UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Doctor of Medicine

FAECAL INTERLEUKIN-8 AND TUMOUR NECROSIS FACTOR α CONCENTRATIONS AND DISEASE ACTIVITY IN CYSTIC FIBROSIS

by Graham Leslie Briars

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CORRECTIONS

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ABSTRACT

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By speculatively using an ELISA technique optimised for measuring cytokine concentrations in cell culture supernatants, I have demonstrated massively elevated faecal IL-8 and TNFα concentrations in 9 UK patients with cystic fibrosis, and have shown a correlation between lung disease severity and faecal IL-8 concentration. Such an assay had the potential to be a repeatable non invasive gauge of the severity of pulmonary inflammation in these patients. However subsequent assays in 14 Australian CF patients detected no faecal cytokines. The assays used proved to be inaccurate and non linear, and I developed and validated an accurate and linear assay protocol by using newborn calf serum as a sample diluent. With this assay patients with crohn's colitis were shown to have high faecal IL-8 and TNFα concentrations, but 19 patients with CF and patients with pneumonia, asthma, bronchiectasis and healthy control children had no detectable faecal cytokine.

Subsequent experiments showed that pancreatic enzyme supplements efficiently digest both cytokines at both pH8 and pH4.5, that CF patients' faecal flora do not have a major digestive effect on these cytokines, and that when gavaged into rat stomach these cytokines do not pass through the intestine in detectable quantities.

The hypothesis that faecal cytokines are produced in response to high lipase pancreatic enzyme therapy in CF was tested by serially measuring cytokine concentrations in cases and controls as this therapy was commenced. TNFα was detected in no patient or control, but low concentrations of IL-8 were found in one pre exposure sample and in two isolated post exposure samples in three seperate patients.

The enzyme dosage in this study was one third of that in the initial UK study and it is possible that a higher enzyme dosage might have replicated those initial results, it is however equally possible that the non linear assay used in the initial study greatly over-estimated the cytokine concentration in that study.

It now seems unlikely that cytokine detected in faeces has it's origins in swallowed pulmonary secretions. It is certain that measurement of faecal IL-8 and TNFα does not give a reliable measure of the severity of pulmonary inflammation in cystic fibrosis.

Contents

Plates

PLATES 1 TO 18 lie after page 173.

They show thumbnail photographs of bacterial culture plates produced in the course of experiments in chapter 7

Accompanying Material

CDROM

A CDROM accompanies this thesis. It contains the image files used to produce plates 1 to 18. They are in TIF format for the PC. They are provided so that the plates can be inspected in detail. It also contains this thesis and the plates in Wordperfect 6.1 format for the PC.

Acknowledgements

Funding

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Access to patients

I am grateful to the patients and their parents for their cooperation, to Drs Paul Francis and Alan Isles at the Royal Children's Hospital Brisbane, and to Dr CJ Rolles and Prof JO Warner at Southampton General Hospital for permission to recruit their patients to the study.

Laboratory Facilities

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Dr Joan Faggolio in the department of Microbiology at the Royal Brisbane Hospital provided counting chambers for the neutrophil migration studies and access to her clinical laboratory.

Mr S Davis at East Glamorgan General Hospital provided laboratory facilities for part of the UK arm of the study.

Practical assistance

Mr Darryl Morris and Dr Alan Clague of the department of biochemistry at the Royal Brisbane Hospital measured the tryptic activity of solutions of commercial pancreatic enzyme supplements. Sandra Klingberg measured faecal $\alpha 1$ antitrypsin concentrations and performed the haemoccult tests. Dr John Masel and his staff in the radiology department at The Royal Children's Hospital conducted the bowel wall thickness measurements.

Academic Supervision

Prof RW Shepherd supervised the practical work in Brisbane and initial drafts of the thesis. Prof JO Warner supervised subsequent preparation of the thesis.

Abbreviations

µg	microgramme
ANOVA	Analysis of variance
C	centigrade
Ca	Calcium
cAMP	cyclicAMP
CF	Cystic fibrosis
CFTR	Cystic fibrosis trans-membrane conductance regulator
cm	centimeter
CTAP-III	Connective tissue activating protein III
CXCR	CXC Chemokine receptor
Da	Dalton
DAG	Diacylglycerol
df	degrees of freedom
Diln	Dilution
DMSO	Dismethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immuno-sorbent assay
ENA-78	Epithelial neutrophil activating peptide 78
F	Female
FEF_{25-75}	forced expiratory flow between 25 & 75% of FVC
FEV_1	forced expired volume in 1 second
fMLP	Formylmethionyl-leucyl-phenylalanine
FVC	forced vital capacity
g	unit of gravitational force
GCP2	Granulocyte chemotactic protein 2
GRO	Growth related oncogene
GTP	Gaunidine triphosphate
HBSS	Hank's blanced salt solution
hpf	high power field
IBD	Inflammarory bowel disease
IL-1	Interleukin 1
IL-8	Interleukin-8
IP3	Inositol triphosphate
Kd	Dissociation constant
kU/l	Thousands of units per litre
LTB_4	Leukotriene B4
M	Male
Mg	Magnesium
ml	Millilitre
mm	millimeter
n/a	not available
NAP2	Neutrophil attractant peptide 2
NBCS	Newborn calf serum

NCS	Newborn calf serum
NIH	National Institutes of Health
NR	Not recorded
NSW	New South Wales
OD	Optical density
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
pg/g	picogrammes per gramme
pM	picoMolar
PMSF	Phenylmethylsulphonylflouride
Pt	Patient
pvp	Polyvinylpyrolidine
QIMR	Queensland Institute of Medical Research
RD5	Proprietary ELISA diluent
RD6a	Proprietary ELISA diluent
RPM	Revolutions per minute
SBTI	Soy bean trypsin inhibitor
SD	Standard deviation
SEM	Standard error of the mean
TNFα	Tumour necrosis factor α
TSB	Tryptone soy broth
UV	Ultraviolet
v/v	volume for volume
Vs	Versus
Wt	weight
yrs	years
z-Score	standard deviation score

Chapter 1
Introduction - General

When Andersen identified cystic fibrosis as an entity distinct from coeliac disease in 1938, she considered it a fatal primary pancreatic disease[1]. She described meconium ileus, bronchitis, bronchopneumonia, bronchiectasis, cirrhosis and osteoporosis as complications. She identified vitamin A and vitamin D deficiencies as complications of pancreatic insufficiency, which she stated required a 90% destruction of pancreatic tissue to occur. She noted the improvement in steatorrhea with a low fat diet, but cautioned against it's use because she felt this would exacerbate the pulmonary disease through worsening vitamin A deficiency. Sixty years later it is now clear that cystic fibrosis is a multisystem disease resulting from a genetically acquired defect of chloride channels in all exocrine glands. The chloride channel protein has been characterised and has been named "cystic fibrosis transmembrane conductance regulator" (CFTR). In September 1989 the mutation in the CFTR gene first known to cause cystic fibrosis was identified. It involved deletion of a phenylalanine residue at amino acid position 508 in the protein, and was named Delta F508. Since that time the gene for wild type CFTR has been sequenced and by January 1998, a total of 622 different mutations occuring in the CFTR gene in CF patients had been identified. Groups of the most common mutations have been characterised by their cellular mode of expression. Delta F508 is amongst a group in which the mutation results in failure of the synthesised protein to reach the cell membrane, and a total absence of CFTR chloride channel activity in the cell membrane. Other groups result in non functioning chloride channels, partially functioning chloride

channels, and decreased populations of chloride channels on the cell membrane. At the tissue level it is therefore possible to quantify the electrochemical severity CF mutations. Whilst partially functioning channels due to R117H and similar mutations are associated with retention of sufficient pancreatic function to avoid the need for pancreatic enzyme replacement therapy, electrochemical severity has not been consistently associated with any other phenotype.

The disease has manifestations in the nose, bronchi, small and large intestines, pancreas, liver, sweat glands and male reproductive tract. The finding of viscous intestinal contents in patients with meconium ileus, and of thick tenacious sputum in patients with chest disease has supported the model that disease manifestations are mediated through an increase in the viscosity of exocrine gland secretions and leads to its alternative name: "mucoviscidosis". This model has pancreatic insufficiency result from the obstruction of pancreatic acini by thickened mucus occasioning a progressive destruction of pancreatic tissue, it has chest disease resulting from the increased physical difficulty in removing respiratory tract secretions from the airway, and azoospermia resulting from a similar viscous blockage of the ductus deferens and seminiferous tubules.

Attempts have been made to establish several genotype-phenotype associations, but with the exception of pancreatic sufficiency and mild chloride channel dysfunction none have proven consistent under scrutiny. There is much variation in pulmonary disease severity within cohorts of patients homozygous for delta F508, and some patients have had azoospermia as their sole manifestation despite carrying mutations that in other patients

have been associated with multisystem disease, and this stretches the credulity of the hyperviscosity model of cystic fibrosis pathogenesis. The description of a form of dibetes mellitus peculiar to cystic fibrosis,[2,3] of fibrosing colonopathy and of an arthropathy[4,5,6] delineates manifestaions of cystic fibrosis not directly related to increased exocrine secretion viscosity.

It is clear that long strands of DNA from dead neutrophils[7] play a major role in the genesis of tenacious sputum.[8] It is also clear that inflammatory cells may be present in the CF lung at a very early stage in the disease,[9,10] and that the relative contribution of inflammatory cells and of changes in the viscoelastic properties of bronchial mucus to this sputum tenacity is uncertain. There is evidence that hyperviscosity of bronchial secretions may be mediated through perturbations in cellular chloride secretion, and that this hyperviscosity reduces pulmonary particle clearance, facilitating pulmonary infection and subsequent inflammation. It is perhaps the indirect relationship between chloride channel function and chest disease that underlies the absence of correlation between chest disease severity and genotype.[11]

Koch and Hoiby[12] have suggested a mechanism whereby impaired airway chloride channel function results in increased viscosity of pulmonary secretions. A physiologic characteristic of the tracheo-broncial tree is that airway surface area increases with each serial branching, so that surface area is maximised at the point of gaseous exchange. In the airway there is a net movement of epithelial fluid away from the alveoli towards the trachea, as a result of cilliary beating, and this aids in the clearance of particulate matter.

Ordinarily when liquid flows from a tributary through a confluence, the rate of flow and the amount of fluid flowing increases progressively downstream. Arguing that the depth of the layer of epithelial lining fluid in the pulmonary airways remains relatively constant they assert that there must be a net secretion of electrolytes and water in the distal airway and a net absorption in the proximal airway. They support their argument with the observations that in the proximal airway CFTR expression is predominantly within the serous tubules of submucosal glands[13], and not in the epithelial cells directly lining the airway.[14] In the proximal terminal and respiratory bronchioles and alveoli however, non cilliated airway lining epithelial cells express CFTR on their apical membrane[15]. These observations are consistent with the normal chloride channel playing a role in chloride secretion and the secondary water secretion into the distal airway but not the proximal airway. Koch and Hoiby assert that an absence of functional CFTR from these airway sites diminishes the distal secretion and proximal reabsoption of epithelial lining fluid in the airway, and they support their assertion with studies of clearance of radiolabelled particles from the airway in which, patients homozygous for the delta F508 mutation have no increase in particle clearance with beta agonist administration, whilst compound heterozygotes expressing delta F508 and another mutation have a blunted increase in particle clearance.[16]

Following the recognition of cystic fibrosis there have been progressive improvements in the management of the disease. In 1958 85% of CF patients died in infancy, but by the 1990s the median survival age was 26 years and the projected median survival age for a child with CF born in that decade had improved to 40 years.[17] This improvement in

prognosis has uncovered CF liver disease and CF diabetes as common complications of the disease, and it seems that as more patients grow older further CF complications will emerge. The improved prognosis has also altered the spectrum of pulmonary disease. Initial pulmonary infection is frequently with *Staph.aureus* or *H.influenzae*[18], subsequently infection with *P.aeruginosa* and *B.cepacia* become more prominent, and allergic bronchopulmonary aspergillosis may develop. Many patients with CF currently suffer from pseudomonal infection, and whilst the impaired mucociliary escalator may be one factor allowing its colonisation, the presence of an inflammatory response to any pre-existing organism may well be another. In contrast to *Staph.aureus* and *H.influenza, Pseudomonas species* are not particularly virulent organisms. In nature they are commonly found in soil, and in rotting matter, and it seems likely that necrotic polymorph neutrophils in a respiratory tract would form a suitable substrate for these organisms.

Aquisition of *Pseudomonas* in the respiratory tract is a milestone in the progression of pulmonary disease. It has long been accepted that once acquired the organism is carried indefinitely, and that therapeutic manouvres result only in a reduction in the size of the colonising population. When colonisation initially occurs the organism is usually in a non mucoid form, subsequently the organism expresses genes for alginate production and microcolonies of *Pseudomonas* grow under a protective film of slime. This change in the morphology of the colonies often coincides with the onset of a humoral response to pseudomonal antigens, and the production of immune complexes which accelerate pulmonary inflammation.[19,20] For those clinicians who draw a distinction between

colonisation and infection, it is the onset of the humoral response that marks the watershed. The change in the organism from smooth to mucoid colonies probably offers the organism protection from antibiotic therapy, and this change seems to be the factor that prevents the effective eradication of the organism from patients with established infection. With regular surveillance for *Pseudomonas* and early aggressive anti-pseudomonal chemotherapy the establishment of chronic carriage has been significantly delayed by somewhere in the region of two years.[21] If this also delays the progression of pulmonary inflammation by two years, it is clearly a useful intervention.

Having initiated pulmonary inflammation, a self amplifying process where inflammatory cells secrete pro-inflammatory cytokines, which stimulate further inflammation occurs. The cytokine profile of infected pulmonary secretions have been studied and elevated sputum concentration of TNFα has been identified.[22] Interleukin-8 has been shown to be the major stimulus for the influx of polymorph neutrophils into the airway,[23] with elevated concentration of the chemokine in serum, sputum and bronchoalveolar lavage fluid.[24] Together these proinflammatory cytokines mediate an active endo-bronchial inflammatory response.

The result of this marked endobronchial inflammatory response has been the overwhelming of the lung's defence mechanism to neutrophil elastase. Quantities of elastase produced by this response easily outstrip the neutralising effect of the lung's production of α-1-antitrypsin, and attempts have been made to supply additional leuco-protease inhibitors,[25] but these have been without dramatic effect.

Additionally the ability of pulmonary macrophages to phagocytose apoptotic polymorphs appears to have been outstripped resulting in their secondary necrosis and a resurgence of inflammatory activity. Ordinarily the acute inflammatory response goes through a phase of neutrophil apoptosis and phagocytosis of the neutrophils by professional and non-professional phagocytes without any further proinflammatory consequences. Neutrophils constitutively activate their apoptotic pathway and generate an "eat-me" signal on their surface, and are phagocytosed by neighbouring cells, so that their mitochondrial products, amongst which are formyl-methionyl peptides, are unable to generate a further inflammatory response. This apoptosis is accelerated by TNFα,[26] and is retarded by bacterial lipopolysaccharide (LPS) and GM-CSF[27], the latter inhibition resulting in an insensitivity to TNFα. Additionally apoptosis is retarded by elevation of cellular cAMP concentration.[28] Whilst there is much TNFα in CF pulmonary exudate there are also high concentrations of LPS and of chemokines which would elevate cellular cAMP in neutrophils still expressing their receptors. These factors conspire to increase the probability the pulmonary neutrophils will undergo a primary or secondary necrosis, and stimulate other cells to secrete further pro-inflammatory chemokines.[29]

The physical clearance of this endobronchial infective exudate by physiotherapy has long been a goal of routine CF treatment and it remains a vital component of disease management. This has been aided recently in some patients with established lung disease, by the use of recombinant human DNase, an enzyme that digests the lengthy strands of neutrophil DNA, reduces sputum viscosity, and in some patients generates a marked improvement in pulmonary health. Others however have deteriorated on the treatment,

and the recent observation that DNase will liberate bound IL-8 from neutrophil DNA might possibly underlie this deterioration. If the DNA bound chemokine was unable to act as a neutrophil chemotaxin, such liberation might allow it to become active again, leading to an increase in inflammatory activity with DNase therapy.

Inflammatory activity is pivotal in the progression of the pulmonary disease. The lung in a newborn CF patient is structurally normal, but the protracted inlammation seen in later life results in the secretion into the lung of elastases and other proteases which destroy pulmonary architecture and permit the development of bronchiectasis. This results in chronic suppuration and is characterised by the daily production of sputum, and it is in the presence of chronic suppuration that DNase and physiotherapy are most easily seen to improve expectoration of sputum. Clearly it would be profitable to interupt this inflammatory process prior to the onset of structural lung damage. Unfortunately patients at this stage of pulmonary disease are difficult to identify. They often produce little sputum, both because they do not have the bronchiectatic reservoirs of inflammatory exudate and because they are often too young to expectorate any sputum that they do produce. It would be possible to identify such pulmonary inflammation by routinely conducting broncho-alveolar lavage in patients at risk, such an approach however is likely to be unacceptable to the majority of patients and their parents. A non invasive method of identifying such patients is desirable and this could be the precursor to a single bronchoscopy and broncho-alveolar lavage, which would allow culture of infecting organisms and the institution of potent and specific antibacterial and anti-inflammatory therapy to retard pulmonary destruction. The description of a new gastrointestinal

manifestation of cystic fibrosis provoked a line of investigation which suggested that such a non invasive test might be developed.

In January 1994 Smyth et al reported a series of cystic fibrosis patients with large bowel obstruction, caused by fibrotic strictures. They associated these strictures with the use of high lipase pancreatic enzyme supplements, which had been introduced into their clinic twelve months before the onset of their epidemic of strictures. In their original report they speculated that high concentrations of enzyme activity in proximity to the colonic mucosa might be the initiating event in the stricturing process.[30] Subsequent investigations into this new disease have lead to the consensus that the most likely initiating factor in these strictures is the methacrylic acid impurities in the coating used on the enzyme microspheres in all but one of the high lipase preparations. Shortly after the publication of Smyth's paper, I reported a case of colonic stricturing in a boy with cystic fibrosis (G542X, 1717-1G>A), in whom it seemed that high strength enzymes were not the only factor predisposing strictures, even though he was taking high doses of one of the enzyme preparations subsequently implicated in stricture pathogenesis. It seemed in his case, that intestinal ischaemia may have played a part.[31]

Shortly after his birth by emergency caesarian section, he developed abdominal distension, with no bowel gas distal to his stomach, and on day 2, a 50 cm necrotic, jejuno-ileal volvulus was excised and white pellets in his terminal ileum were flushed out. He suffered a postoperative stricture of the healed anastomosis but was fully enterally fed and taking pancreatic enzyme supplements by the age of 1 month.

He had been taking a high lipase preparation (Nutrizym 22) for 1.7 years when at the age of 4.6 years he presented with daily abdominal pains and was found to have a smoothly tapering stricture in the distal transverse colon and splenic flexure (figure1.1). At laparotomy the fibrotic stricture extended from the transverse to the mid descending colon and the left and middle colic arteries were one-quarter normal calibre. It seemed likely, in view of the narrowness of the colic arterial supply, that this stricture originated at the time of his perinatal illness, and that the pancreatic enzymes were not the sole cause of the stricture.

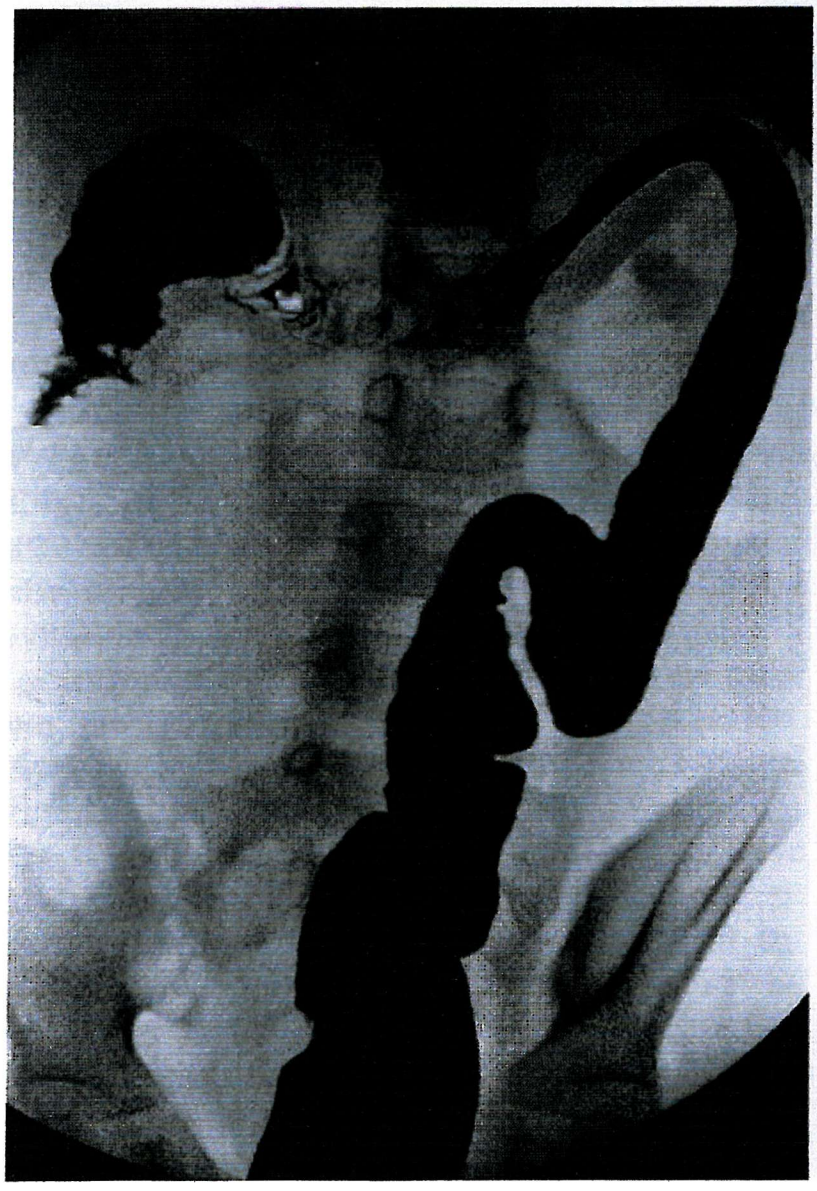


Figure 1.1

Smyth et al.'s suggestion that an agent taken by mouth might pass through the gut unaltered and in sufficient quantity to cause disease was thought provoking.

