled water.
- **Diluent :** 9g sodium chloride, 60g PEG 3350 and 1g sodium azide in 1 litre solution distilled water.
- **Anti human alpha 1 antitrypsin** - goat: (Protein Reference Unit - Sheffield).
- **A1AT antibody reagent:** Dilute antisera 1 in 50 with the PEG reagent on the day of assay.
- **Anti human albumin (sheep):** (SAPU - code S034-205).
- **Albumin antibody reagent:** Dilute sheep anti human albumin serum 1 in 50 with the PEG reagent on the day of assay.

Haemoglobin Reagents

All reagents are analar grade and obtained from Merck (BDH) unless otherwise stated.

- **Oxalic Acid Reagent:** 4.0 g oxalic acid (analar) is made up to a 10 ml volume with distilled water, and dissolved in a water bath at 100C. 0.31 g FeSO₄ is added to the oxalic acid reagent which is returned to 100 C. 0.10 g uric acid and 0.11 g mannitol are then added with mixing, final volume adjusted to 10 ml and the reagent left at 100 C for 5 minutes. Any undissolved reagent is left to settle out. The supernatant is pipetted while hot in the fume cupboard. The reagent is prepared fresh before use.
- **Ethyl acetate/acetic acid 10/1 v/v:** Add 50 ml glacial acetic acid to 500 ml ethyl acetate in a fume cupboard.
- **3. 3M Potassium Acetate (294 g/l):** Dissolve 29.4 g potassium acetate (BDH GPR) in 100 ml distilled water. Store at room temperature.

- **4.3M Potassium Acetate in 1M Potassium Hydroxide (56 g/l):** Dissolve 147 g potassium acetate in 300 ml distilled water, add 28 g 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 distilled water (fume cupboard). When cool, make up to 500 ml with distilled water and add 58 ml glacial acetic acid.
- **PEG 4000 reagent:** Dissolve 60 g PEG 3350, 9 g NaCl, and 0.2 g sodium azide in 1 litre of distilled water. Use to make Drabkins reagent.
- **Drabkins Reagent:** (Sigma cat. no. 525-2) 6 vials.

Each vial contains 1 g sodium bicarbonate, 0.2 g potassium ferricyanide, and 0.05 g sodium cyanide.

Take 1 vial and reconstitute to 1 litre with PEG 4000 reagent.

PEG Assay Reagents (all BDH Biochemicals)

- **Acacia (gum arabic):** 9 mg/l distilled water
- **Barium Chloride:** 11.72 g BaCl₂.2H₂O per 100 ml distilled water
- **Barium Hydroxide:** 47.332g Ba(OH)₂.8H₂O per litre distilled water
- **Zinc Sulphate:** 5 g ZnSO₄.7H₂O in 100 ml distilled water
- **Trichloroacetic acid (TCA)/ BaCl₂:** 30 g TCA plus 5.86 g Ba(OH)₂.8H₂O

IL-8/RANTES Reagents

Included in the kit.

IL-8/RANTES Microtitre Plate

IL-8/RANTES Standard (recombinant human IL-8/RANTES), lyophilised

IL-8/RANTES-horseradish peroxidase conjugate

Wash buffer concentrate

Colour reagent A - stabilized hydrogen peroxide

Colour Reagent B - Stabilized chromogen (tetramethylbenzidine)

Substrate solution - equal volumes of colour reagent A and colour reagent B

Stop solution - 2N sulphuric acid

IL-1 β Reagents

All included in the kit.

Microtitre plates coated with monoclonal IL-1 β

Wash Buffer made up 1:5 with distilled water

Standard/sample diluting buffer

Recombinant IL-1 β standard, stock solution made up to 50 ng/ml

IL-1 β antiserum (rabbit)

Conjugate diluting buffer

anti-rabbit IgG-Horse radish peroxidase conjugate (dilute 1:100 for the 2 hour assay described here)

Tetramethyl peroxidase , Solution A

Tetramethyl peroxidase, Solution B

Chymotrypsin Assay Reagents:

- **Solvent** - Boehringer Mannheim (BM) cat. no. 718 238

0.7% lauryl trimethylammonium chloride

500 mM sodium chloride

100 mM calcium chloride

- **Substrate** = succ-ala-ala-pro-phe-p.nitroaniline. Sigma Chemical Co. Cat. no. S7388.

- **Buffer** = Tris buffer pH 9.0

Tris 100 mM

Calcium chloride 20 mM

Sodium chloride 250 mM

- **Laboratory substrate**

Dissolve 0.025g substrate in 75 ml buffer. Aliquot into 1.1 ml samples (each sufficient for 1 assay). Store aliquots at -20°C.

Non-specific protease Reagents

- **Azocoll** (Sigma A 9409) is suspended in PBS.

ECP Reagents

Included in the kit

- ECP (human) Standards 0, 2, 5, 15, 100, 200 µg/l
- ECP-¹²⁵I
- Anti-ECP
- Decanting suspension (Sepharose® anti-rabbit IgG raised in sheep)

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

Sonicator:

Soniprep 150 (Sanyo Galenkamp PLC)

Spectrophotometer:

Pye Unicam PU 8610 UV/VIS (Philips)

Vortex Mixer:

Rotamixer Deluxe (Hook & Tucker)

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