This coupled with the idea that pancreatic enzymes may not be the sole cause of fibrosing colonopathy generated the hypothesis that large quantities of proinflammatory cytokines produced in the lung and then swallowed might pass through the gut to exert a pro-inflammatory effect in the colon. This was not a completely speculative hypothesis as TNFα is present in high concentration in CF sputum[32] and it has been associated with the production of fibrosis in chronic inflammation. Chronically elevated TNF-α concentration does stimulate fibroblasts to proliferate, probably through an autocrine loop involving stimulated secretion of platelet derived growth factor (PDGF) from fibroblasts.[33] There was therefore sufficient reason to explore the possibility that pulmonary cytokines might be swallowed and traverse the gut intact.

There had been a sustained interest in the chemokine interleukin-8 in the Southampton CF clinic, and an in house ELISA for IL-8 had been developed. This in-house ELISA and a commercially produced ELISA for TNFα were used to detect these two cytokines in faeces from a group of CF patients attending the Southampton CF clinic.

Interleukin-8 concentration in faeces had not been reported in any condition, but tumour necrosis factor α concentration had been assayed in faeces in a number of inflammatory and infectious colitides, and in healthy controls[34,35,36] and an assay protocol derived from the protocol used in these studies was used.

Chapter 2

Introduction 2: Faecal cytokines

Assays of IL-8 and TNFα in CF patients in the Southampton CF clinic.[37]

Opportunity to conduct a study

At the time the question of faecal cytokine analysis arose we had a number of faecal samples from cystic fibrosis patients in deep freeze, following their analysis for a study investigating nutrient digestion and absorption. Fortuitously two samples were available for the patient described above who had developed a colonic stricture.

These samples had been collected for a study of the digestion of triglyceride labelled with a stable carbon isotope ^{13}C. The study was conducted by Dr J.L.Murphy in the department of Human Nutrition at Southampton University. Patient selection was deliberately biased towards patients who were high lipase users. On the day of the test subjects were given a slice of buttered toast containing the labelled triglyceride of palmitic acid, a single capsule of Creon 25,000, and an oral carmine marker. Stools were collected for five days between carmine markers, and a three day weighed food intake diary was commenced.

In order that faecal bacteria did not alter the carbohydrate, fat, protein balance in the sample after production, the faecal samples were frozen immediately after production. Energy absorption was calculated from the ingested energy and the stool excreted energy, determined by bomb calorimetry. Stools were then refrozen and stored for future

reference. In addition to the faeces samples, breath samples were taken for the measurement of $^{13}CO_2$. In that study the triglyceride initially selected probably had a melting point too high for our purpose and well over 90% of the ingested ^{13}C was excreted in the stool. The experimental work for that study completed, the frozen faecal samples were available for analysis for faecal cytokine concentration.

Pathophysiological considerations

In the design of this initial assay, it is necessary to include a measure of pancreatic function and we had already determined percentage energy absorption for the parent study and Schwachman score for clinical assessment. It would also be necessary to include some measure of pulmonary disease activity, because if any faecal cytokine does originate from pulmonary secretions, higher faecal cytokine concentrations might occur in patients with more severe pulmonary disease. Therefore spirometric measurements and respiratory tract secretion culture results were noted.

Patients & Controls

Twenty four hour stool samples were collected from 9 pancreatic insufficient patients with Cystic Fibrosis. Their pancreatic enzyme preparation was Creon 25000 (Duphar), except for patient 1 (Creon (Duphar)) and patient 7 (Nutrizym 22 (Merk)). Control samples were collected from clinically healthy children in the community. The children were age matched and paired with patients before analysis of the samples. However their samples had not been collected between carmine markers.

Four patients(1-4) had established Pseudomonal colonisation and had suffered exacerbations of chest disease. Two patients (5 & 8) had never grown Pseudomonas, and two patients (9 & 6) had grown it on a single occasion from a throat swab 1 and 2 years before the study respectively. Patient 7 had isolated Pseudomonas from a throat swab twice: two years and then two months before the study. No study patient had ever been culture positive for Burkholdaria cepacia. Patient 3 had cirrhosis and portal hypertension; no other patient had liver disease. No control had any concurrent disorder.

Methods

Stool samples were homogenised to a manageable consistency with water. Aliquots of the homogenate were frozen at -20°C and stored. The homogenised stool was thawed and 1 gram was added to 1ml of phosphate buffered saline. This was vortexed for two minutes and centrifuged at 20000g for 15 minutes at 4°C. The aqueous supernatant was removed and frozen at -70°C until analysis. The IL-8 assay has been described elsewhere.[38] TNF-α was assayed using the Quantikine kit (R&D systems, Abingdon, Oxon) according to the manufacturers instructions. All cytokine concentrations were expressed as pg per gram of wet stool.

The patients were assessed by Shwachman score, apparent energy absorption, pancreatic enzyme dosage, and in those old enough to cooperate by spirometry. Weighed food intake was recorded for five days as described by Marr[39] and gross energy intake calculated.[40] A three day stool collection between carmine markers was homogenised and faecal energy was measured in a ballistic bomb calorimeter. Apparent energy absorption was expressed as a percentage of intake. Comparative dosages of pancreatic enzymes were expressed as thousands of European units of lipase activity per kilogram body weight per day. Shwachman scores were calculated at a full clinical review in advance of the assays.

Results

Patients are described in table 1, and faecal cytokine concentrations are shown in table 2. The median (range) stool IL-8 and TNF-α concentrations in patients was 32113 pg/g

(21656 to 178128) and 3187 pg/g (368 to 17611) respectively. In controls cytokine concentration was below the detection limit of the assay in seven of nine IL-8 samples and two of nine TNF-α samples.

Figure 2.1 Southampton CF patients
Disease severity

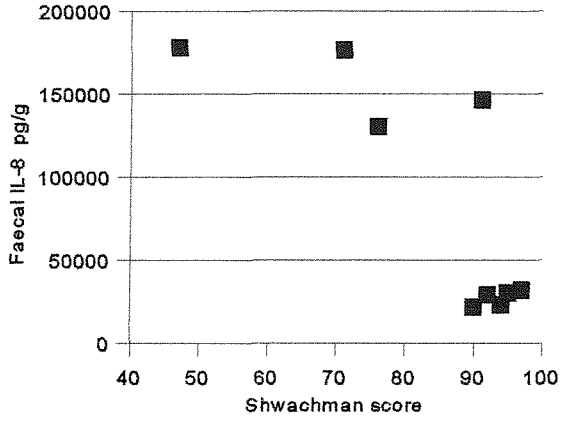


Figure 2.2 Southampton CF patients
Enzyme consumption

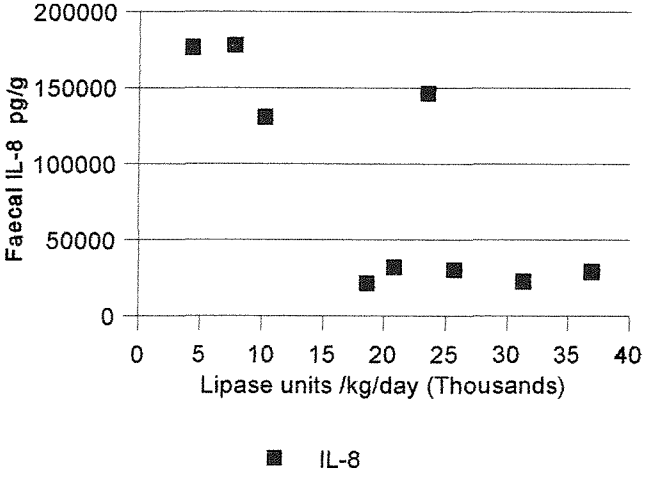


Figure 2.3 Southampton CF patients
Respiratory Function

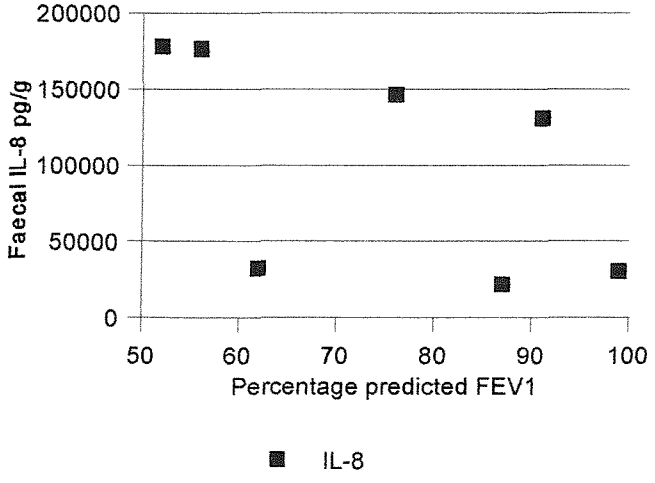


Table 1 Patients Characteristics

No	Shwachman score	Lipase dosage†	% Energy absorption	FVC % predicted	FEV_1 % predicted	FEF_{25-75} % predicted
1	47	7.7	87.2	52	22	7
2	71	4.3	81.5	56	29	10
3	91	23.5	83.6	76	70	52
4	76	10.2	91.2	91	53	19
5	97	20.8	88.7	62	59	47
6	95	25.7	85.6	99	84	47
7	92	37	61.3	-	-	-
8	94	31.3	89.6	-	-	-
9	90	18.6	92.4	87	74	44

† Thousands of European Lipase Units /Kg body weight /day.

TABLE 2 Faecal IL-8 and TNF-α concentrations as pg/g of wet stool

	CONTROLS			**PATIENTS**		
No	Age	IL-8	TNF-α	Age	IL-8	TNF-α
1	13.74	<33	66	12.88	178128	3187
2	21.60	<22	37	20.64	176497	5897
3	9.47	<31.5	<0.26	9.70	146438	17611
4	15.17	<43.3	155	14.29	130725	10422
5	7.59	496.2	<0.68	3.72	32113	4472
6	11.54	<53.7	123	7.44	30439	1075
7	5.11	4079.3	129	3.88	29169	1497
8	7.87	<56.6	231	5.26	22872	368
9	9.68	<43.5	99	7.80	21656	998

Assuming the respective concentrations to be at their detection limits, the median (range) stool IL-8 and TNF-α concentrations in controls are estimated at <43.5 pg/g (<22 to 4079) and 99 pg/g (<0.26 to 231).

The median (95% CI) difference in stool cytokine concentrations (CF population - non CF population) was 87072 pg/g (25090-154388) for IL-8 and 3705pg/g (925-10267) for TNF-α. Faecal cytokine concentration was greater in patients with, than in those without established Pseudomonal colonisation: median difference 134583pg(IL-8)/g and 5740pg(TNF-α)/g.

In patients, IL-8 concentration was negatively correlated with Shwachman score (r=-0.79, 95%CI -.95 to -.27, p<0.02) (Figure 2.1) and lipase usage (r=-0.77, 95%CI -.95 to -.21, p<0.02)(Figure 2.2), but not with energy absorption. Seven patients were able to perform simple spirometry. Faecal IL-8 concentration was negatively correlated with FEV_1 (r=-0.78, 95%CI -0.97 to -0.06 ,p<0.05)(Figure2.3). The correlation between IL-8 concentration and FEF_{25-75} (r=-0.73), and FVC (r=-0.57), did not reach statistical significance. Faecal TNF-α concentrations did not correlate with lung function, Shwachman score, lipase usage or energy absorption.

Six months after the study, patient 7 became consistently Pseudomonas colonised, on the basis of repeated throat swab isolates and increased symptoms. He was receiving 54 thousand units of lipase per kilo per day and faecal analysis after one week of intravenous antibiotic showed little change in cytokine concentrations: 22562pg/g (IL-8) and 2052pg/g (TNF-α). Four months later he presented with a splenic flexure colonic stricture.[41] A child selected as his control (Age 5.18) had asthma, was receiving Sodium Cromoglycate, and was asymptomatic. His faecal concentrations were 9712.8pg/g (IL-8) and 247pg/g (TNF-α).

Discussion

In this experiment faecal IL-8 and TNF-α concentrations were raised in patients with cystic fibrosis, when compared with healthy children. Differences in cytokine concentration between disease and control groups were greater for IL-8 than TNF-α when they were expressed weight for weight. These differences are even greater when expressed in molar units, given the small molecular weight of IL-8.

TNF-α concentrations in control samples were comparable with those of the normal control children used by Nichols et al[42] (12-130pg/g) and by Braegger et al[43] (40-84pg/g). The faecal TNF-α concentrations in these CF patients are similar to those of active Ulcerative Colitis and Crohns disease patients. None of them had inflammatory bowel disease clinically, but sub-clinical intestinal inflammation can not be excluded as intestinal biopsy specimens were not taken. Indeed the patient who subsequently developed a colonic stricture did have a mild chronic inflammatory infiltrate at the time of his colectomy, but his IL-8 concentrations were only moderately raised. If faecal cytokines originate from chronic inflammation accompanying colonic stricture, more strictures would have been expected in the study population. In the current study one patient was taking low strength pancreatic enzyme, and his faecal IL-8 concentration was the highest recorded. The negative correlation between faecal IL-8 concentration and pancreatic enzyme dosage does not support the idea that the enzyme causes sub-clinical intestinal inflammation. It is possible that a neutrophil infiltrate occurs in CF bowel as a

result of the high lumenal IL-8 concentration, if this IL-8 is biologically active. Potentially lumenal IL-8 could be the cause rather than the result of sub-clinical intestinal inflammation.

Sheron et al[44] have demonstrated raised IL-8 concentrations in the serum and liver tissue of adults with acute severe alcoholic hepatitis which is characterised by an intense tissue neutrophilia. Hepatic tissue concentrations in that group of patients were one to two orders of magnitude greater than the faecal concentrations recorded here. If hepatic tissue IL-8 concentration in CF liver disease was comparable with that in acute severe alcoholic hepatitis, liver disease could be a major source of faecal cytokines. Whilst the possibility that hepatic disease may contribute to stool IL-8 concentrations can not be discounted, it is unlikely to be it's major source as eight of nine patients had no evidence of liver disease. An alternative source for these high cytokine concentrations must be postulated.

The large difference in IL-8 concentration between patients with and without established Pseudomonal colonisation and the negative correlation of Shwachman score and lung function with faecal IL-8 concentration are consistent with a pulmonary source for this cytokine.

Greally et al[45] have described TNF-α concentrations between 10 and 1988pg/ml in the sputum of 16 patients with CF. The faecal TNF-α concentrations in our patients are slightly higher (368 to 17611 pg/g), although they are expressed differently. High IL-8 concentrations occur in CF sputum, serum and broncho-alveolar lavage fluid, with

recorded concentrations lying between 620.5 and 92628 pg/g wet weight of sputum.[46] Faecal concentrations in the present study are in excess of this using an identical assay in the same laboratory. If TNF-α and IL-8 simply pass through the GI tract, the high concentrations in the dietary residue imply that the largest part of daily sputum production is swallowed rather than expectorated, and that volumes of sputum are much higher than has been appreciated, even in patients without overt chest symptoms.

The results suggest that faecal IL-8 concentration may reflect severity of pulmonary inflammation, and this possibility deserves further consideration because measurement of faecal IL-8 is easily repeated, non-invasive and could be applicable to patients who are too young to expectorate sputum. Having serendipitously arrived at this conclusion, using samples that were collected for another purpose, it was necessary to repeat the experiment in another population that was representative of the CF population in general, and not biased towards high lipase users. The opportunity arose to conduct such a study in Queensland.

Initial Study in Australian CF patients

The outline of the investigation was to recruit children with CF who had been diagnosed by the neonatal screening program, have them stratified for age and follow their serial feacal cytokine concentrations for two years thereby gaining an insight into the changes in faecal cytokine concentration that occur with progression of the disease. There were to be several other investigations including the effect of intravenous antibiotics on faecal cytokine concentration. However the initial part of the study was designed to confirm that the Southampton results could be obtained elsewhere. As the final objective of the study was now to develop a technique which could be applied in a provincial hospital, the equipment used for the study was modified to be representative of equipment typically available in such a hospital. In particular the centrifuge used to obtain a faecal homogenate supernatant was of a type used routinely to seperate serum from blood cells.

Method

Thirty six faecal specimens from patients with cystic fibrosis, and one from a boy with severe crohns colitis were collected into one litre purpose designed paint cans and frozen within 1 hour of being produced. They were thawed overnight and homogenised in distilled water to a manageable consistency. Homogenates were centrifuged at 1000g and the supernatants were refrozen at -70°C until assayed. Samples were assayed using commercial ELISAs for TNFα and IL-8: Quantikine Kits(R&D Minneapolis), according to the manufacturers instructions for culture supernatant. Samples were diluted in the

supplied diluent so that IL-8 concentrations similar to those measured in the original study, would give aliquot concentrations in the middle of the assay's range.

In eight patients who had samples assayed for both IL-8 and TNFα, disease severity was assessed by NIH score and in those old enough to perform it simple spirometry.

Results

Interleukin-8

No sample had IL-8 immunoreactivity, either at the original dilution or when the assay was repeated with minimal sample dilution. Assuming all these samples to have an IL-8 concentration of 32pg/ml, the minimum standard concentration in the assay; the maximum possible faecal Il-8 concentrations for these patients were estimated to lie between 93.65pg/g and 1129pg/g, values that lie within the range seen in healthy controls in the Southampton study. In contrast the Crohn's colitis sample had an aliquot concentration above the range of the assay, and by extrapolation the faecal IL-8 concentration was estimated to be 120,011pg/g.

Tumour Necrosis factor α

Faeces from 14 Cystic Fibrosis patients, and one Crohn's colitis patient were assayed. No CF stool had detectable TNFα immunorecativity, whilst the sample from the Crohn's colitis patient had a TNFα concentration of 6269pg/g.

Clinical characteristics

The mean (SD) NIH score for the eight patients who had this assessment performed and who had had stools assayed for both cytokines was 79.6 (18.8). Their mean (SD) height and weight z-scores were -0.975 (1.03) and -0.811 (0.50) respectively. Four of these patients had spirometry performed and their mean (SD) FEV_1 and FVC as a percentage of that predicted from height age and sex according to Polgar[47] were 44% (19.6) for FEV_1 and 59% (14.5) for FVC.

Discussion

These initial Australian assays have failed to reproduce the results seen in the original Southampton study, despite the fact that the study included patients with advanced pulmonary disease. Apart from the fact that the patients in the two studies were residing in different countries with widely different environmental conditions, there were several differences between the studies that might possibly account for these differences.

Patients

The Southampton patients had been recruited into a study measuring breath ^{13}C carbon dioxide following a meal containing triglyceride whose fatty acids were ^{13}C palmitic acid. Their selection had been deliberately biased towards patients on high dosage of pancreatic enzymes, and maldigestion may have been more common in this group than in the Australian group who were not so selected. All Southampton patients had their faeces

collected between Carmine markers, whilst none of the Australian patients received Carmine, as they were having only a single sample collected.

Specimen collection and processing

Samples in the current study had been collected into metal paint cans in contrast to polyethylene containers in Southampton. Equally Southampton homogenates were centrifuged at 20,000g whilst Brisbane samples were centrifuged at 1000g. A number of the Brisbane samples were opaque, and on microscopy this was shown to be a result of a high viable bacteria count. Finally whilst the TNFα assays were identical in the two studies, the IL-8 assays were entirely different. The Southampton assay was developed in-house from monoclonal goat and sheep antibodies donated by Sandoz (Austria), which have not been marketed and are now unavailable, whilst the Brisbane assay was a commercial kit assay developed from murine monoclonal antibodies.

The assay technique is a double antibody sandwich technique. Murine monoclonal anti-IL8 antibody is coated onto the microtitre plate. When the sample is added to the well IL-8 binds to this antibody, and the supernatant is drained and the plate washed so that any unbound proteins are removed. A polyclonal anti IL8 antibody conjugated to horseradish peroxidase is then added and the concentration of IL8 determined through the differential development of colour from a chromogenic horseradish peroxidase substrate.

Considering the possibility that failure to detect cytokine in the Australian assay was a result of technical artefact, the capture of the IL-8 onto the coating monoclonal antibody

is step in the assay most likely to be subject to interference. Possibilities are:

1 The IL-8 antigenic site recognised by the monoclonal antibody is anavailable as a result of binding to some other ligand.

2 The monoclonal binding site is similarly unavailable.

3 An enzyme present in the faecal sample might liberate the capture antibody Fab site from its Fc fragment. Alternatively the entire capture antibody might be freed from the assay plate.

The limited published work on ELISAs for cytokines in faecal material[48,49,50,51] suggested that like our initial study, they had been performed in a speculative manner and that none of the groups had validated their assays for use in faecal samples. Personal communications with two of these authors confirmed that neither had performed such validation.

In order to make a meaningful investigation into faecal cytokine concentrations, the issues of assay accuracy, reproducibility and linearity required formal exploration, as did aspects of sample collection, handling and processing. Once these studies had been completed, the biological influences on faecal cytokine concentrations could be investigated.

Furthermore evidence of biological IL-8 activity in faeces could be sought, to dispel the possibility that either any IL-8 immunoreactivity in stool was due to cross reaction with one of the myriad of antigens present in faeces, or that IL-8 immunoreactivity had been lost in stool by molecular modification, whilst chemotactic activity remained.

Before such experimental measurements of faecal chemotactic activity for neutrophils are made, it is necessary to consider those feacal constsituents that might also cause neutrophil chemotaxis.

Chapter 3

Introduction 3: Neutrophil chemotaxins in faeces

My initial experiments have relied on cytokine binding to a monoclonal antibody directed against the cytokine, in the setting of an ELISA, to infer that the cytokine is present in faeaces. It is desirable to conduct confirmatory studies using a functional assay for the cytokines of interest. Techniques for quantifying neutrophil chemotaxis are well established, and I therefore propose to describe such studies using faecal supernatants as the chemotaxin, in order to provide independent evidence of IL-8 activity in stool. Such studies would be invalidated if faecal supernatant contained chemotaxins other than interleukin-8. There has been limited published work on the chemotaxin content of faeces, and it is logical to review the biology of known neutrophil chemotaxins, with a view to speculating which chemotaxins might exist in faecal supernatant.

Neutrophil Chemoattractants in the Intestinal Lumen

Neutrophil chemoattractants can logically be divided into several groups. These are serum complement factors, bacterial formyl-methionine compounds, lipid mediators, chemokines and platelet alpha granule proteins, although these last two groups are not mutually exclusive.

Complement

C5a is chemotactic for neutrophils. It is produced in the complement pathway as a consequence of C5 convertase activity on C5 (molecular weight 190 kDa) giving C5b (mw 75 kDa) which goes on to form the basis of the membrane attack complex, and C5a (mw 125 kDa). Both classical and alternate complement pathways produce C5 convertase activity. In the classical pathway this action is fulfilled by the C4b2a3b complex, whilst in the alternate pathway the C3bBb3b complex has this role. C5a mediated chemotaxis could result from IgG or IgM antigen antibody complexes via the classical pathway, or form bacterial polysaccharide via the alternate pathway. Lumenal C5a activity might occur as a consequence of leakage of C5a from the lamina propria in inflamed mucosa following the ingress of antigen. Alternatively it might follow plasma leakage into the lumen and insitu activation. It is a markedly larger protein ~~that~~ than the chemokines, and this should allow C5a chemotactic activity to be distiguished from that of the chemokines.

Formyl-methionyl peptides

Prokaryotic organisms commence protein translation with formylated methionine, coded for by the triplet "AUG", and the resulting lead peptides are cleaved from the cytoplasmic proteins post-translationally.[52] This characteristic is shared with eukaryotic mitochondria.[53] Formylated methionine and small formylated peptides are released into bacterial culture medium in vitro. Schiffmann et al.[54] noted that substances produced by

Escherishia coli are potent chemoattractants for neutrophils and set about a systematic study of the chemotactic activities of the N-Formylmethionyl peptides. He showed that N-formyl methionine was chemotactic for neutrophils at 0.1 to 10mM concentration, and that corresponding dipeptides and tripeptides were active at much lower concentration. The most potent chemoattractant he found was fMet-Phe which was active at nanomolar concentrations.

N-Formylmethionyl-leucyl-phenylalanine is the cardinal chemotactic peptide emanating from *E.Coli*[55]. It has been the subject of much study, and has been a popular reference chemotaxin for studies of human neutrophil function.[56,57,58,59] It is likely that other *E.coli* neutrophil chemotaxins exist and some may be more potent than fMLP. Two tetrapeptides fMet-Leu-Phe-Ile and fMet-Leu-Phe-Phe that have been synthesised in the laboratory have been demonstrated to be 20 and 10 times more potent respectively, in stimulating lysosomal enzyme release in rabbit neutrophils; a process mediated through the same neutrophil receptor as chemotaxis.[60]

A recent investigation[61] into the environmental sources of fMLP identified it in cultures of *E.coli* from an air sample in a "sick building" and from a urinary tract infection, and in cultures of *Pseudomonas aeruginosa* from a tracheal washing in a patient with cystic fibrosis. They were unable to identify it in *E.coli* cultures from pig intestine, in *Burkholdaria cepacia* cultures from a "sick building" air sample or from cultures of *Yersinia pseudotuberculosis, Staphylococcus aureus, Pseudomonas mallei, Bacillus sp., Streptomyces sp., Enterococcus faecalis, Micrococcus sp., Aeromonas hydrophilus, and*

Clavibacter michiganese from various other sources. They tried several techniques for fMLP detection and had success with high performance liquid chromatography, but even this was hampered by lability of the ligand.

fMLP production within the bowel lumen can be anticipated given the frequency with which E.coli is isolated from it, although it might be present in low concentrations. Marasco et al[62] estimated concentrations in supernatant from a 24 hour pure culture of *E.coli* to be $1.1x10^{-9}$M. Cole et al[63] have investigated the possibility that alterations in the bowel's defence to the peptide may be a factor in maintaining intestinal inflammation in inflammatory bowel disease. These peptides all have molecular weight below 1kDa and can thus be differentiated from chemokines.

Lipid Chemoattractants

Leukotriene B_4

Leukotriene B_4 in contrast to the other leukotrienes is mostly a product of polymorphonuclear leucocytes. In common with them it is produced through the lipoxygenase pathway from arachidonic acid. It acts as a stimulant of neutrophil chemotaxis and chemokinesis[64], and as such may serve to amplify the inflammatory process. The possible amplification of inflammation through LTB_4 has been considered as a possible factor in maintaining inflammation in a number of inflammatory conditions including Ulcerative and Crohn's colitis[65] and cystic fibrosis.[66,67] This may underlie the the therapeutic benefit of fish oil supplementation, which reduces LTB_4 production by

neutrophils.[68] LTB_4 may be present in stool, but this would probably require a degree of intestinal inflammation, and the preparation of faecal supernatant as an aqueous phase should minimize the LTB_4 concentration.

Platelet Activating Factor

Platelet activating factor is also a lipid mediator which is produced in mast cells, basophils and vascular endothelial cells. It is synthesised by the acylation of lysoglyceryl ether phosphorylcholine, which is derived from membrane phospholipid by phospholipase A_2 release of a fatty acid. It is rapidly broken down by enzymes in plasma, casting doubt on its efficacy as a neutrophil chemotaxin in vivo.

Chemokines

Recently a number of small (8-10KDa), inducible secreted pro-inflammatory cytokines have been grouped together under the name chemokines. This name being a shortened version of chemotactically active cytokines. This family have in common a four cysteine motif. They are divided into two families, the C-C or β chemokines which have their cysteine residues adjacent and are chemotactic for macrophages and the C-X-C or α chemokines which have an intervening amino acid, and this sub family contains the chemokines that are chemotactic for neutrophils. Those chemokines have a glutamic acid-leucine-arginine sequence near the amino terminal, immediately preceding the first cysteine residue. These chemokines are IL-8, GRO α, ß and γ, ENA-78, GCP-2 and platelet basic protein and its proteolytic derived products CTAP-III, ß-thromboglobulin

and NAP-2.

Interleukin-8 (IL-8)

Interleukin-8 was the first of the α chemokines to be identified. It is a potent neutrophil chemotaxin and activator, but it is not chemotactic for macrophages. All previously identified neutrophil chemotaxins were chemotactic for the cardinal cells of both acute and chronic inflammation, and it is this specificity of IL-8 that makes it the prime suspect for causing neutrophil infiltration in acute inflammation. IL-8 has subsequently been shown to be chemotactic for T Lymphocytes, basophils and eosinophils.[69] Whilst it was originally identified as a product of monocytes, it has been produced by eosinophils, T lymphocytes, natural killer cells, neutrophils, fibroblasts, endothelial cells, keratinocytes, hepatocytes, astrocytes and chondrocytes. It's production is stimulated by lipopolysaccharide, tumour necrosis factor-α, and interleukin-1.

Interleukin-8 plays a central part in the pathogenesis of a wide spectrum of inflammatory conditions, and it has been of particular research interest in cystic fibrosis, inflammatory bowel disease, psoriasis, arthritis, pyogenic meningitis,[70] cystitis, acute severe alcoholic hepatitis, antral gastritis,[71] respiratory distress syndrome, adult respiratory distress syndrome, and chronic neonatal lung disease. It seems likely that it is a major mediator of acute inflammation.

GROα, ß and γ

GROα, ß and γ expression has been reported in monocytes, fibroblasts, melanocytes, keratinocytes, neutrophils, mammary epithelium and umbilical endothelial cells.[72,73,74,75,76,77,78] It's expression is induced by PDGF or by a variety of inflammatory mediators including IL-1 and TNF-α. It gains it's name from the gene that encodes it: growth related oncogene. Mature GRO-α is produced from a 107 amino acid precursor by the cleavage of it's N-terminal 34 amino acids. It has been extracted from human psoriatic scales.[79,80,81] GROα, ß and γ are potent chemoattractants for human neutrophils, they also induce shape changes, granule exocytosis, respiratory burst activity and a transient increase in cytosolic Ca^{2+} concentration.

In the upper gut GRO alpha expression has been associated with helicobacter pylori infection.[82,83] In the lower gut GRO alpha and GRO gamma expression has been demonstrated in colonic epithelial cell lines following cytokine stimulation, or infection with *Salmonella dublin.*[84] GRO alpha immunoflouresence has been demonstrated in neoplastic colonic mucosa, whilst GRO gamma immunoflouresence has been seen in both neoplastic and healthy colonic mucosae.[85] Thus a proportion of faecal supernatant neutrophil chemotactic activity might possibly be ascribed to GRO alpha, beta or gamma.

Epithelial neutrophil activating peptide-78 (ENA-78)

Epithelial neutrophil activating peptide-78 (ENA-78) is small polypeptide of 78 amino acid residues, with a molecular weight of 8.3kDa. It has 22% amino acid homology with

IL-8, and 52%, 48% and 51% homology with GRO α, ß and γ respectively. It is expressed in neutrophils and the monocyte macrophage series on lipopolysaccharide stimulation. It is highly inducible by IL-1 in endothelial cells, vascular smooth muscle cells, pulmonary epithelial cells and pulmonary fibroblasts.[86,87] Similar to other α-chemokines it is a neutrophil attractant and activator, and it's effect is mediated through a CXC receptor.

ENA-78 is produced in gastrointestinal mucosae. Immunoflourescence and in-situ hybridisation studies have shown ENA-78 in intestinal epithelial cells in Crohn's disease, ulcerative colitis and acute appendicitis tissue.[88] This observation is supported by further immunoflourescence studies and studies on colonocyte cell lines stimulated with IL-1ß or TNFα which have produced ENA-78 eight to twenty four hours after stimulation.[89] ENA-78 can be expected to be present in faecal supernatant particularly when inflammation is present.

Platelet basic protein

Platelet basic protein is believed to be biologically inactive[90], but removal of amino acids does produce biologically active agents CTAP-III, ß-thromboglobulin and NAP-2. Removal of the first nine amino acids from the molecule generates connective tissue activating protein III (CTAP-III) which stimulates chondrocyte proliferation and glycoaminoglycan production.[91] Removal of a further four N-terminal amino acids results in the potent fibroblast and weak neutrophil chemotaxin ß-thromboglobulin.[92]

Proteolytic removal of a further eleven amino acids gives the more potent neutrophil chemotaxin neutrophil attractant peptide II (NAP II).[93] Walz et al.[94] showed that NAP-2, like IL-8 induced a concentration dependent neutrophil chemotaxis between 0.03 and 1nM concentrations. In contrast 10 to 100nM concentration was necessary before CTAP-III and platelet basic protein showed the slightest chemotactic activity, PBP being a little more active than CTAP-III. They concluded that NAP-2, IL-8 and C5a were equipotent chemotaxins.

Granulocyte chemotactic protein-2 (GCP-2)

GCP-2 fulfils the criteria for being an alpha chemokine. It is estimated to be a molecule of 6kDa size. Its maximal effect in producing chemotaxis is comparable with IL-8, but its minimum effective dose is approximately one tenth that of IL-8. It does not seem to be produced widely and to date has only been identified from cultured and stimulated human osteosarcoma cells.[95]

Platelet alpha granule proteins

These include platelet factor 4, platelet-derived growth factor and ß-thromboglobulin. Platelet factor 4 is a potent chemotactic factor for monocytes and neutrophils in concentrations (1-5µg/ml) observed in peripheral blood.[96] Platelet derived growth factor (PGDF) is maximally chemoattractant for monocytes at 0.7pM and for neutrophils at 32pM.[97] ß-thromboglobulin is chemotactic for fibroblasts.[98]

The non IL-8 CXC chemokines, and the platelet alpha granule proteins, can be expected to produce neutrophil chemotaxis that would be difficult to differentiate from that of IL-8. Whilst there is currently no reason to predict the existence of GCP-2, Platelet basic protein, or platelet alpha granule proteins within the gut lumen, the likelihood of ENA-78 and of GROα, ß and γ being present in the gut lumen will need to be considered in the interpretation of chemotactic experiments using faecal supernatant an the chemotaxin.

Chemotaxin receptors

Two distinct human IL-8 receptors have been identified the type 1 receptor IL-8RA and the type 2 receptor IL-8RB. Il-8 binds to both receptors with high affinity and induces rapid elevation of cytosolic Calcium^{2+} concentrations. The type 1 receptor is highly specific for IL-8. It is now termed CXCR2. The type 2 receptor is bound by interleukin-8 and also by NAP2, ENA78 and GROα, ß and γ. It is now termed CXCR1. Three other CXC chemokine receptors CXCR3, CXCR4 and CXCR5 are not activated by interleukin-8.

Additionally a promiscuous chemokine receptor has been identified on the erythrocyte.[99,100,101] It binds many α and ß chemokines without effect on the erythrocyte. IL-8, GRO and NAP2 are among the chemokines that will bind to it. This receptor which is the Duffy blood group antigen is the portal of entry to the erythrocyte for the vivax malaria parasite Plasmodium vivax.[102,103] It provides a sink for chemokines that diffuse into the circulation, and it has been proposed that this allows chemotactic gradients to be

maintained, a function that has also been ascribed to IgG IL-8 autoantibodies.[104] The Duffy antigen has a K_d for binding with IL-8 of 5 x 10^{-9}M, whilst the IgG anti-IL8 antibody binds with a K_d of approximately 10^{-11}M. These data imply that when IgG anti-IL8 antibody is present, it will bind free intravascular IL-8, leaving the Duffy antigen redundant in this respect. Sylvester et al.[105] detected the auto-antibody complexed to IL-8 in 18 of 26 serum samples from healthy donors. Free anti IL-8 IgG was detected in the three donors without complexes that were tested. These findings have not however been universably repeatable and it remains uncertain whether anti IL-8 antibodies play a role in bufferring chemokine that has diffused into the plasma space.

fMLP has its own receptor. It is a peptide of 350 amino acids, with a molecular weight of 30 - 50kDa and in common with the chemokine receptors it is a G protein coupled receptor.[106] Anton et al[107] have shown increased fMLP receptors on circulating neutrophils of patients with Crohn's disease, but not Ulcerative colitis.

C5a, PF4 and the lipid attractants LTB_4 and PAF have other receptors. There is however interaction between activators of neutrophils. Despite having separate receptors, IL-8, fMLP, C5a, PAF and LTB_4 share a common signal transduction pathway, that continues to be activated whilst the ligand is in contact with its receptor.[108] The pathway involves GTP binding proteins, as the GTP binding protein inhibitor *Bordetella pertussis* toxin abolishes activation of the neutrophil by chemoattractants.[109] Activation of Phospholipase C follows and generates inositol triphosphate (IP_3) and diacylglycerol (DAG) as second mesengers. The inositol triphosphate binds to receptors on calcium

containing organelles and mediates an increase in cytosolic Ca^{2+} concentration, whilst the diacylglycerol remains membrane associated and activates protein kinase C, which mediates the respiratory burst.

Stimulation of one receptor will desensitise others, such that C5a, fMLP and IL-8 desensitise each other's calcium mobilising responses, but not that of the lipid mediators PAF and leukotriene B_4.[110] Despite the degree of commonality in their signalling pathways there are marked differences in the ability of receptor stimulation of the various receptors to provoke adhesion and chemotaxis.[111] The interaction between IL-8 and other chemotaxins on neutrophil chemotaxis is not easily predicted. Fortunately the interaction between fMLP receptor stimulation and IL-8 receptor stimulation has been examined specifically in respect of effects on leukocyte adhesion and chemotactic responses. IL-8 pre-treatment had no effect on fMLP stimulated adhesion and migration, but fMLP pre-treatment totally abolished these responses to IL-8,[112] demonstrating the primacy of chemotactic responses to the bacterial product chemotaxin that is most likely to coexist in our faecal supernatant samples.

Implications for neutrophil chemotaxis experiments

One general feature of bioassays is an inability to reliably show small differences in biological response, as a consequence of inherent biological variability. Neutrophil migration studies are burdened with this characteristic. In the current investigation this is a handicap as quantities of chemotaxin in the experimental fluid other than the one of interest, may act to contaminate the results. Efforts will be necessary to negate the effects

of non IL-8 chemoattractants in the faecal supernatant fluid.

Of the non IL-8 chemotaxins, the lipid factors are likely to be removed in the preparation of aliquots of faecal supernatant. They are most likely to reside within the lipid fraction that floats on the aqueous phase after centrifugation, a phase that is not aliquoted. Complement components might be present in the intestinal lumen if they leak from the circulation, but require activation to produce their chemotactic products, and the intestinal lumen would not be an ideal environment for such activation.

In contrast the formyl methionyl peptides are highly likely to be present in the intestinal lumen in concentrations that are well capable of stimulating a near maximal neutrophil chemotaxis. Fortunately these peptides are less than 1kDa in size as opposed to 6kDa for the smallest chemokines. This difference allows separation of these chemotaxins through a chromatography column.

One further bacterial product deserves mention. Bacterial lipopolysaccharide should be present in the supernatant in significant concentrations. Whilst this is not directly chemotactic for neutrophils, it is a potent stimulus for the production of IL-8 and other pro-inflammatory cytokines from macrophages, and of complement activation. Separation of neutrophils from peripheral blood can not be gauranteed to totally exclude macrophages, and it is possible that these are stimulated to produce chemotaxins. Inactivation of lipopolysaccharide with polymyxin B should exclude this potential confounder.

Experiments using chromatography processed samples and polymyxin B coincubated samples can be expected to negate the effects of two of the most likely non IL-8 contaminating neutrophil chemoattractants. These techniques will however not negate the effect of other CXC chemokines. The use of monoclonal antibodies directed against these chemokines, to block the chemotaxis might achieve this, but with the close amino acid sequence homology between many CXC chemokines even this can not be certain. However monoclonal antibody blocking of the CXCR1 receptor would eliminate the influence of the non IL-8 chemokines.

Ligation of the CXCR1 receptor would abolish non IL-8 chemokine neutrophil chemotactic activity. This would not be sufficient to demonstrate that the chemotactic activity remaining was due to IL-8 because formyl methionyl peptides and complement effects would not have been ablated. It will therefore be necessary to conduct studies that can first isolate neutrophil chemotactic activity due to agents in the chemokine molecular weight range.

Chapter 4

Methods 1: Development of ELISA protocol

Having seen disparate results in the initial UK and Australian experiments, that used ELISAs which had not been validated for faecal specimens, it behoves me to validate these assays for this use. Several authors have reported elevated Interleukin-8 or TNFα concentrations in faeces and faecally derived material.[113,114,115,116,117,118,119,120] There is no clear evidence that these enzyme linked immunoassays have been validated for use in such samples. Personal communications with two of the authors confirm that their assays had not been validated.

Here I describe the assay characteristics in assays of faecal IL-8 and TNFα using an unmodified commercial ELISA, subsequent modifications of the assay protocols and validation of the modified protocols. The chapter is divided into five sections. In the first the characteristics of the assays initially adopted in Australia are explored. In the second the assay anomalies detected are investigated, and modifications to the assay protocol suggested. The third section describes the validation of the modified assay protocol. The fourth section investigates the intra and inter assay variation of the modified assay, whilst the final section explores the potential of using other commercially available cytokine ELISAs for faecal samples.

The issues addressed are linearity, accuracy and reproducibility, and the methods used in the five sections are similar.

Linearity is investigated by measuring cytokine concentrations at multiple dilutions, and examining the variability of the calculated sample cytokine concentration from the various dilutions. Accuracy is assessed by measuring the cytokine concentration in a sample that has previously been shown not to contain cytokine immunoreactivity, which is then spiked to a known concentration, using cytokine supplied with the ELISA which is intended for construction of the assay's standard curve. For economy, accuracy and linearity were frequently assessed in parallel in the same assays. Reproducibility was evaluated by repeatedly assaying aliquots of the same sample, both within one assay and in multiple assays, allowing the calculation of intra and inter-assay coefficients of variation.

In general linearity and accuracy experiments used spiked samples from patients with cystic fibrosis and reproducibility experiments used unspiked samples from patients with inflammatory bowel disease.

Common methods

Sample collection and processing

Faecal samples were taken from patients with Cystic Fibrosis or Inflammatory Bowel Disease attending the Royal Children's Hospital Brisbane whose parents had given informed consent. Stools were homogenised in either distilled water or Phosphate Buffered Saline (PBS) to achieve a manageable consistency. Samples from the homogenates were diluted further in PBS, centrifuged at 20000g for 15 minutes, and

supernatant was frozen at -70°C until the assays were performed.

Assay techniques (sections 1 &2)

Thawed samples were assayed in the appropriate R&D Quantikine kit, catalogue number DTA50 for TNFα and D8000 for IL-8, according to the manufacturer's instructions for non-serum samples. Briefly 50 or 100µl of assay diluent was placed in each well of a pre-coated 96 well plate. An equal volume of standard or sample was added to the appropriate wells in duplicate, covered and incubated at room temperature for 2 hours. Wells were emptied washed three times and 200µl of horse-radish peroxidase conjugated secondary antibody was added and incubated for 1 or 2 hours. Wells were again emptied and washed thee times, and 200µl of substrate was added and wells were incubated for 20 minutes at room temperature. Finally 50µl of 2N sulphuric acid stop solution was added and the optical density of each well was determined at 450nm in a plate reader with optical correction at 570nm. Absorbances were calculated and cytokine concentrations were determined using a regression equation derived from the standard and blank wells.

Section 1: Characteristics of Quantikine TNFα and IL-8 ELISA kits

Introduction

Interleukin-8 has been assayed in the initial UK and Australian faecal samples, with conflicting results. The IL-8 ELISA used in the UK was an in-house assay and it's components were not readily available in Australia. The TNFα ELISA used in the two countries was identical. As the cytokine of greatest interest in the current study was IL-8, the locally available IL-8 ELISA was investigated first. The first step in investigating the disparity is to show that the assay employed accurately measures sample IL-8 concentration, irrespective of sample dilution. On the one hand assay accuracy throughout the assayable range was investigated with variable spiking of one aliquot, and on the other hand by spiking variable dilutions of aliquot to the same spike concentration. Assay linearity was then assessed in samples from a range of patients. Finally these samples were assayed in a similar manner for TNFα, to allow comparisons to be made between the two assay systems.

Methods

IL-8 linearity and accuracy exploratory experiment

Aliquots of faecal supernatant, from two patients with advanced Cystic Fibrosis chest disease, duplicates of which had previously been shown to have no IL-8 immunoreactivity, were subjected to serial 10 fold dilutions in the diluent (RD5)

provided for this purpose in the Quantikine kit. These dilutions were then spiked to a concentration of 500pg/ml IL-8 prior to assay. Additionally, neat aliquots from these patients were spiked with serial 2 fold dilutions of IL-8 to achieve aliquot concentrations ranging from 1500 to 93.75pg/ml.

IL-8 linearity confirmatory and TNFα linearity experiments

To confirm that any observed assay characteristics were not solely idiosyncrasies of these two patients, repeat aliquots from these two patients, aliquots from seven other children with cystic fibrosis, one healthy control and one boy with severe Crohn's colitis in both the acute phase and in remission were selected for linearity assays for IL-8. These aliquots were 6 fold dilutions of faeces (i.e. 6 grams of supernatant was produced from 1 gram of faeces). Duplicate samples were either spiked to a concentration of 1000pg/ml IL-8 neat, or diluted x100 and spiked to a concentration of 500pg/ml IL-8 before assay. All selected aliquots had been shown to have no significant IL-8 or TNFα immunoreactivity. In the case of the acute crohn's colitis sample this had followed a prolonged period of incubation at room temperature. Duplicates of eight of the aliquots assayed with IL-8 spikes were spiked with TNFα to a concentration of 125pg/ml, they were additionally diluted x100 in the supplied diluent and spiked to a concentration of 62.5pg/ml before assay. Recovery of spiked cytokine is expressed as a percentage of that administered.

Results

IL-8 accuracy exploratory experiment

Tables 4.1 & 4.2 (Figs 4.1 and 4.2) show the recoveries of IL-8 achieved when faecal supernatant was assayed as a non serum sample at multiple dilutions in the two initial CF patients.

Variable dilution

The mean(SD) spike recoveries were 32.8 (12.1)% for samples spiked whilst neat and 156.2 (69.0)% for those spiked following dilution in the supplied diluent. There was a maximum recovery of 46% of administered spike in the former samples, but apparent recoveries approached 200% in the latter samples from both patients.

Variable spike

No clear trend was seen in the relationship between spike dosage and spike recovery.

Figure 4.1

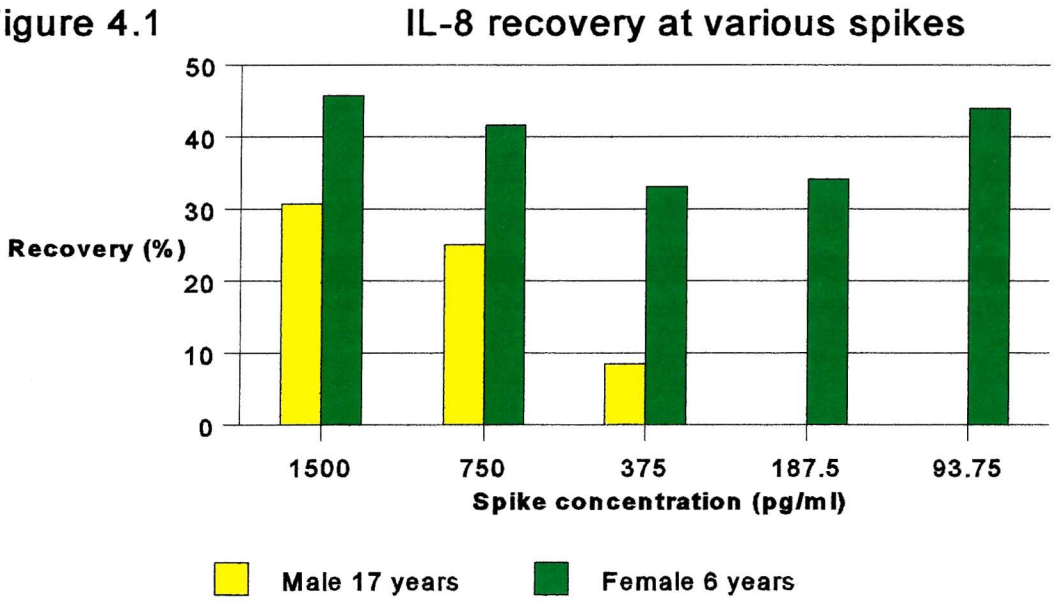


Table 4.1

RD5	Male age 17 years		Female age 6 years	
IL-8 spike pg/ml	Measured IL8 pg/ml	Recovery %	Measured IL8 pg/ml	Recovery %
1500	460.32	30.68	686.15	45.74
750	187.97	25.06	312.61	41.68
375	31.84	8.49	124.05	33.08
187.5	0	0.00	63.95	34.11
93.75	0	0.00	41.25	44.00

Figure 4.2 Recovery of 500pg/ml IL-8 spike at various dilutions

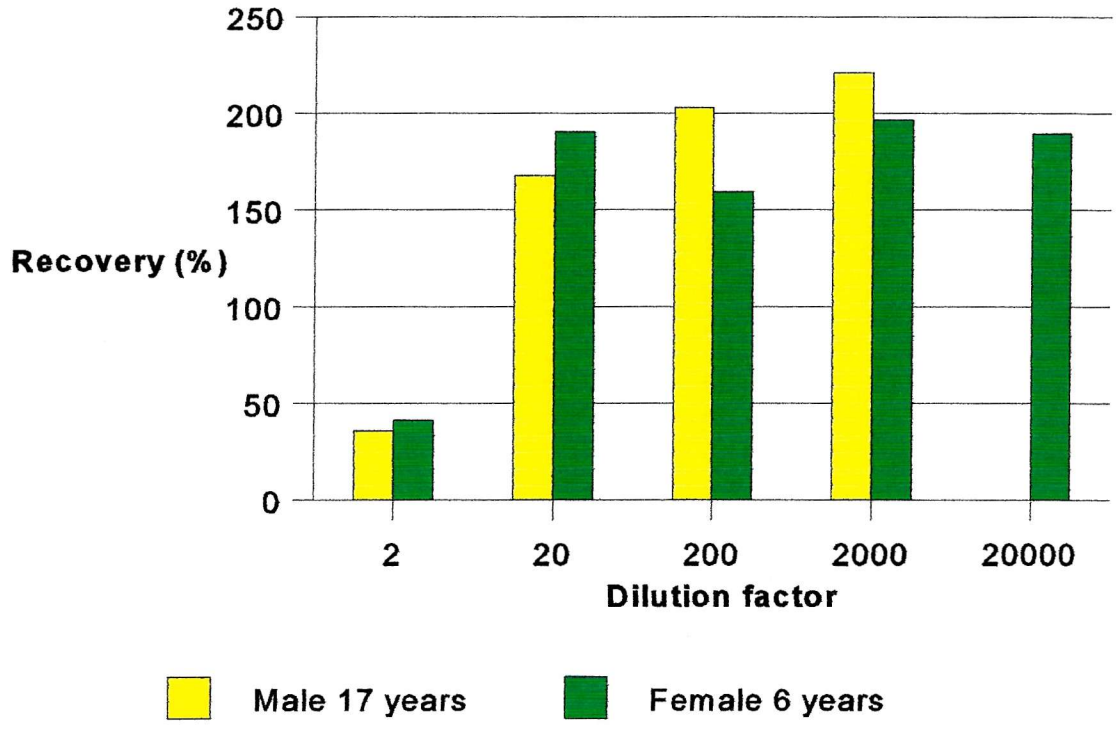


Table 4.2

500pg/ml spike	Male age 17 years		Female age 6 years	
Diln aliquot (%)	Measured IL8 pg/ml	Recovery %	Measured IL8 pg/ml	Recovery %
50	184.47	35.89	207.07	41.41
5	839.10	167.82	953.39	190.68
0.5	1015.16	203.03	797.59	159.51
0.05	1105.16	221.03	983.56	196.71
0.005	not performed		947.67	189.53

IL-8 linearity confirmatory experiment

Interleukin-8 spike recoveries for the samples from the other patients are shown for both dilutions employed in table 4.3. With the exception of the acute Crohn's colitis sample, where recovery fell from 108% to 79% with dilution, percentage recovery of the IL-8 spike was greater in all prediluted samples than their neat counterparts. In contrast to the samples from the initial two CF patients, recoveries did not approach 200%. Recoveries

for the 9 CF patients are illustrated in figure 4.3. Mean (SD) spike recovery in the diluted samples of 107.5 (11.6)% was greater than the corresponding value of 45.6 (27.0)% in the neat samples (t=6.14, p=0.0007, paired two tailed t test). On average recovery in the more dilute sample was 1.27 times greater than the recovery for the more concentrated sample.

Figure 4.3 Two dilution IL8 recoveries

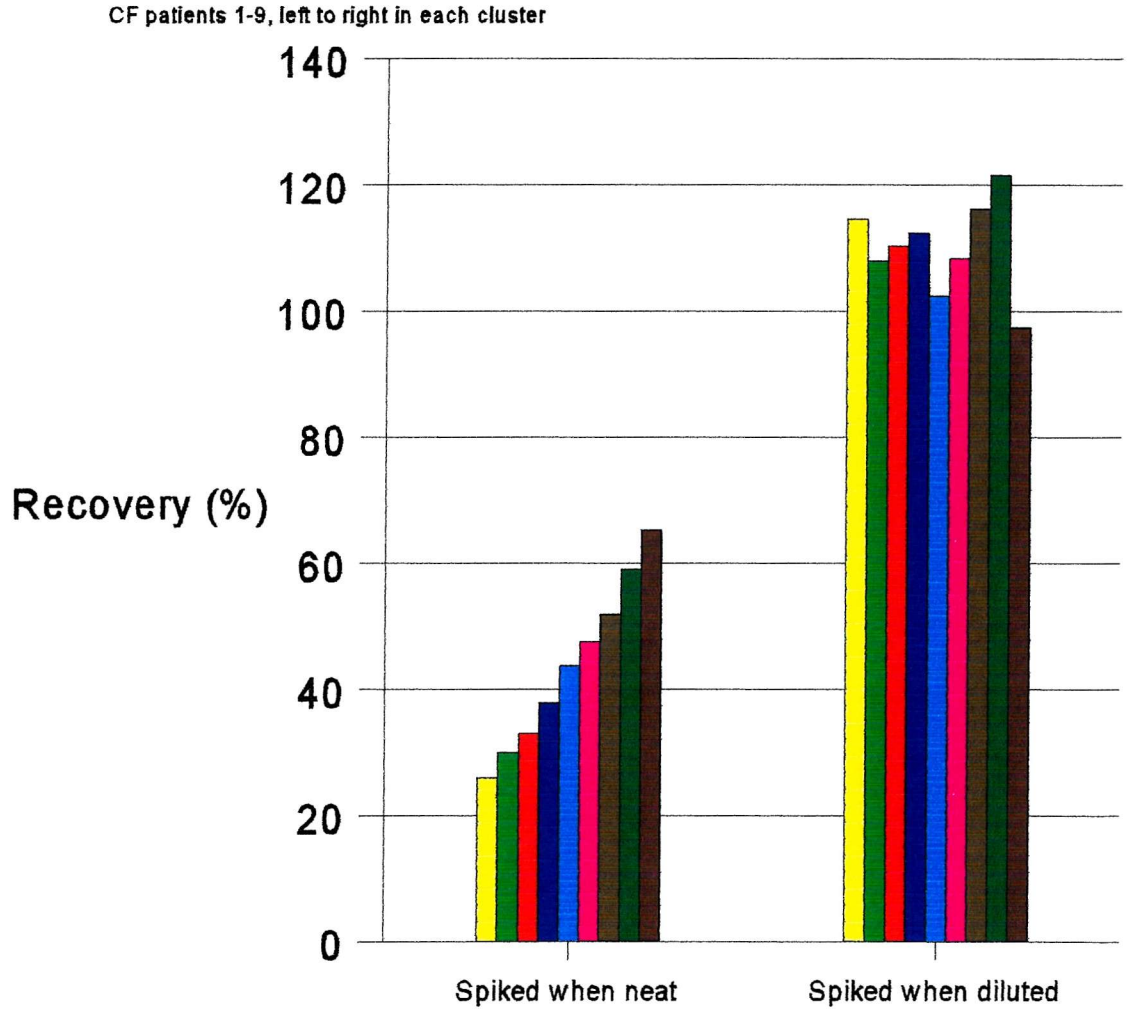


Table 4.3 Two dilution Il-8 recoveries

		spiked to 1000pg/ml		spiked to 500pg/ml	
No	Patient	[IL8]	Recovery %	[IL8]	Recovery %
492	Healthy male	17	1.7	574	114.8
145	Male Crohn's (Acute)	1082	108.2	384	76.8
513	Male Crohn's (Convalescent)	184	18.4	537	107.4
31	Female CF 1	260	26.0	573	114.6
427	Male CF 2	300	30.0	540	108.0
16	Male CF 3	331	33.1	552	110.4
634	Female CF 4	379	37.9	562	112.4
382	Female CF 5	438	43.8	512	102.4
456	Male CF 6	476	47.6	542	108.4
537	Female CF 7	519	51.9	581	116.2
494	Male CF 8	591	59.1	608	121.6
665	Female CF 9	653	65.3	487	97.4

linearity of the Quantikine DTA50 TNF alpha ELISA

Cytokine recoveries are shown in table 4.4 and figure 4.4. The mean (SD) spike recovery in the prediluted samples of 211 (16.0)% was significantly greater than the spike recovery from the neat samples 64.8 (30.9)% (t=18.7, p=0.0000003, paired two tailed t test). The diluted sample recovery was a mean of 3.83 times that in the concentrated sample.

Figure 4.4 Two dilution recovery of TNF alpha spikes

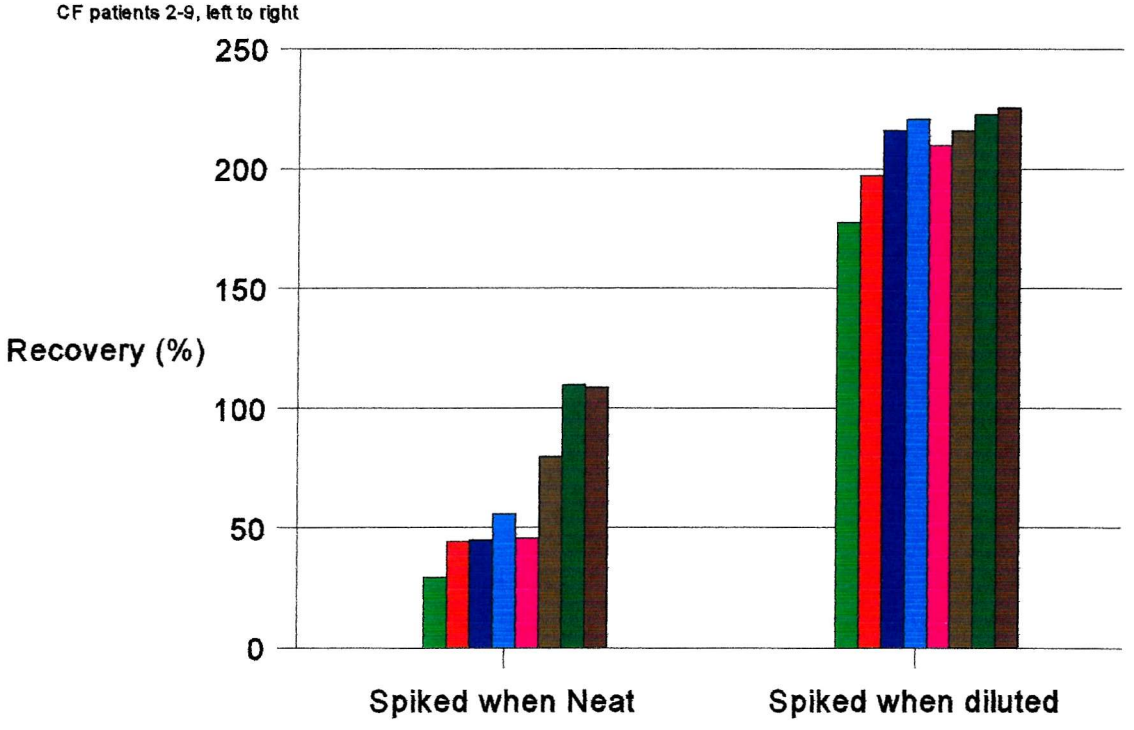


Table 4.4 Two dilution TNFα recoveries

Patient	No	Neat spiked to 125pg/ml		Diluted spiked to 62.5pg/ml	
		Measured TNFα	Recovery %	Measured TNFα	Recovery %
Male CF 3	17	55.36	44.29	123.2	197.12
Female CF 5	383	69.90	55.92	138.0	220.80
Male CF 2	428	36.60	29.28	111.0	177.60
Male CF 6	457	57.14	45.71	131.0	209.60
Male CF 8	495	137.20	109.76	139.1	222.56
Female CF 7	538	99.70	79.76	135.0	216.00
Female CF 4	635	56.10	44.88	135.0	216.00
Female CF 9	666	136.00	108.80	141.0	225.60

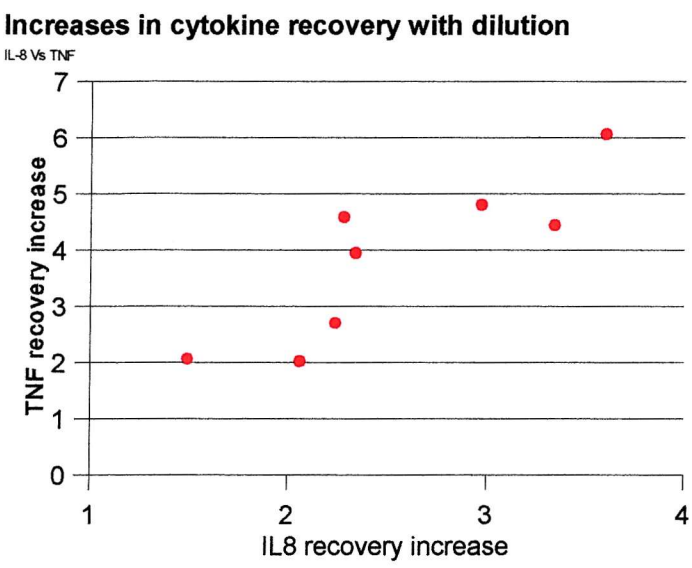
Discussion

These strikingly non linear assay characteristics were not idiosyncrasies of the initial two CF patients, having been reproduced in several other patients. They are however highly unusual. Control samples had no IL-8 immunoreactivity, therefore the 200% recoveries seen here relate to assay malfunction rather than a high cytokine concentration prior to spiking. Knowledge of the mechanism leading to these erroneous results might allow this phenomenon to be overcome.

In this ELISA there is the potential for interference in cytokine to capture antibody binding in the initial incubation phase, however once the assay plate has been washed to remove any unbound sample, conditions are highly controlled. The secondary antibody requires bound cytokine in the 96 well plate to facilitate its own binding, and when apparent spike recoveries greater than 100% are achieved, it is likely that the amount of bound secondary antibody has been artefactually increased. A contaminant in the sample might achieve this either by binding to the primary antibody or the cytokine molecule or by adhering to the plate and resisting the post sample incubation plate wash. The persistence of the effect at high dilution suggests that any binding between such a substance and the cytokine is likely to be strong. A similar pattern was seen in the TNFα assay.

If a single substance were to be responsible for non linearity of both IL-8 and TNFα assays then the effects of this substance in the one assay might be proportional to its

effects in the other. When the factors by which recovery increased in the IL-8 assay were compared with the corresponding factors in the TNFα assay. There was a significant correlation between these two improvement factors (r=0.86 95%CI 0.39-0.97).



The presence of an effect of proportional magnitude in both assays makes substances specific to either cytokine unlikely to be responsible for these effects. Anti IL-8 autoantibodies and soluble TNFα receptor, which could both avidly bind their respective cytokines were therefore unlikely to be responsible for these phenomena.

Both assays have in common the dilution of samples by a factor of 100 and the use of a 50% spike reduction in the dilute sample. Either dilution or reduced spike dosage could influence the recovery, but an effect of an aliquot constituent would better account for variations in the relationship between recovery in the IL-8 and TNFα assays. The persistence of an apparent 200% spike recovery at dilutions of 1:2,000 and 1:20,000 in the initial IL-8 linearity experiment make a weak and non specific binding agent unlikely to be responsible for these data. At these dilutions, the chemical constitution of the assay well contents would be similar to that of the standard wells.

One interpretation of this is that the assay plate blocking protein may have been enzymatically removed from the plate allowing excessive secondary antibody binding to the plate after the washing step. Whilst this would be a high affinity non cytokine specific process, it is not fully consistent with the observations in this study, because an apparently high cytokine concentration would have been expected even in the unspiked and cytokine free samples, and this was not seen. If cytokine binding with the polystyrene plate were responsible, no cytokine immunoreactivity would be expected in unspiked samples. This mechanism implies that the Kd for cytokine to plate binding was of the order of twice that for cytokine antibody binding. However it does not in itself, explain the consistent reduction in cytokine recovery seen in the neat samples. There may be more than one factor in the faecal supernatant contributing to the anomalous results. For example an agent which enzymatically removes the plate blocking protein might have pH critical activity. In this case reduced cytokine recovery in neat samples could result from suboptimal antigen antibody binding conditions, and in samples diluted in buffer, both antigen antibody binding and proteolysis of the blocking protein might increase.

Alternatively these results could be consistent with the presence of a superantigen in the faecal supernatant. Superantigen could bind to the Fab portion of the capture antibody outside of the antigen binding folds, and act to cross link with the secondary antibody, allowing an increased secondary antibody binding with apparently increased cytokine immunoreactivity. A superantigen would not normally require the presence of another antigen binding to the antibody for its own activity, and this hypothesis does not explain why no apparent cytokine immunoreactivity was seen when unspiked controls were

assayed in this environment, unless under these circumstances, in the absence of competing antigen, the superantigen crosslinks multiple capture antibodies and renders them unable to present their Fab regions to secondary antibody.

Five molecules are known to bind the Fab fragments of human immunoglobulins.[121] Protein A binds to the VH3 domain of the heavy chain, whilst Protein G binds to the CH1 domain of the heavy chain. These two proteins have been extensively studied and are widely used in the laboratory purification of antibodies. Protein L also binds to the CH1 domain whilst Protein P binds to immunoglobulin polymers. Protein Fv binds to the VH domains[122], but when bound does not diminish antigen antibody binding. It binds to all antibody classes across all taxonomic classes with the exceptions of horse, sheep, goat and cow antibodies where binding is prevented by the immunoglobulin's Hydrogen bonds.[123] It came to prominence as an agent giving false positive results in an ELISA for Hepatitis E associated antigen in faeces.[124] Whilst initially thought to be secreted in patients with hepatitis, it has subsequently been demonstrated in the gut of normal individuals.[125] Here it is saturated with six Fab sites bound to a single Fv molecule, and it has been suggested that this allows cleaved immunoglobulin Fab sites to bind antigen, whilst in the intestinal lumen, providing one line of mucosal defence.

Four of the five known Fab binding proteins are bacterial products and might be expected to be present in faeces, whilst the fifth is secreted into the gut and is known to have characteristics that could account for the unreliable ELISA results seen here.

Potentially, the activity of these molecules could be removed with antibody pretreatment of the faecal supernatant samples. The exploration of this possibility is described in section 2.

Section 2: Investigating a possible superantigen content of the faecal supernatants

Introduction

A selection of human antibodies were available from commercial suppliers and a solution of Human IgG was selected as experimental agent purely on the basis of cost. Whilst it was logical to use the supplied assay diluents as one control, a negative control was also desirable. Calves do not acquire IgG by trans-placental transfer, but do so orally after birth by suckling colostrum. In their post natal period their gut is permeable to macromolecules until gut closure occurs late in the first week of life.[126,127,128] Newborn calf serum was an appropriate negative control. The most simple experimental design would involve addition of the diluent under investigation to the faecal supernatant aliquot, and this was adopted in this experiment.

Methods

A human IgG concentrate (Sigma immunochemicals, St Louis, MO,USA, catalogue number I-8640), the diluent supplied for serum samples (RD6a) and newborn calf serum (Sigma) were selected as diluents for the samples. Two, four and eight fold dilutions of faecal supernatant from a single 17 year old male patient with end-stage CF lung disease, and of phosphate buffered saline were produced. These were spiked with IL-8 to 500pg/ml and assayed according to the manufacturers instructions. Control samples were

of neat aliquot. Additionally an eight fold dilution of PBS and supernatant was spiked to 250pg/ml and assayed for each diluent, in order to separate the effects of sample predilution from those of spike dosage.

The experiment was repeated for TNFα, omitting the supplied diluent (RD6a), using a standard TNFα spike concentration of 83.3pg/ml and 41.6pg/ml for the additional eight fold dilution sample.

Statistical Analysis

Recovery of spiked cytokine is expressed as a percentage of that administered. Analysis of variance has been used to detect significant differences between recovery of cytokine from the diluents, with t-Tests to compare subgroups. The null hypothesis for these tests was that the diluents were equally close to ideal diluent behaviour, and it was the differences between observed and expected cytokine concentration that was subjected to analysis of variance.

Results

Fig 4.5a Human IgG as diluent

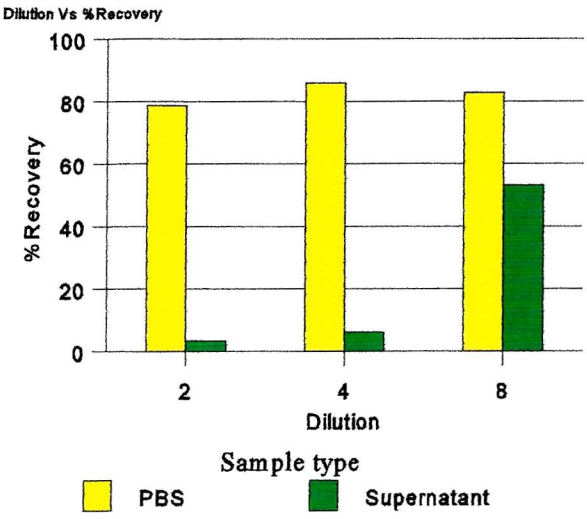
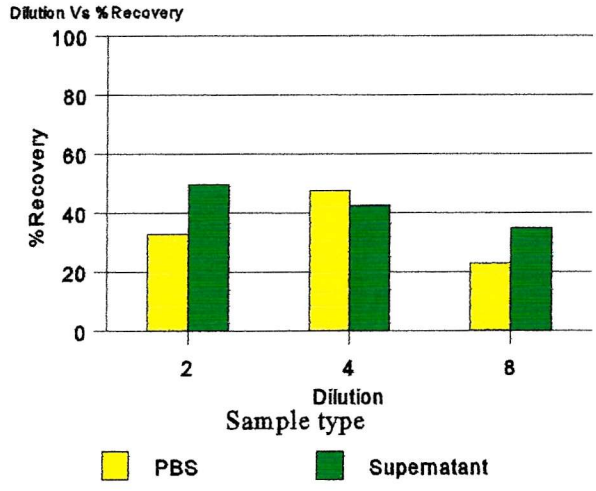


Fig 4.5b RD6a as diluent



Interleukin-8 assay

Results of the IL-8 spikes in the new diluents are shown in table 4.5 and figures 4.5a-c. The mean recoveries of the 500pg/ml spike of IL-8 in phosphate buffered saline were 82.5% (Human IgG), 34.5% (RD6a) & 100.1% (Calf serum). In dilutions of faecal supernatant mean spike recoveries were 20.9% (Human IgG), 42.4% (RD6a) & 96.9% (Calf serum). By ANOVA there were significant differences amongst these means ($F=9.97$ $p=0.0028$). Spike recovery was greater in newborn calf serum dilutions of faecal supernatant samples, than both RD6a ($t=7.98$ $p=0.0013$) and Human IgG dilutions ($t=4.46$ $p=0.011$). There was no significant difference between the supplied diluent RD6a and Human Immunoglobulin cytokine recoveries ($t=1.286$ $p=0.267$).

In phosphate buffered saline samples spike recovery with RD6a dilution was significantly lower than with IgG (t=6.45 p=0.003) but not calf serum (t=0.92 p=0.4) dilution.

Mean spike recovery for 8 fold dilutions was 74.1% for all 500pg/ml spikes and 58.6% for 250pg/ml spikes. These were not significantly different (t=0.9 p=0.39), implying that any effect of spike dosage on percentage spike recovery is small relative to the effect of aliquot predilution.

Fig 4.5c Newborn Calf serum as diluent

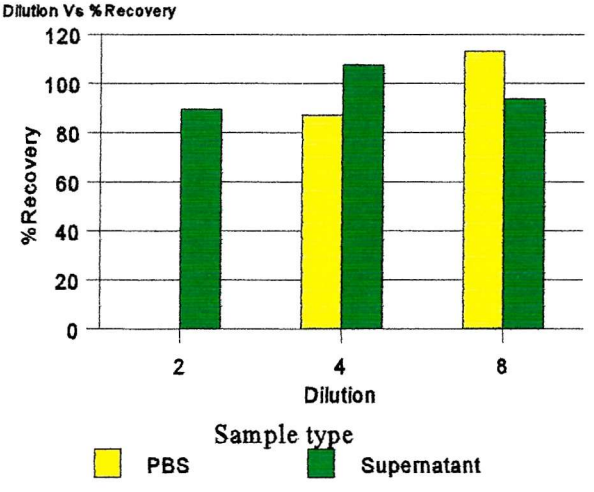


Table 4.5

Diln	Spike	Well IL-8	Recovery %	Well IL-8	Recovery %
		PBS		Supernatant	
Human IgG as Diluent					
2	500	394.0	78.80	16.5*	3.30
4	500	430.0	86.00	30.9*	6.18
8	500	414.0	82.80	266.0	53.20
8	250	195.0	78.00	86.4	34.56
RD6a as Diluent					
2	500	165.0	33.00	248.0	49.60
4	500	238.0	47.60	213.0	42.60
8	500	115.0	23.00	175.0	35.00
8	250	86.4	34.56	78.8	31.52
Newborn Calf Serum as Diluent					
2	500	Failed		448.0	89.60
4	500	436.1	87.22	537.0	107.40
8	500	565.7	113.14	468.8	93.76
8	250	209.9	83.60	223.1	89.24

TNFα assay

Fig 4.6a Human IgG as diluent

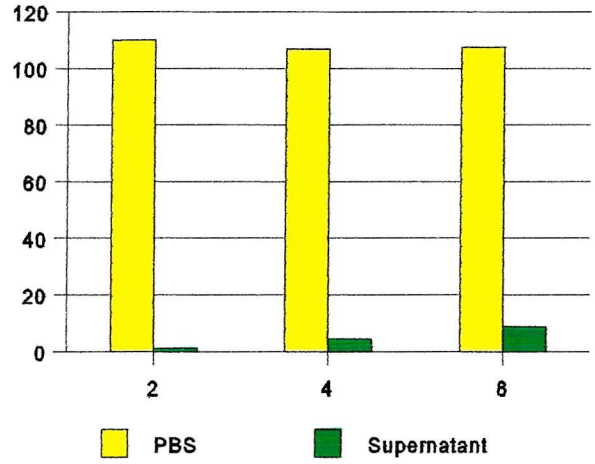
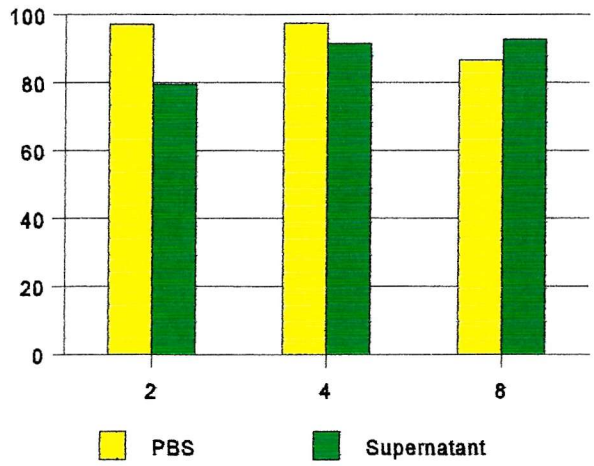


Table 4.6 and figures 4.6a & b show spike recovery results for the TNFα assay. The mean recoveries of the 83.3 pg/ml spike of TNFα in phosphate buffered saline were 108.2% (Human IgG) & 93.8% (Calf serum). In dilutions of faecal supernatant mean spike recoveries were 4.8% (Human IgG) & 87.9% (Calf serum). By ANOVA there were significant differences amongst these means ($F=130.9$, $p=3.8\times10^{-7}$). Calf serum dilution resulted in a greater spike recovery than human IgG dilution in both faecal supernatant ($t=11.37$ $p=0.0003$), and PBS samples ($t=4.2$ $p=0.013$).

Fig 4.6b Newborn calf serum as diluent



For the eight fold dilutions the 74.0% mean spike recovery for the 83.3 pg/ml spike was not significantly different from the 67.1% mean spike recovery for the 41.65 pg/ml

spikes (t=0.23, p=0.82).

Table 4.6

Diln	Spike	Well TNFα	Recovery %	Well TNFα	Recovery %
		PBS		**Supernatant**	
Human IgG as diluent					
2	83.3	91.7	110.08	0.83	0.99
4	83.3	89.1	106.96	8.31	4.54
8	83.3	89.7	107.68	16.30	8.91
8	41.6	35.1	84.27	3.86	4.22
Newborn Calf Serum as diluent					
2	83.3	81.03	97.27	66.20	79.47
4	83.3	80.17	97.44	76.20	91.48
8	83.3	72.14	86.60	77.30	92.79
8	41.6	39.24	94.21	35.66	85.61

Discussion

Spike recovery of both IL-8 and TNFα from Human IgG diluted phosphate buffered saline samples was close to 100%, but was markedly reduced in faecal supernatant samples. Whilst there was increasing recovery of cytokine with dilution, and therefore greater concentration of IgG, in both assays, this concentrated IgG solution has not significantly reduced superantigen binding. Either this model of the mechanism of faecal supernatant interference in the assay is not correct, or administration of this formulation of human IgG can not counteract it. Whilst these issues could be explored further, this will not be done here.

Newborn calf serum was not expected to have any better linearity characteristics than the supplied diluent. Fortuitously it achieved spike recoveries close to 100% irrespective of dilution, cytokine, spike dosage, or whether samples were faecal supernatant or phosphate buffered saline. This suggested that it might be a suitable diluent for use in faecal supernatant cytokine ELISAs.

These IL-8 and TNFα assays have been conducted using samples from a single patient, and the extent to which these observations are representative of other paediatric cystic fibrosis faecal supernatant samples requires formal testing. Intra and inter assay variability must also be determined. These experiments will be described in sections 3 and 4.

Section 3: Validity of Newborn Calf serum as an assay diluent

Testing Newborn Calf Serum diluent in spiked samples

Introduction

In order to investigate linearity and spike recovery in a range of patients, their samples were to be assayed neat without spiking as a control and spiked and diluted at two serial dilutions. This approach allows the calculation of mean percentage spike recovery, whilst the mean variation in calculated spike concentration from results for the serial dilutions gives a numerical indication of the overall linearity of the assay. All selected dilutions were to fall within the assayable range for the ELISA. In the expectation of a 100% spike recovery, IL-8 spikes of 2000, 500 and 125pg/ml and TNFα spikes of 250, 62.5 and 13.125pg/ml, or of 500, 125 and 32.5pg/ml, would satisfy these constraints.

Methods

IL-8 assay

Aliquots from eight patients with cystic fibrosis were diluted 1:1 in Newborn calf Serum as controls & 1:1:1 Aliquot :NCS: 6000pg/ml IL-8 spike solution, to produce a three fold dilution of aliquot containing 2000pg/ml IL-8. This spiked solution was then subjected to two serial four fold dilutions in NCS. The spiked aliquots were incubated at room temperature for 1 hour prior to assay in parallel with unspiked controls.

TNFα assay

The experiment was repeated for TNFα, with the modification that the initial spike solution of 250pg/ml was produced by adding 1 volume of 1000pg/ml TNFα standard to 1 volume of NCS and two volumes of Aliquot.

Results

No cytokine immunoreactivity was detected in the control unspiked samples in either assay. The measured recoveries for the IL-8 assay are shown in table 4.7 & figure 4.7 and corresponding data for the TNFα assay are shown in table 4.8 & figure 4.8. The mean (SD) spike recovery was 92.12 (12.48)% for the IL-8 assays and 91.48 (5.57)% for the TNFα assays. By two way ANOVA there were no significant differences in recovery from the three separate dilutions in either the IL-8 assay ($F=0.229$, $p=0.80$), or the TNFα assay ($F=0.89$, $p=0.43$), or between the various patients' samples in the IL-8 assay ($F=1.80$, $p=0.16$). There were however significant differences between the recoveries of TNFα spike from the supernatant samples of the various patients ($F=5.58$, $P=0.003$).

One sample's dilution series (Aliquot 430 in figure 4.7) is noteworthy as an outlier. In this series IL-8 spike recoveries of 83.2% and 93.28% were achieved at 3 fold and 48 fold dilution, whilst a recovery of 42.06% was observed at the intermediate 12 fold dilution. It is this result which accounts for the larger standard deviation of the IL-8 assay's recoveries, when compared to the standard deviation in the recoveries in the TNFα assay.

Figure 4.7 Newborn calf serum as diluent for IL-8

Recoveries at 3 dilutions in 8 patients

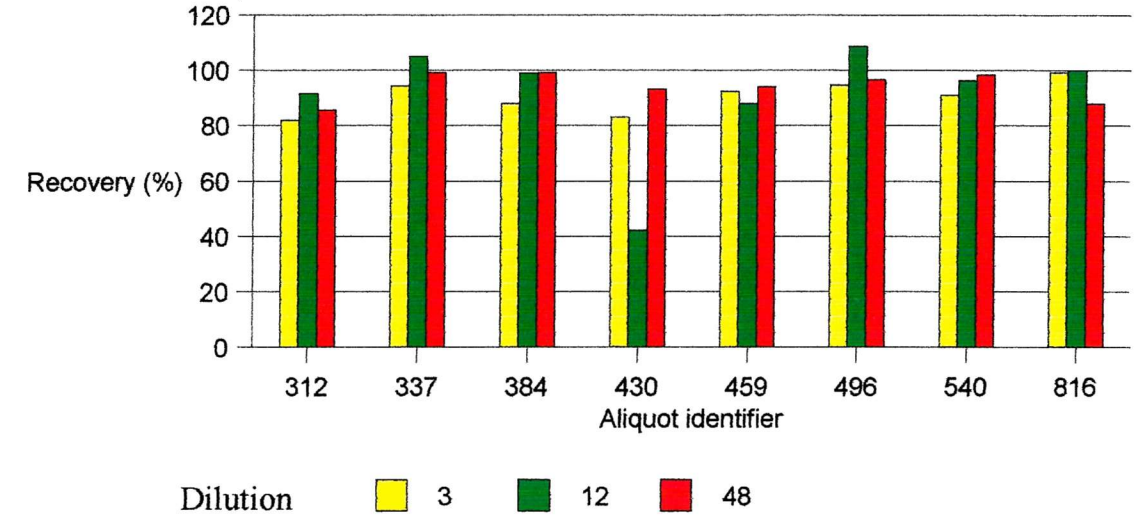


Figure 4.8 Newborn calf serum as diluent for TNF alpha

Recoveries at three dilutions from eight patients

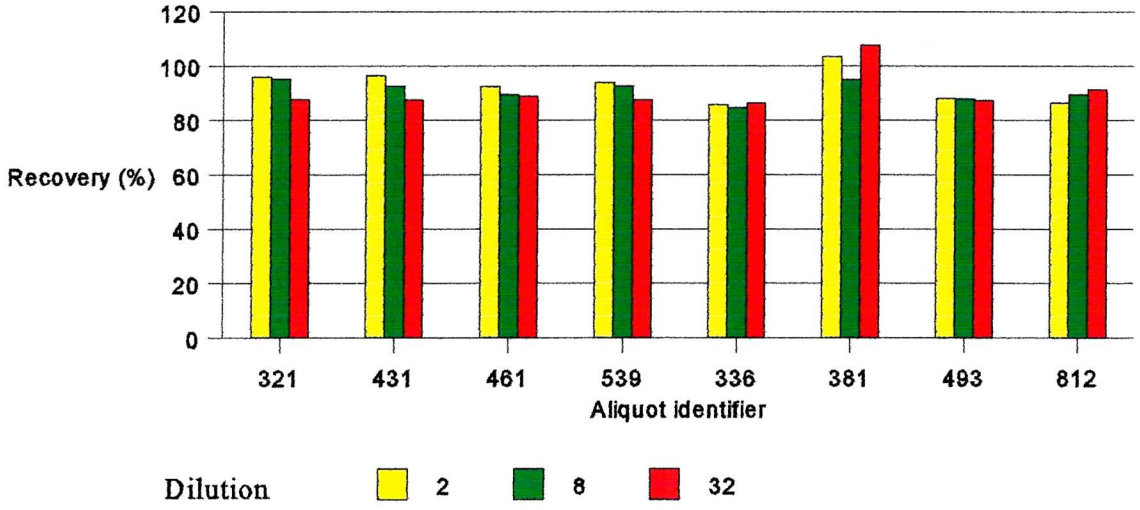


Table 4.7 (Part 1) IL-8 recoveries newborn calf serum as diluent

Aliquot	Diln	Spike	Well IL-8	Recovery %
312	2	0	*2.02	
	3	2000	1640.00	82.0
	12	500	459.00	91.8
	48	125	107.00	85.6
337	2	0	*2.02	
	3	2000	1889.00	94.45
	12	500	525.00	105.0
	48	125	124.00	99.2
384	2	0	*1.16	
	3	2000	1760.00	88.0
	12	500	495.00	99.0
	48	125	124.00	99.2
430	2	0	*22.94	
	3	2000	1664.00	83.20
	12	500	210.40	42.06
	48	125	116.60	93.28

* Value calculated by extrapolation

Table 4.7 (Part 2) IL-8 recoveries newborn calf serum as diluent

Aliquot	Diln	Spike	Well IL-8	Recovery %
459	2	0	*0.0	
	3	2000	1848.00	92.40
	12	500	439.90	87.98
	48	125	117.70	94.16
496	2	0	*3.26	
	3	2000	1896.00	94.80
	12	500	544.00	108.80
	48	125	121.00	96.80
540	2	0	*3.91	
	3	2000	1822.00	91.10
	12	500	482.00	96.40
	48	125	123.00	98.40
816	2	0	*7.01	
	3	2000	1985.00	99.25
	12	500	500.00	100.00
	48	125	110.00	88.00

* Value calculated by extrapolation

Table 4.8 (Part 1) TNFα recoveries newborn calf serum as diluent

Aliquot	Diln	Spike	Well TNFα	Recovery %
336	2		1.09	
	2	500	428.88	85.78
	8	125	105.89	84.71
	32	31.25	27.02	86.46
381	2	0	0.43	
	2	500	517.34	103.47
	8	125	118.81	95.05
	32	31.25	33.68	107.78
493	2	0	1.99	
	2	500	440.97	88.19
	8	125	109.94	87.95
	32	31.25	27.32	87.42
812	2	0	1.09	
	2	500	433.08	86.62
	8	125	111.96	89.57
	32	31.25	28.54	91.33

Table 4.8 (Part 2) TNFα recoveries newborn calf serum as diluent

Aliquot	Diln	Spike	Well TNFα	Recovery %
321	2		0.00	
	2	250	240.20	96.08
	8	62.5	59.60	95.36
	32	15.625	13.40	87.86
431	2	0	0.00	
	2	250	241.00	96.40
	8	62.5	57.90	92.64
	32	15.625	13.70	87.68
461	2	0	0.00	
	2	250	231.00	92.4
	8	62.5	56.00	89.6
	32	15.625	13.90	88.96
539	2	0	0.40	
	2	250	235.00	94.00
	8	62.5	57.90	92.64
	32	15.625	13.70	87.68

Discussion

With the single exception of the outlying IL-8 recovery of 42%, these results show consistently good and consistently linear assay characteristics. It is fascinating in this outlier that spike recovery should fall when the spiked sample has been diluted x4 only to rise again when this sample is again diluted x4. This observation suggests that whilst the use of newborn calf serum as a diluent has improved the characteristics of the assay it has not eliminated the possibility of aberrant results. Further evidence in favour of diminution rather than elimination comes from the observation that there are significant differences in TNFα spike recovery between the various patients. Further modifications to the assay protocol are necessary to render it reliable for experimental use.

It will be necessary to conduct each experimental assay at more than one dilution to confirm that the assay has had linear characteristics in the sample under investigation. To minimise assay costs I elected to assay at two dilutions and to accept the result for an individual sample if the two individual estimates of cytokine concentration were within 10% of their mean. The standard deviation for recoveries in the TNFα assay, which had no outlying values, was 5.57%. The 10% linearity constraint is therefore simple and approximates to +/- two standard deviations of the expected recovery.

Section 4: Intra and inter-assay variation

Introduction

Having arbitrarily elected to assay cytokine concentration at two dilutions and accept the assay as linear if the results from these dilutions lie within 10% of their mean, it is necessary to assess the repeatability of this assay protocol, if the results of repeated assays are to be correctly interpreted.

This was achieved by measuring intra and interassay variation in repeated assays for both cytokines. Here the coefficients of variation were taken as the standard error of the mean expressed as a percentage of the mean.

TNFα assay Intra and Inter assay variation

A single sample of faecal supernatant from a patient with severe Crohn's colitis was assayed ten times at two dilutions in one assay. The aliquot cytokine concentration was calculated for each dilution pair. For all pairs the difference between the apparent aliquot cytokine concentration, calculated from the neat sample, was within 10% of that calculated from the dilute sample. Whilst 10% had been selected as an arbitrary limit for quality control, in fact the maximum difference was 5.77% implying good linearity in this sample in this assay. The mean (SEM) aliquot TNFα concentration for these 10 pairs was 1032.738pg/ml (47.15). The intra-assay coefficient of variation (SEM expressed as a percentage of the mean) was therefore 4.57%.

A further sample was assayed in two separate assays, it's mean (SEM) TNFα

concentration was 931.1 pg/ml (155.2). The inter-assay coefficient of variation was 6.92%.

IL-8 assay Intra and Inter assay variation

Seven of eight assay pairs for one sample from a boy with crohn's colitis passed the linearity constraints when assayed in a single assay repeatedly. The mean (SEM) IL-8 concentration in the sample was 6444pg/ml (233.3). The intra assay coefficient of variation was 3.46%.

A further sample from this patient was assayed in five separate assays. All five assay pairs passed the linearity constraints. The mean (SEM) IL-8 concentration for this sample was 2835pg/ml (194). The inter assay coefficient of variation was 6.85%.

Conclusion

By using newborn calf serum as the sample diluent in conjunction with assays at two dilutions, an assay protocol sufficiently robust for experimental use has been developed, with low variability and high spike recovery. Section 5 goes on to explore the applicability of these assay modifications to other commercially available IL-8 ELISAs.

Section 5: Non- validity of alternative commercial IL-8 ELISAs

Introduction

In September 1996 R&D announced to its distributors that it would be changing its interleukin-8 ELISA kit. This announcement was to allow customers to perform comparative studies in advance of the change. Unfortunately the Australian distributors chose not to pass this announcement on to all of their customers for these kits. By early 1997 when my validation of the Quantikine IL-8 ELISA kit (catalogue number D8000) had just been completed, the validated kit became unavailable. It was necessary to revalidate the replacement assay kit (catalogue number D8050).

In the new Quantikine kits the sample size was reduced by a factor of two, and once samples and standards had been added to the 96 well plate, the IL-8 antibody conjugate was added immediately and the plate was incubated for three hours before it was aspirated and washed six times. This changed the assay from a two incubation stage assay to a single incubation stage assay. Additionally indicator dyes were added so that the colour of the well contents changed as the enzyme conjugated antibody was added, in an attempt to lessen the risk of double loading a well with conjugated antibody.

The manufacturers have presented data showing that in their laboratory there had been no difference between the results obtained from old and new assays when serum samples

and cell culture supernatant samples were assayed according to their instructions. Revalidation of the new kit for use in faecal supernatant samples was necessary, and in the event of failure of this validation a reserve assay would be valuable. For this reason a two stage modification of the Quantikine (D8050) assay, using assay components some of which had been used in the previously validated assay, was developed and tested. Additionally two alternative commercial IL-8 ELISAs were also evaluated. These were the Pelikine human IL-8 ELISA kit, which is produced by The Central Laboratory of The Netherlands Red Cross Blood Transfusion Service which is a two stage assay, and the one stage Biotrak Human IL-8 ELISA kit (RPN2176), (Amersham International plc, Buckinhamshire, England).

The number of assay kits available for assessment was different for each of the kit types, and it was not possible to conduct all experiments on all kits. However all kits were tested for linearity in measuring IL-8 concentration in IBD samples with newborn calf serum as the diluent. Two were tested for linearity in IBD samples using the supplied diluent. Two were tested with unspiked and two with spiked CF samples in newborn calf serum

Methods

IBD samples with Newborn calf serum as diluent

Faecal homogenate supernatant samples were prediluted in newborn calf serum to bring their cytokine concentration into the assayable range for each assay. A further 4 fold dilution in newborn calf serum was made, and both dilutions were assayed according to the manufacturers instructions in the various assay kits. For each dilution the respective undiluted sample cytokine concentration was calculated. The mean of these was calculated for comparison with other assay kits when identical samples had been assayed in different kits. Linearity was assessed by calculating the percentage variation from the pair's mean for the two dilutions. Values less than 10% would have satisfied the linearity standard adopted in the initially validated assay protocol, whilst the numerical values give an opportunity to directly compare linearity between assay types objectively.

Twelve samples were assayed with the Pelikine kit, 8 with the Amersham kit, 3 with the D8050 Quantikine kit and 3 with a two stage modification of that kit.[I]

[I]The laboratory instructions for the modified D8050 assay were:

1 Add 100µl of assay diluent into each well
2 Add 50µl of sample or standard into the wells.
3 Cover and incubate at room temperature for 2 hours.
4 Wash three times
5 Add 100µl of conjugate.
6 Cover and incubate at room temperature for 2 hours.
7 Wash three times
8 Add 200µl of substrate.
9 Incubate for 20 minutes
10 Add 50µl of stop solution.
11 Measure the OD in a plate reader.

IBD samples with supplied diluent

Samples were prediluted in the supplied diluent to bring the cytokine concentration into the assayable range, and a further four fold dilution was made in this diluent. Both dilutions were assayed according to manufacturers instructions, and mean sample cytokine concentration and percentage variation was calculated for the two dilutions.

Ten samples were assayed using the Pelikine kit and three with the D8050 Quantikine kit.

Spiked and unspiked CF samples in newborn calf serum

R&D Quantikine IL-8 Kits (D8050) unmodified

Two aliquots that had been previously shown to contain no immunoreactive IL-8 were spiked to a concentration of 1000pg/ml. These spiked samples were assayed at dilutions of x4 and x16 using newborn calf serum as the diluent. For controls the two samples were assayed unspiked but at only one dilution.

Two stage assay with R&D Quantikine IL-8 kit (D8050)

Four non IL-8 containing aliquots from CF patients were spiked to an IL-8 concentration of 1000pg/ml, assayed neat and at x2 and x8 dilutions in newborn calf serum.

The Amersham Human IL-8 ELISA kit (RPN2176)

Aliquots of faecal supernatant from twelve patients with cystic fibrosis, all of whom had no IL-8 immunoreactivity in identical aliquots assayed in the validated R&D Quantikine (D8000) assay, were assayed unspiked at twofold and eightfold dilution.

Results

IBD samples with Newborn calf serum as diluent

Table 4.9 summarises the linearity performance of the four assays tested. Figures for two IBD samples also assayed in the validated D8000 assay are shown for comparison. In newborn calf serum none of the four tested kits approached the standard of linearity achieved with the original Quantikine kits (catalogue number D8000), with mean percentage variation lying above 32% for all of them. This is despite the fact that even the two samples from the D8000 assay quoted for comparison, would not have been accepted as valid according to the linearity constraints applied in validation of the D8000 assay, and are therefore poor results for that assay.

Table 4.9	Number of samples		Percentage variation of dilution result from mean	
Assay Kit	Assayed	Within assayable range	Mean variation (%)	SD of percentage variation
Quantikine D8000	2	2	19.85	5.55
Quantikine D8050	3	3	32.48	9.53
Two stage D8050	3	1	36.18	
Amersham	8	8	36.64	20.4
Pelikine	12	11	73.89	31.91

Table 4.10 shows the mean IL-8 concentrations of identical samples according the dilution pairs in the various assay kits. The percentage variation from this mean of the individual dilution result is shown in parentheses.

Table 4.10	IL-8 concentration by assay /pg/ml (%variation)				
Number (Homogenate)	D8000	D8050	Two- stage D8050	Amersham	Pelikine
47	10428 (14.3)	3015 (45.32)	-	-	615 (53.2)
102	23759 (25.40)	29287 (29.62)	-	-	-
103	-	-	-	-	633 (35.7)
106	-	-	-	-	111 (69.9)
107	-	-	-	-	206 (146.2)
112	-	-	-	-	14 (65.0)
131	-	6076 (22.5)	6335 (36.18)	3920 (23.5)	-
132	-	-	-	-	173 (71.3)
137	-	-	-	421 (81.8)	8 (35.6)
145	-	-	-	1941 (20.5)	38 (61.8)
168	-	-	-	509 (53.0)	16 (107.3)
169	-	-	-	657 (42.0)	10 (86.7)
170	-	-	-	1533 (20.0)	34 (80.6)

IBD samples with supplied diluent

Quantikine kit

In the D8050 Quantikine assay, only one of three aliquots assayed in the supplied diluent had both dilutions results lying in the assayable range. This aliquot had a mean IL-8 concentration of 1938 pg/ml, and the variation of the individual dilution values from this mean was 48.1%

Pelikine kit

In the Pelikine assay ten of twelve assayed samples had both dilutions lying within the assayable range and here the mean (SD) variation from calculated IL-8 concentration was 31.3 (27.4)%. Mean IL-8 concentrations and dilution variations from this mean are shown in table 4.11 .

Table 4.11 Pelikine kit linearity (Supplied diluent)

Homogenate number	Mean [IL-8] pg/ml	Percentage variation
47	12154	11.21
131	1274	29.33
168	21	95.14
169	45	53.73
170	549	34.94
145	218	22.98
102	40727	0.04
103	19473	7.99
106	3027	19.48
107	5641	38.23

Spiked CF samples in Newborn Calf serum

R&D Quantikine IL-8 Kits (D8050) unmodified

Table 4.12 shows the spike recovery and linearity data for the two samples assayed in newborn calf serum after spiking. Cytokine recovery is shown as a percentage of that expected taking the sample dilution into account. The mean IL-8 concentration is calculated from the assay measured IL-8 concentration and the dilution for both dilutions, and given that these samples were produced by making a 1:1 dilution of faecal supernatant aliquot in 2000pg/ml IL-8 this calculation should give 1000pg/ml for aliquots not containing any pre-existing IL-8 at the time of spiking. Control unspiked samples did not have detectable IL-8 in this assay.

Table 4.12 Spiked aliquots (one stage assay) R&D Quantikine D8050

Aliquot	Dilution	Newborn Calf Serum			
		[IL8] pg/ml	Recovery %	Mean [IL-8] pg/ml	Variation %
188	4	90	18	5529	93.5
	16	668	534		
356	4	602	120	3068	21.5
	16	233	80		

Two stage assay with R&D Quantikine IL-8 kit (D8050)

Spike recoveries are shown in table 4.13, where they demonstrate non linear characteristics. Once again the mean IL-8 concentration should equate to 1000pg/ml. All four values are almost double this, and variation between the cytokine concentrations calculated from the individual dilutions is not close to the 10% value accepted in the validated assay. The non linearity of the assay can be seen in figure 4.11 where cytokine recoveries are expressed as percentage spike recovery for the two dilutions.

Figure 4.11 Spiked CF samples

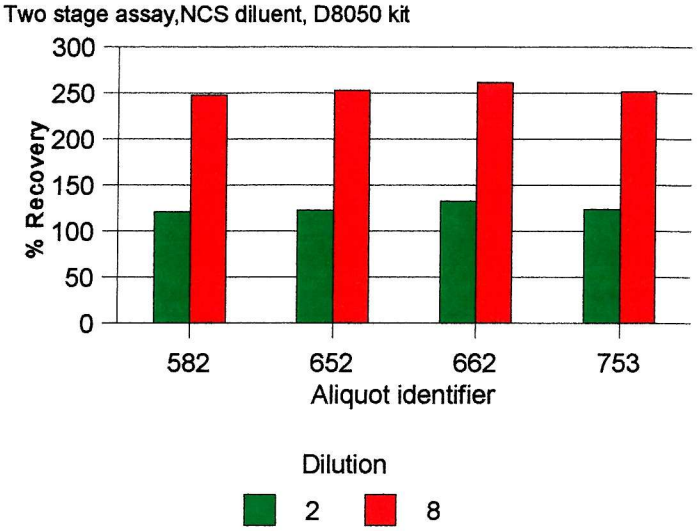


Table 4.13 Spiked CF samples, 2 stage D8050 assay

Aliquot Number	IL-8 concentration pg/ml			Variation (%)
	x 2 Diln	**x 8 Diln**	**Mean***	
582	605.17	311.12	1850	34.6
652	616.88	316.83	1884	34.5
662	665.40	328.66	1980	32.8
753	622.36	315.48	1884	34.0

*Mean of (Dilution [IL-8] x dilution)

Unspiked CF samples in Newborn Calf serum

The Amersham Human IL-8 ELISA kit (RPN2176)

None of the samples assayed in this assay had any spike of cytokine added to them, and all had been shown with the validated assay to contain no cytokine immunoreactivity. However all demonstrated apparent interleukin-8 content, which paradoxically increased in concentration with sample dilution. This is shown in figure 4.12. Table 4.14 shows the respective raw data, and presents the numerical measures of linearity for the individual sample dilution pairs. In these samples the mean (SD) variation in cytokine concentration between the two dilutions is 74.5 (7.0)%.

Figure 4.12 Apparent IL-8 concentration in 12 CF patients

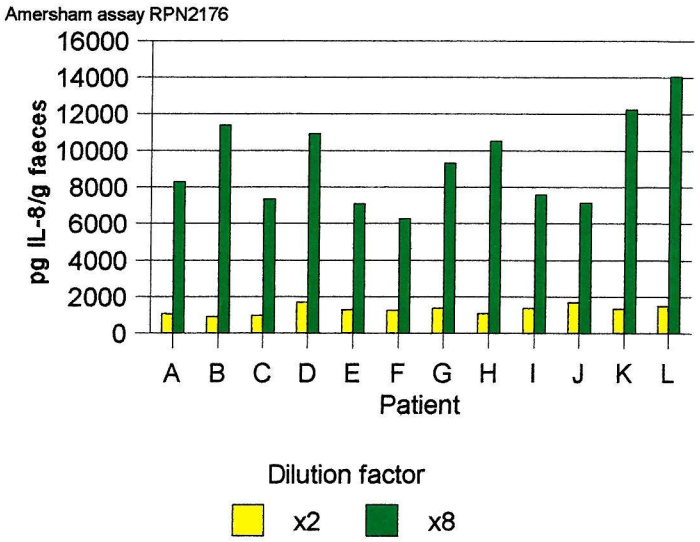


Table 4.14

Results of the one step Amersham assay

RPN2176 Unspiked CF samples

Patient	Faecal [IL-8]/pg/g according to the sample at:			
	Twofold dilution	**Eightfold dilution**	Mean faecal [IL-8]	Variation %
A	1,077	8,294	4685	77.0
B	915	11,402	6158	85.1
C	974	7,353	4163	76.6
D	1,713	10,941	6327	72.9
E	1,248	7,092	4170	70.0
F	1,276	6,270	3773	66.2
G	1,394	9,303	5348	73.9
H	1,098	10,534	5816	81.1
I	1,400	7,606	4503	68.9
J	1,713	7,163	4438	61.4
K	1,349	12,256	6802	80.2
L	1,498	14,074	7786	80.8

Discussion

The failure of the alternative IL-8 ELISAs to show consistent linearity and good spike recovery makes them all unsuitable alternatives to the validated D8000 assay in newborn calf serum.

These further experiments do however shed some light on the mechanism of the anomalous ELISA results. Whilst newborn calf serum as a diluent has made the D8000 assay linear it has not had the same effect on the other assays, confirming that the perturbations of the assays are not solely an effect on the cytokine in the environment of faecal supernatant. A significant disruption to these assays, in the assay plate, is occasioned by the faecal supernatant in one way or other.

Furthermore one assay has measured high cytokine concentrations in samples containing no cytokine, which paradoxically increase with dilution. This underlines the importance of this validation process and might offer an artefactual explanation for the high faecal cytokine concentrations observed in the initial Southampton study, if the assay we used there proved to behave similarly.

Summary

The work described in this chapter has demonstrated that the measurement of IL-8 and TNFα in faecal supernatant samples is not straightforward. Any ELISA for these cytokines that is intended for use in faecal samples requires specific validation for faecal samples. Furthermore the variable nature of the constituents of the faecal material from different individuals contrasts with the constancy of serum contents which are subject to homeostatic control, and with experimental conditions in cell culture, and mandates that sufficient dilutions of the sample concerned are assayed in each experiment to show that idiosyncratic faecal components have not invalidated the assay.

By diluting faecal homogenate supernatant 1:1 in newborn calf serum and conducting subsequent dilutions in this diluent, I have developed assay protocols which give acceptable spike recovery and repeatability both within and between assays for both IL-8 and TNFα in faecal samples. I have also shown that this modification is not applicable to any other commercial ELISA for IL-8.

I am therefore indebted to R&D, who having withdrawn from sale the ELISA (catalogue number D8000) which I had validated for use in faecal samples, agreed to manufacture and supply as a one off package sufficient D8000 kits to allow me to complete the series of experiments described subsequently in this thesis.

Chapter 5

Methods 2: Chemotaxis studies

Neutrophil migration studies

Introduction

In order to detect the presence of functional IL-8 in faecal samples without relying upon binding of the chemokine to an antibody, I have elected to perform biological assays by looking for IL-8 mediated chemotaxis, stimulated by faecal homogenate supernatant. The principal difficulty is that such material also contains bacterial formyl peptides which are also strongly chemotactic for neutrophils. In order to separate these chemotaxins, I planned to pass the supernatant through a chromatography column, and separate the chemotaxins on the basis of molecular weight.

This chapter describes techniques adopted to this end in the current investigation, which are common to subsequently described work. It is divided into three sections. The first section describes the development of techniques for quantifying neutrophil chemotaxis. The second describes the column chromatography techniques used, and the final section describes the calibration of the column to separate the chemotactic activities of IL-8 and fMLP.

Section 1: Chemotaxis methods - Background

Neutrophil preparation

In his trail-blazing work Boyden harvested rabbit peritoneal cavity neutrophils stimulated by a three hour intra-peritoneal exposure to oyster glycogen and demonstrated their chemotaxis in the Boyden chamber to chemotaxins.[129] Such a technique could only be applied to experimental animals.

Investigation of human neutrophil migration required techniques for separating viable neutrophils from whole blood, and these were subsequently developed. The separation of centrifuged whole blood into erythrocytes, a buffy coat and serum provided the impetus for this development. Of all the leukocytes, granulocytes are the most dense, and the density of the individual cells increases with maturity.[130] Mature granulocytes have a density comparable with that of erythrocytes[131], posing a problem in the separation of these cell types with simple centrifugation.

Sedimentation of a whole blood mixture with dextran or methylcellulose[132] improve neutrophil yield, but not purity. A two stage technique was developed by Böyum.[133] He first separated mononuclear cells from peripheral blood and then granulocytes from erythrocytes, but his yield was low and there was still erythrocyte contamination of the granulocyte harvest.

Dewar[134] improved on this method by centrifuging diluted whole blood over a mixture of the sucrose polymer Ficoll and sodium metrizoate, the mixture having a density of 1.077g/ml. She then washed the erythrocyte and granulocyte pellet, added group AB negative plasma and sedimented this mixture in Dextran. Finally she lysed any remaining red cells in a solution of ammonium chloride, and resuspended the granulocytes in Hank's balanced salt solution. With this technique she achieved a neutrophil yield of 70.9% and a viability greater than 98% as assessed by trypan blue exclusion.

Ferrante and Thong[135] experimented with mixtures of Hypaque 85% (Sodium Diatrizoate (3,5-Bis[acetylamino]-2,4,6-tri-iodobenzoic acid)) and Ficoll in different concentrations and centrifuged heparinised whole blood at 200g. Using a Ficoll concentration of 8.2% and a density of 1.114g/ml at room temperature, they achieved a clear separation of polymorphonuclear leukocytes from monocytes and red cells.

A later improvement in technique was a modification of Ferrante and Thong's method. Kalmar et al[136] used two commercially available Ficoll-Hypaque mixtures (Mono-Poly resolving medium, Flow laboratories USA, density 1.114g/ml and Histopaque 1.077, Sigma Chemical Company, USA, density 1.077g/ml). When they layered these over each other, they achieved a more consistent separation of cells, and a greater separation in the centrifuge tube. This made it possible to extract neutrophils from smaller samples.

I selected this rapid and effective technique for the neutrophil separation in the current study, but added two hypotonic washes after centrifugation to remove any contaminating

erythrocytes. This method is now described.

Preparation of Neutrophils for Boyden Chamber Studies

Equipment

A Beckman GPR benchtop centrifuge fitted with a GH-3.7 horizontal rotor loaded with stainless steel buckets was used for neutrophil separation.

Method

One ml of Histopaque 1.077 was layered over 3mls of Mono-poly resolving medium, and 5 mls of freshly collected whole blood anticoagulated with EDTA was layered over this preparation. These tubes were centrifuged at 500g (1479 RPM) for thirty minutes in swing out buckets at room temperature (20-24°C). Neutrophils were removed with a sterile glass pasteur pipette, resuspended in 10 mls of ~~HBBS~~ HBSS (-CaMg), cetrifuged at 300g (1146 RPM) for ten minutes at room temperature, and the supernatant was removed.

Remaining red blood cells were lysed by adding 2.5ml of 0.2% sodium chloride to the pellet, followed by 2.5 ml of 1.6% sodium chloride. The centrifuge tube was made up to 10 ml with HBSS (-CaMg), and centrifuged at 500g (1479 RPM) and 4 deg C for ten minutes. This red cell lysis step was repeated and the final pellet was suspended in 1ml of HBSS (+CaMg) and stored on ice.

A 10 microlitre aliquot of neutrophil cell suspension was stained with an equal volume of 0.4% trypan blue, and a viable cell count made using a haemocytometer. If the cell count

was greater than one million cells per millilitre, it was corrected to this by dilution in HBSS with calcium and magnesium. When the resulting volume of final cell suspension was less than 2.4mls, it was made up to this volume, to allow inoculation of all 48 wells in the modified boyden chamber.

Neutrophil migration studies

In Boyden's chamber, a device consisting of an upper and lower chamber separated by a filter, a solution containing chemotaxin is placed in the lower chamber. This is covered with the filter membrane and the upper chamber is placed on the filter and is inoculated with the suspension of neutrophils. The chamber is then incubated at 37°C in 5% CO_2, and after removing neutrophils that have settled on the upper surface of the filter, it is examined under the microscope and the number of neutrophils in a selected sample of high power fields is used as an index of neutrophil chemotactic activity. Whilst this device was revolutionary it has a filter that is 13mm in diameter and it requires 1.5mls of chemotactic solution and 3.6mls of neutrophil suspension.

With these difficulties in mind Falk et al[137] designed a device with 48 chemotaxis wells, and a single filter. Their modified Boyden chamber requires only 25µl of chemoattractant, and a 30µl sample of cell suspension per well. They found that this micro-chamber gave similar results to the original chamber, but that the distribution of the migrant cells was more even in the new chamber.

Other investigators[138,139] have used an agarose technique, where linear sets of three 2.5mm diameter wells are cut in the agar, with their outer edges 2.5mm apart. Each well takes a volume of 5µl, and neutrophils are placed in the middle well with

chemoattractant in one well and control substance in the third. Chemotaxis is quantified by measuring the distance travelled towards the chemoattractant by the ten fastest moving cells, using an inverted microscope and an eyepiece graticule. This method has the advantage of small sample sizes, but it is not as statistically robust as the modified Boyden chamber, relying as it does on a smaller number of counted migrated cells. I therefore adopted this modified Boyden chamber technique for these experiments. The technique is descibed below.

Modified Boyden Chamber technique

Aliquots of faecal supernatant will be placed into the lower well of a modified boyden chamber to be used as stimuli for neutrophil migration across the chamber's membrane. IL-8 will be a positive control. Negative control will be a zero IL-8 standard or Hanks balanced salt solution.

Reagent preparation

Aliquots of 4000pg/ml IL-8 were produced by diluting 10μg/ml stock IL-8 in Phospate Buffered saline with Human serum albumin. These aliquots were used as control chemoattractants.

0.2% and 1.6% Saline solutions were produced from saline solutions provided for addition into intravenous fluids. The resulting saline solutions were autoclaved on a wet autoclave cycle.

Formyl-methionyl-leucyl-phenylalanine solutions were produced from lyophilysed fMLP

purchased from Sigma. The dry reagent was reconstituted in DMSO at a concentration of 10mg/ml. This was diluted in phosphate buffered saline to achieve a concentration of 2.28 x 10^{-7}M fMLP.

Sample preparation

Samples were diluted 50:50 in Hank's balanced salt solution (HBSS) prior to loading them into the lower wells of the chemotxis chamber.

Preparation and incubation of chamber

Thirty µl of chemoattractant or sample was inoculated in each lower well producing a slight positive meniscus. A pvp free 5µ polycarbonate membrane (Porvair Filtronics Ltd, Middlesex, UK) was placed over these wells, followed by the silicone gasket, and the top plate. Thumbnuts were tightened and 50µl of cell suspension was added to each well. The modified Boyden chamber was incubated at 37°C with 5% CO_2 for one hour. The chamber was then disassembled, unmigrated cells were removed from the upper aspect of the membrane and it was fixed in methanol and stained with the Haema Gurr stain (BDH Poole) according to manufacturers directions.

Cell counting

The stained and mounted membrane was examined under an oil emersion lens. Six high power fields were counted in each well. In order to minimise observer bias the high power fields to be counted were selected according to a predetermined plan. The well was identified on low power examination and the lens was then changed to the high

power lens. The field visible at this time was the first field to be counted. The remaining counting fields were taken in the pattern of the six dots on a dice, taking the initial field as the bottom right dot. The microscope stage was moved under direct vision in the direction of the next dot in the pattern.

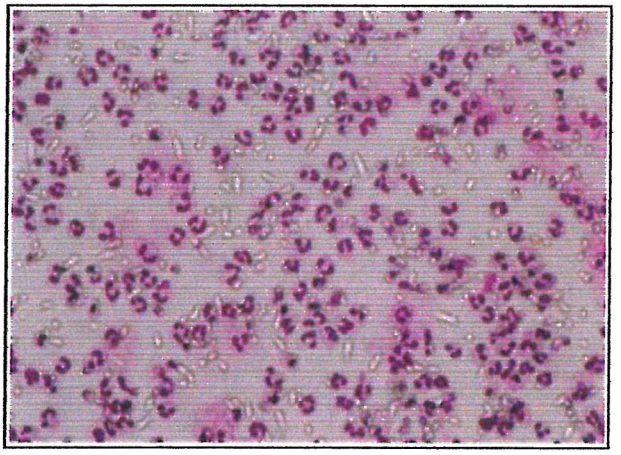


Figure 5.0 Stained neutrophils on Boyden chamber filter (Haema Gurr)

When the stage came to a stop, it was moved further in this direction until all of the membrane pores or cells (figure 5.0) that were visible when the stage first came to rest had been moved out of the field of vision. Cell counts were then recorded. Total cell counts for the six fields were calculated, and summary statistics for each set of repeated wells were determined. The significance of differences between neutrophil migration in the well sets was assessed using parametric tests; ANOVA and t-tests.

Resolution of Neutrophil migration studies as an IL-8 bioassay

To explore the resolution of neutrophil migration studies as a bioassay for IL-8 concentration, duplicate neutrophil migration studies were performed for serial two fold dilutions of IL-8 from 1000 to 31.25pg/ml to mirror the ELISA adopted elsewhere in this study. These were designated studies 1 &2. Additionally ten fold dilutions of IL-8 from 2000 to 20pg/ml and a negative control were performed. These were designated studies 3 & 4.

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Results

Results for the two fold Il-8 dilution series are shown in table and figure 5.1. Within these series, studies 1 and 2 the neutrophil count in the upper chambers was 1 x 10^6 cells per ml. The IL-8 containing wells and the blank wells were subjected to ANOVA. There were significant differences amongst these groups in both study1 ($F=10.93$, $p=0.00039$) and study 2 ($F=5.56$, $p=0.007$). Figure 5.1 demonstrates that although there is a dose reponse curve differences between migrated neutrophil counts for any two consecutive dilutions were not clearly different. In contrast in figure 5.2 and table 5.2 which show the dose response curve for ten fold dilutions of Il-8 in studies 3 & 4, consecutive dilutions have clearly different migrated neutrophil counts.

Table 5.1

Sample	Mean neutrophils per six high power fields (SEM)	
	Study 1	**Study 2**
Blank samples		
Blank 1	34.00 (6.48)	65.67 (20.74)
IL-8 standards		
1000pg/ml	123.33 (87.67)	282.5 (11.5)
500pg/ml	78.67 (11.61)	268.0 (76.38)
250pg/ml	93.67 (10.34)	271.33 (64.86)
125pg/ml	81.67 (7.41)	160.67 (32.43)
62.5pg/ml	47.00 (11.46)	159.0 (32.17)
31.25pg/ml	60.33 (9.43)	178.67 (18.21)

Figure 5.1 Neutrophil migration with IL-8

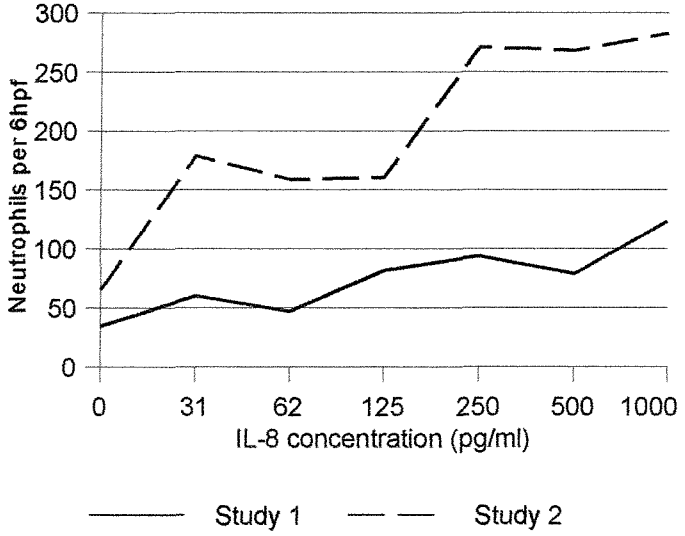
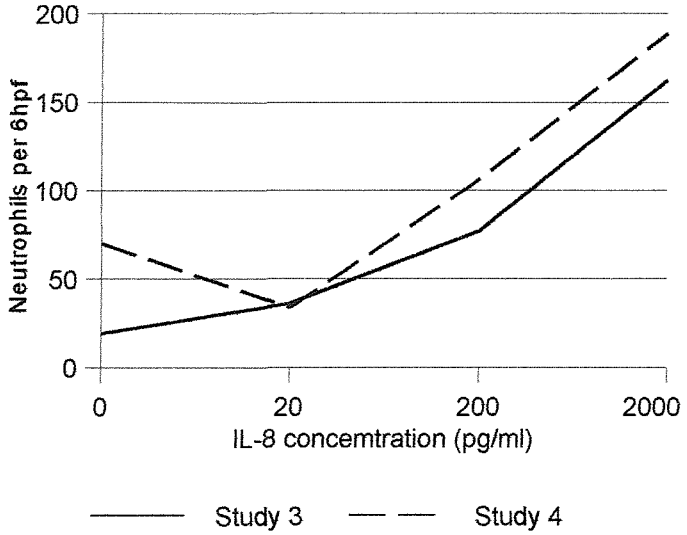


Table 5.2

Sample	Mean (SEM) Migrated Neutrophil count	
	Study 3	Study 4
2000pg/ml IL-8	162.3 (18.35)	188.7 (62.83)
200pg/ml IL-8	77.0 (10.80)	106.0 (19.82)
20pg/ml IL-8	36.0 (6.35)	33.7 (20.76)
HBSS	19.0 (6.38)	70.0 (7.12)

Figure 5.2 Neutrophil migration with IL-8



Conclusion

Whilst migrated neutrophil count may serve as an index of IL-8 concentration, its resolution is less than that of the ELISA. These studies will therefore be interpreted as qualitatative rather than quantitative indicators of neutrophil chemotactic activity.

Section 2: Column Chromatography

Column chromatography is a well established technique for performing separations of molecules in solution by virtue of their molecular size. The column contains a gel which effectively produces pores in the medium through cross linking of adjacent parts of the gel material. These pores allow entry of the smaller molecules in solution whilst prohibiting entry of larger molecules. This increases the volume of distribution of the smaller molecules, so that when sample is applied to one end of the column, and the flow of eluent is sufficiently slow, larger and smaller molecules are eluted from the column in separate fractions. This separation is greater when the eluent flow is slow and the column height is large.

A large array of Gels is available for such studies and each is characterised by a range of sizes of molecules which will be separated by it, and by the range of eluents than can be used with it. Molecules larger than the upper limit of the gel pass through the column at the same rate as the solvent used as eluent, and they are eluted when sufficient solvent to fill the liquid phase space of the column has passed through it. This volume is known as the void volume. After the void volume the larger molecules appear and are followed by smaller molecules.

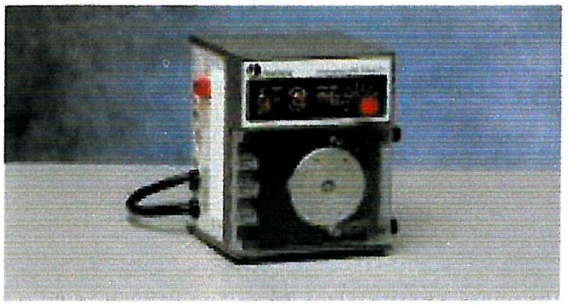
Sephadex G-10 was selected as the gel for these studies. It retards the passage of molecules with a molecular weight below 700Da, allowing larger molecules to appear in the void volume. fMLP which is one of the larger formyl methionyl compounds has a

molecular weight below 400Da, whilst the smallest chemokines have molecular weights of 6kDa. Phosphate buffered saline was chosen as the eluent, so that all fractions would contain only solutes that would not interfere with neutrophil migration in the subsequent Boyden chamber studies. Six molar urea or sodium dodecyl sulphate would have denatured the proteins and improved the accuracy with which the molecular weight of proteins in a particular fraction, could have been estimated. With such a large difference between the molecular weights of the proteins of interest, I felt that this extra accuracy would not be necessary.

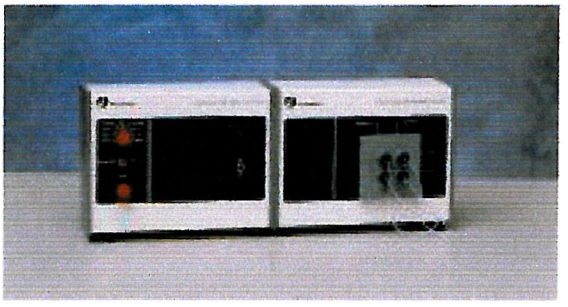
Equipment

The column selected was a 20 cm column of 16mm internal diameter C16/20 (Pharmacia). Samples were added to a 0.5ml capillary tubing loop, which could be added into the solvent delivery circuit when the apparatus had stabilised. Degassed phosphate buffered saline was delivered to the column by a P1 peristaltic pump (Pharmacia), to achieve a linear flow of solvent. The column was orientated so that solvent flow through the column was in an upward direction. The eluent passed through a UV detector with an absorbance filter set for 280nM (Pharmacia). Absorbances were recorded on a penchart recorder (Lloyd Instruments, Southampton), and fractions were collected into test tubes

P1 peristaltic pump

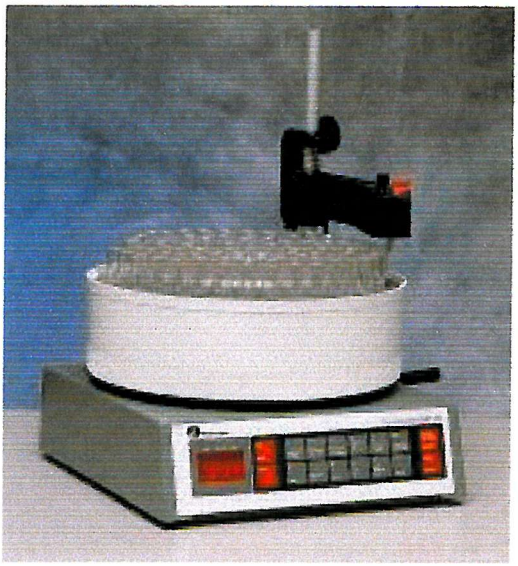


UV Detector



loaded into a Frac 100 fraction collector (Pharmacia).

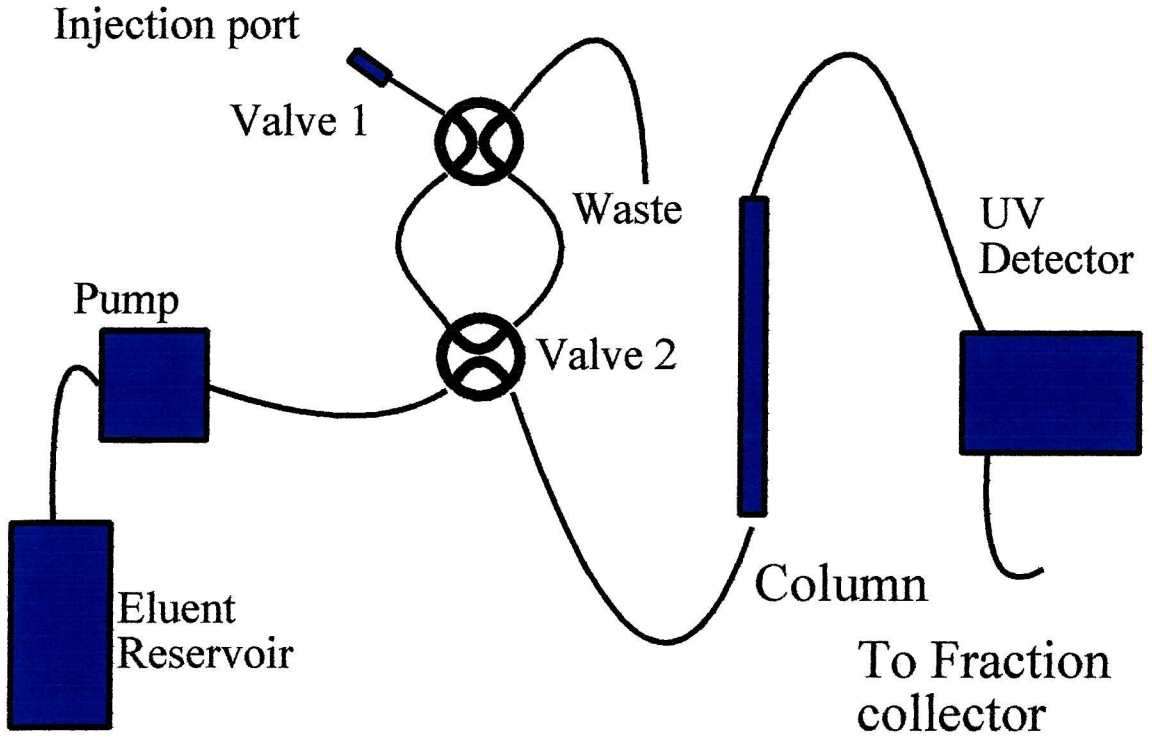
Fraction collector



Section 3: Column calibration

Void volume was initially determined to be between 15 and 16 mls using a flow rate of 45mls/hr and Evan's blue as the sample. Both interleukin-8 and larger proteins were identified in this fraction, and this was confirmed when an IL-8 concentration of 120pg/ml was measured in a fraction contained within the elution peak at this volume. However because the administered concentration had been 6000pg/ml, it proved necessary to use a greatly

Fig 5.3a Chromatography circuit: sample injection mode



reduced flow rate, in order to achieve greater focusing of the IL-8 peak. This would be necessary if an IL-8 concentration great enough to provoke neutrophil migration was to be present in the eluate for samples containing concentrations of IL-8 in the range 1000 to 2000 pg/ml.

Detection of the fMLP elution volume was more problematical. The fMLP was supplied as a powder, and this powder is not directly soluble in water. It is necessary to produce a stock fMLP solution either in ethanol or in Dimethylsulfoxide (DMSO), and then to dilute this in more physiological solvents. I performed the initial dilution in DMSO and made a further ten fold dilution in phosphate buffered saline. A series of chromatography runs with this solution produced small positive and negative deflections of absorbance at elution volumes of 15.6 and 23.9mls respectively.

As a next step spectra were obtained for solutions of DMSO in PBS with and without dissolved fMLP, using the Lambda 2 UV spectrophotometer (Perkin Elmer). Relatively small peaks were obtained in the spectrum for fMLP at 241.49, 248.18 and 222.89nm (figure 5.3), and when the fMLP solution was diluted to the concentrations expected in a fraction from the column, the peaks were barely perceptible. The minimum size of these fractions was dictated by the minimum size of sample necessary for the spectrophotometer. Accordingly when fractions from the column were analysed in the spectrophotometer at the three most prominent wavelengths in the spectrum, no fMLP was evident. Two alternative filters, other than the 280nm filter, were commercially available for the UV detector used to monitor the output from the column. These had

wavelengths of 254 and 405nm. Inspecting the spectrum for fMLP minima were evident at all three wavelengths for which filters were available.

The predicted protein concentration in fractions was below accurate detection limits for standard laboratory protein assays. Chromatography runs had collected up to ninety fractions on a single run, and it was necessary to subject each of these fractions to a neutrophil migration study.

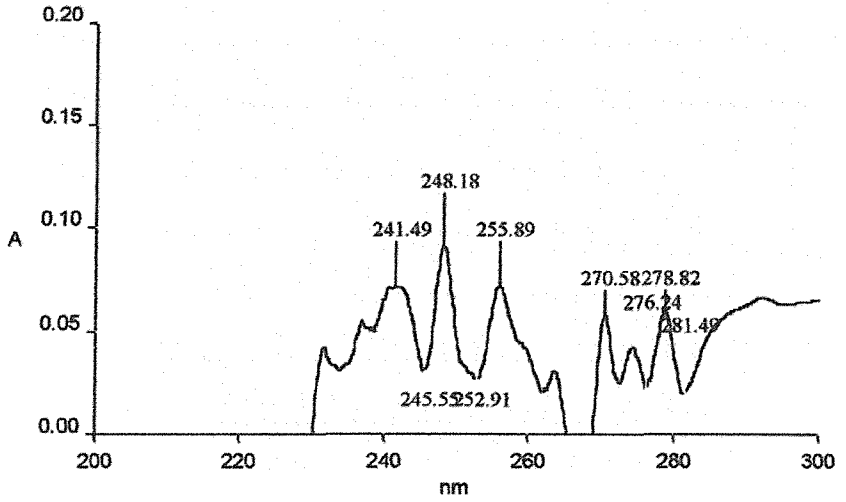


FIGURE 5.3 FMLP SPECTRUM

Column calibration with fMLP by neutrophil migration

Method

Stock fMLP in DMSO was diluted 1:9 v/v in PBS to produce a 1mM solution. A 0.5ml aliquot was injected into the column sample loop and the remainder of the solution was left on the bench in proximity to the column. Phosphate buffered saline was used as an eluent at 6.35mls/hr. Once the sample loop was included into the eluent circuit and applied to the column 15 minute fractions were collected for 18 hours.

Fractions 1 to 36 were diluted 50:50 in Hanks balanced salt solution and a single well of the 48 well modified Boyden chamber was inoculated for each fraction. Controls and standards were inoculated into wells in triplicate after 50:50 dilution in HBSS. These solutions were, IL-8 at 2000pg/ml, 1mM and 0.1mM fMLP, and HBSS as a control.

Neutrophils were separated from the peripheral blood of a healthy adult male. However yield and viability (74.4%) were lower than expected and the neutrophil count in the wells was correspondingly low at 720,000 viable neutrophils/ml.

Six high power fields were counted for each fraction well and the mean cell count for each fraction was calculated. Cell count per high power field was selected as the index of neutrophil migration so that within and between fraction variability could be tested by ANOVA, all 36 fractions having been incubated with the same preparation of

neutrophils in the same experiment. Neutrophil migration in the control and standard wells was similarly expressed as neutrophils per high power field.

Results

Mean(SEM) migrated counts per high power field were 29.33 (8.42) for 2000pg/ml IL-8, 45 (10.99) for 1mM fMLP, 56.78 (11.59) for 0.1mM fMLP and 20.72 (4.15) for Hanks balanced salt solution. Amongst the 36 fractions there were highly significant differences in migrated neutrophil counts ($F=55.5$, $p=7.7 \times 10^{-79}$). Full results for the fractions are shown in the table 5.4 and illustrated in figure 5.4. These data show that fMLP chemotactic activity resides in fractions 11 to 19.

Figure 5.4

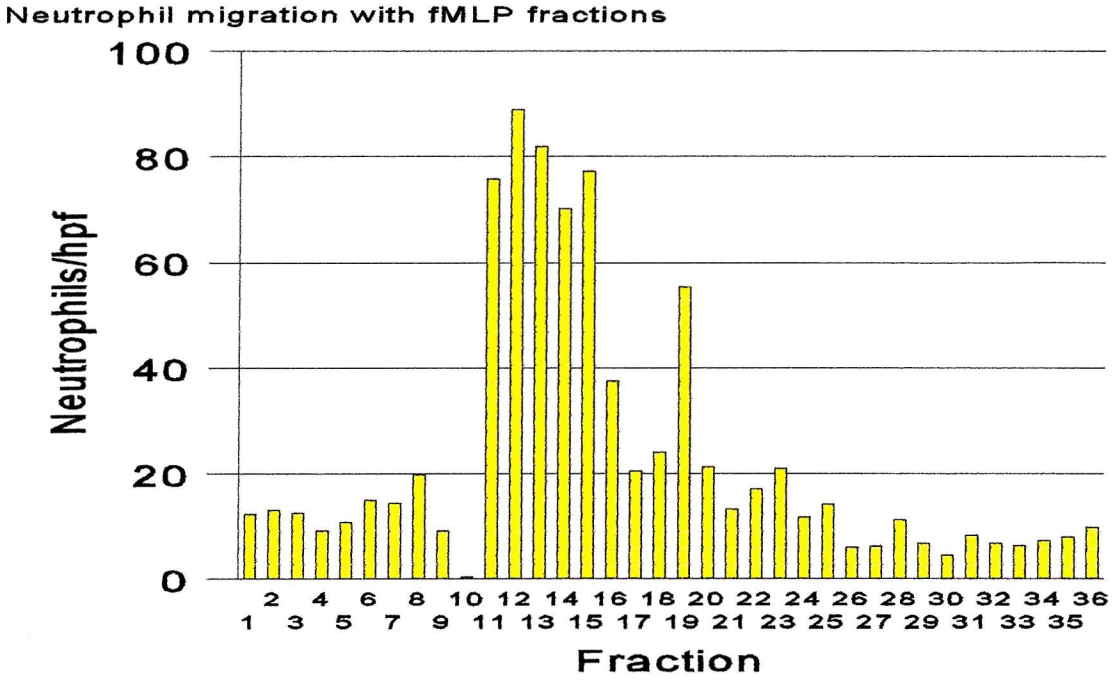


Table 5.4

Fraction Number	Neutrophils /hpf	SEM
1	12.3	4.45
2	13.0	2.08
3	12.5	1.50
4	9.2	1.67
5	10.7	3.03
6	15.0	2.58
7	14.3	3.19
8	19.8	6.09
9	9.2	2.03
10	0.3	0.47
11	75.8	13.66
12	89.0	10.87
13	82.0	12.54
14	70.2	22.49
15	77.3	18.30
16	37.5	7.27
17	20.5	6.24
18	24.0	6.24
19	55.3	14.25
20	21.3	4.14
21	13.2	4.56
22	17.0	5.35
23	21.0	4.69

Table 5.4

Fraction Number	Neutrophils /hpf	SEM
24	11.7	3.81
25	14.2	4.91
26	6.0	4.47
27	6.2	2.27
28	11.2	4.14
29	6.8	1.86
30	4.5	1.80
31	8.3	4.81
32	6.7	3.54
33	6.3	4.15
34	7.2	3.13
35	8.0	4.32
36	9.8	3.80

Column calibration with IL-8 by neutrophil migration

Method

Using the same column, eluent and flow rate as the fMLP experiment, 0.5mls of a 1μg/ml solution of IL-8 in PBS containing 0.5% Human serum albumin was loaded onto the column and eluted into 39 fifteen minute fractions. An absorbance peak corresponding to fractions 6 & 7 was observed. As this may have been the result of dissolved IL-8 or albumin it was assumed to represent the void volume. This gives a void volume for the column between 7.9 and 11.1 mls.

A 2000pg/ml IL-8 standard and fractions 1 to 14 were diluted 50:50 v/v in Hanks balanced salt solution and loaded into the lower wells of the modified Boyden chamber in triplicate. HBSS triplicate wells served as a control.

Results

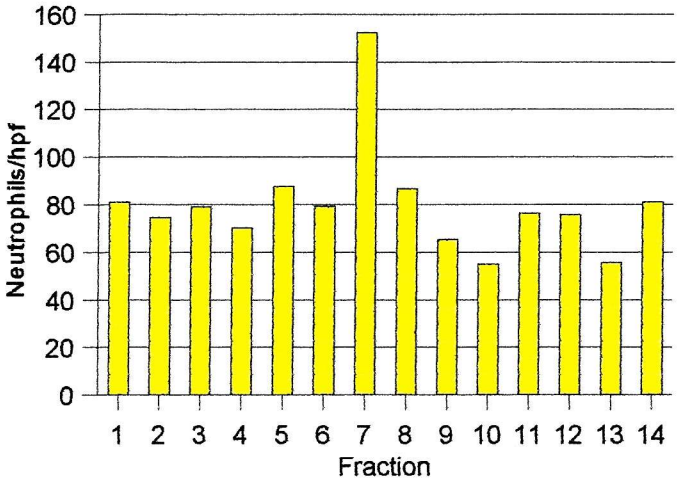
Amongst the 14 fractions there were significant differences in mean neutrophil count by ANOVA ($F=6.72$, $p=1.3 \times 10^{-5}$). A subsequent t test showed that the mean neutrophil count in fraction seven (152.3/6hpfs) was significantly greater than the blank sample (101.7/6hpfs) ($t=-3.32$, $p=0.029$), and from fraction 1 an alternative control (81.0/6hpfs), ($t=-5.49$, $p=0.005$). Neutrophil counts for all samples are shown in table 5.5.

Table 5.5

Sample	Mean neutrophils /6 hpfs	SEM
IL-8 Standard		
2000pg/ml	505.0	73.38
Blank		
HBSS	101.7	12.66
Fractions		
1	81.0	5.72
2	74.7	11.09
3	79.0	4.55
4	70.3	1.70
5	87.7	10.37
6	79.3	10.34
7	152.3	17.44
8	86.7	25.49
9	65.3	7.59
10	55.0	6.68
11	76.3	10.87
12	75.7	14.84
13	55.7	8.18
14	81.0	19.13

Figure 5.5

Neutrophil migration with IL-8 fractions



Conclusions

These two calibration experiments demonstrate that this Sephadex G10 column will when run under these conditions separate IL-8 from fMLP chemotactic activity with IL-8 appearing in the void volume in fraction 7 and fMLP appearing after fraction 10. The column can now be used at the current flow rate with phosphate buffered saline as the eluent to separate fractions of faecal supernatant containing fMLP from those containing chemokines.

Chapter 6

Faecal IL-8 and TNF alpha in CF patients, healthy controls and disease controls

Introduction

Having developed linear and accurate assay protocols for measuring faecal IL-8 and TNF alpha, I have used these protocols to conduct cytokine concentration measurements in faecal supernatant from Australian CF patients, from healthy control children and from disease control children with intestinal or pulmonary disease. Here I describe the execution and results of these studies.

The chapter also gives an account of neutrophil migration studies on faecal supernatant from these patients with cystic fibrosis. These studies were conducted to explore the possibility that ELISA undetectable but functional IL-8 was present in stools which contained no measurable IL-8. These studies were conducted in three stages.

Firstly neat aliquots were assayed for chemotactic activity, then fractions of these aliquots were subjected to neutrophil migration studies to see if neutrophil chemotactic activity resided in fractions that would contain IL-8 size molecules. Finally aliquots were coincubated with Polymyxin B to negate effects of bacterial endotoxin as an exploratory experiment seeking effects of other void volume agents on neutrophil migration. Endotoxin was selected because it has a direct effect on macrophages,[140] stimulating them to produce pro-inflammatory cytokines including IL-8, it stimulates neutrophil

chemotaxis through activation of complement[141], but may also have an inhibitory effect on complement mediated chemotaxis.[142]

Patients

Eleven patients attending the Royal Children's Hospital Brisbane, CF clinic were recruited to the study. They were selected to have a range of pulmonary disease severity and ages. Recruitment was biased towards patients who had previously consented to be subjects in research studies, but no other bias was wittingly applied.

Control subjects were ten healthy children free from any respiratory infection recruited from the community. These were relatives and social contacts of members of the hospital staff. Disease controls were consecutive consenting patients admitted to the Royal Children's Hospital with acute severe asthma (n=4), with pneumonia (n=6), two children with bronchiectasis secondary to congenital hypogammaglobulinaemia, one boy with idiopathic constipation (increased whole gut transit time), one with short gut syndrome (decreased whole gut transit time) who had also been exposed to carmine, and five children with inflammatory bowel disease.

Methods

Patient Assessment

NIH scores[143] were determined at routine clinic visits for the CF patients. Control Patients were accepted as having pneumonia if they had cough, radiological evidence of consolidation in at least one segment, and no wheeze. Acute severe asthma was defined as a wheezing illness requiring hospital admission, oral steroid treatment and salbutamol nebulisation more frequently than two hourly, by the treating paediatrician. Inflammatory bowel disease was defined as biopsy proven intestinal inflammation in a clinical setting consistent with Inflammatory bowel disease.

Faecal sample collection & processing and ELISA procedure

These are described in chapter 4.

Neutrophil migration studies of faecal supernatant

Stage1: Untreated feacal supernatant

Supernatant samples identical to those used in the cytokine ELISAs were subjected to neutrophil migration studies according to methods described in chapter 5, using a Hanks balanced salt solution (HBSS) as a negative control and 1000pg/ml IL-8 as a positive control. The numbers of migrating neutrophils in each sample were expressed as mean cells per 6 high power fields per well, and as a percentage of the corresponding value for

both the positive and negative controls.

Stage2: Fractionated feacal supernatant

Six aliquots of faecal supernatant, identical to samples used in the ELISAs of over 250μl were selected; aliquots for the other five ELISA assayed patients had been exhausted in other experiments. These studies were conducted over three neutrophil migration experiments. In the first two, multiple fractions of both single aliquots of more than 500μl were studied, whilst in the third, selected fractions of the four remaining smaller aliquots were studied. For two patients (CF01 and CF04, aliquots 1114 & 1092), a sample of the aliquot was chromatographed overnight through a Sephadex G-10 column, and the remainder was left on the bench adjacent to the column. Fractions 3-14 were diluted 50:50 v/v in HBSS and loaded into the lower wells of the modified Boyden chamber as described previously. Triplicated controls contained 2000pg/ml IL-8,HBSS and the unchromatographed aliquot remnant, all diluted 50:50 in HBSS. These two samples were to serve as a check that chemotaxins in the void volume, remained in fraction 7 in the presence of componenets of faecal supernatant that were additional to those of the spiked PBS in the calibration studies.

The aliquots of less than 500μl, were diluted 50:50 in PBS before loading onto the column. Over a period of 36 hours these diluted aliquots were run through the column at flow rates described above. Each aliquot having a run time of at least 5 hours. They were loaded onto the column in the order 1080, 1084, 961, 940. Fractions 7 & 8 were selected from each aliquot run, as a result of the two check studies described above. They were

stored at 4°C for 3 days before being studied in the chemotaxis chamber. Each aliquot 7 & 8 was diluted 50:50 in HBSS and loaded into the lower well of the chemotaxis chamber.

Stage3: Neutrophil migration with polymyxin b coincubation

Aliquots of faecal supernatant were incubated at 37°C for 1 hour either with or without 10μg/ml Polymyxin B before being loaded into the lower wells of the chemotaxis chamber. The positive control was 2000pg/ml IL-8 and the negative control was HBSS. The chemotaxis experiment was conducted over two runs using different aliquots of faecal supernatant in each.

In the first run neutrophil yield had been lower than anticipated (viability 85.5%) and the neutrophil count administered to the upper chamber was 3.76×10^5 viable cells/ml. In the second run the yield had been higher despite a viability of 74.7%, and the administered neutrophil count in this experiment was 1.66×10^6 viable cells/ml.

Differences between Polymyxin B and non Polymyxin B co-incubated sample mean migrated neutrophil count was tested using a two tailed t-test for each pair.

Statistical methods

The mathematical manipulation of absorbances from the plate reader which allow calculation of cytokine concentration in the ELISAs is described in Chapter 4, and the

statistical tecniques used in the neutrophil migration studies is described in Chapter 5.

A further statistical test will be applied here to aid in the interpretation of any ELISA samples that have absorbances below that of the least concentrated cytokine standard. In such samples it is of interest whether they show more cytokine immunoreactivity than the blank well in the assay.

In these assays every sample and standard are assayed in two adjacent wells, additionally all faecal supernatant samples are assayed at x2 and x8 dilution. As all samples for all eleven CF patients were assayed in a single plate for each cytokine assay, there are three variables in this group of samples which may produce variations in the recorded absorbance. These are firstly random variations between identical adjacent wells. Secondly variations between the x2 and x8 dilutions and finally variations between the individual samples and the blank wells. Whilst the first source of variation is of little interest, a statistically significant difference in absorbance between dilutions or between samples would imply cytokine immunoreactivity. Therefore two way ANOVA with one replication was applied to the recorded absorbances for all of the samples that had absorbance less than the low standard. The absorbances from the blank well were included as a sample in both the x2 and x8 dilution columns.

Results

ELISA: Non Inflammatory Bowel Disease controls

The mean (sd) age for controls was 2.58 (0.91) years with asthma, 2.90 (2.67) years with pneumonia, 8.71 (4.79) years with bronchiectasis and 8.13 (5.52) years from the community. The boys with short gut syndrome and with constipation were 1.69 and 8.0 years old respectively.

A single two fold dilution of an aliquot from a 3.75 year old girl with bronchiectasis had an IL-8 concentration in the assayable range. The corresponding faecal IL-8 concentration was 885pg/g. However the eightfold dilution for this sample had an apparent concentration below the assayable range, and as the two apparent aliquot concentration values were 16% either side of the mean concentration, this sample is considered to have failed quality control.

Otherwise no non-IBD control had any assayable IL-8 or TNF alpha in their faecal samples.

ELISA: Inflammatory Bowel Disease Controls

The mean (sd) age of the Inflammatory bowel disease patients was 15.29 (1.87) years. All had Crohn's disease. Three had acute Crohn's colitis, one was in clinical remission from Crohn's colitis, and the final patient had small bowel disease and had previously had a subtotal colectomy. Her samples had been collected from her ileostomy.

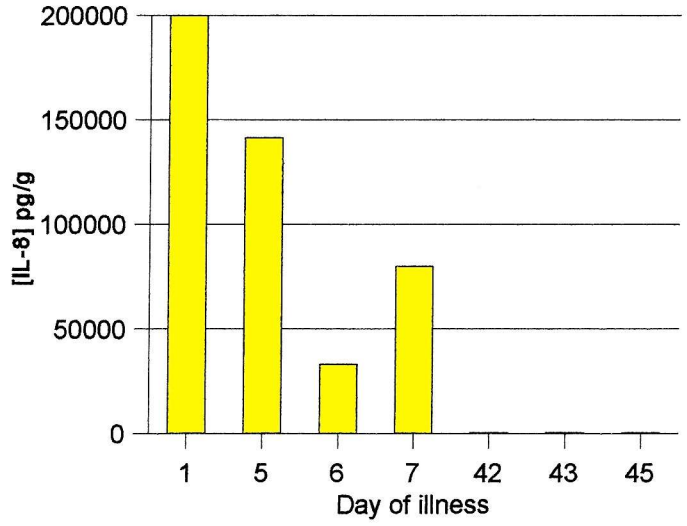
IBD faecal TNF alpha concentrations

One sample from a boy with severe colitis, had a faecal TNFα concentration of 14,396 pg/g. Two samples from the girl with small bowel disease and one from the patient in clinical remission had no assayable TNFα. Serial samples from two patients with colitis were available, and all had lower than expected TNFα concentrations such that none had more than one dilution with a TNFα concentration in the assayable range. Thus neither patient had measurable faecal TNFα concentrations. TNFα immunoreactivity was present in the first patient on days 4,5 and 16 but not 3 and in the second patient on days 5,6 and 7 when she had active disease but not on days 43, 44 and 45, when she was in clinical remission.

IBD faecal IL-8 concentrations

A sample from the boy with severe colitis had an apparent IL-8 concentration above 100,000pg/g, but it failed quality control. No samples from the girl with small bowel disease were available for assay. One sample from a girl in remission had an IL-8 concentration of 16,168pg/g. Samples taken serially from a boy with severe colitis had IL-8 concentrations of 36,387 and 16,655 and 17,324 pg/g on days 1, 2 and 16 of his illness respectively. There were seven serial samples assayed from the final patient (figure 6.1), samples from days 5 & 6 had IL-8 concentrations of 141,647 and 33,165 pg/g, remission samples from days 42, 43 and 45 had an IL-8 concentration less than 384pg/g: the lower limit of the assayable range for this assay. Samples from days 1 and 7 had apparently high IL-8 concentrations; around 200,000pg/g on day 1 and around 80,000pg/g on day 7, but both of these samples failed quality control.

Figure 6.1 Serial faecal IL-8 in IBD



ELISA: Cystic Fibrosis patients

The mean (sd) age of the cystic fibrosis patients was 12.9 (3.5) years. Their mean (sd) NIH score was 76 (13), and mean (sd) percentage predicted FEV1 was 65(18)%. Patient details are shown in table 6.1. No CF patient had an IL-8 or TNFα concentration in the assay wells approaching the lower limit of the assayable range. The corresponding faecal cytokine concentrations for these patients were therefore much below 384pg/g for IL-8 and 172pg/g for TNF alpha in all cases.

In the TNFα assay there was no significant difference in TNFα immunoreactivity between the blanks and the samples ($F=1.235$, $p=0.32$), the two dilutions ($F=0.365$, $p=0.55$), or their interaction ($F=0.339$, $p=0.97$).

In the IL-8 assay absorbances from sample wells were all marginally greater than in the blank wells,implying IL-8 immunoreactivity. There were significant differences between absorbances for the blank wells and the individual samples ($F=19.31$, $p=2.6\times10^{-9}$). There were also significant differences between the absorbances for the x2 and x8 dilutions ($F=19.28$, $p=0.0002$). However, in contrast to the expectation for samples containing minute amounts of chemokine, in 9 of the 11 dilution pairs absorbance was greater in the x8 dilution than in the corresponding x2 dilution.

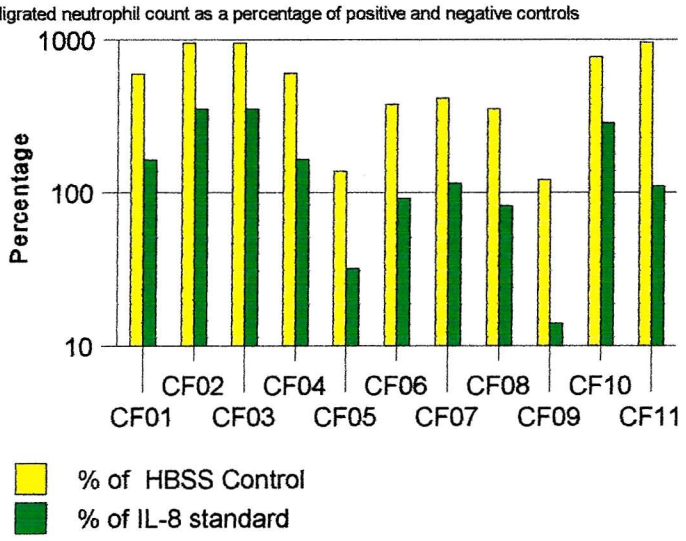
Table 6.1 Cystic Fibrosis patient characteristics

Pt	M F	Age	NIH Score	Respiratory function tests (% predicted)		
				FEV1	FVC	FEF25-75
CF01	F	17.18	57	69	74	56
CF02	F	9.40	61	32	57	8
CF03	F	6.46	95	72	68	62
CF04	F	14.11	73	80	74	98
CF05	M	12.24	90	87	83	83
CF06	F	8.06	88	60	82	38
CF07	M	15.07	86	76	81	67
CF08	M	13.68	69	46	48	37
CF09	F	16.81	58	50	73	22
CF10	M	15.55	79	57	69	35
CF11	F	13.37	80	>90	NR	NR

CF patients: Neutrophil migration studies

Stage1: Neutrophil migration studies with native faecal supernatant

Figure 6.2 Native faecal supernatant



Migrated neutrophil counts for the unaltered CF faecal supernatants are shown in table 6.2 and illustrated on a logarithmic scale in figure 6.2 as a percentage of the relevant positive and negative control samples. Supernatants from two patients (CF05 and CF09) had migrated neutrophil counts that were not significantly different from that of the Hank's balanced salt solution negative control. All other samples had markedly greater migrated neutrophil count than the negative control. This was always at least 3.5 times the negative control values and up to 3.5 times the corresponding value for the IL-8 positive control.

Table 6.2 Migrated neutrophil counts for CF faecal supernatants

Patient	Aliquot Number	Mean (SEM) neutrophils / 6 hpf			t test p values	Aliquot neutrophils as	
		Aliquot of faecal supernatant	HBSS Blank	IL-8 Standard	Aliquot Vs Blank	% of Control	% of standard
CF01	1115	202.00 (40.41)	34.00 (6.89)	123.33 (87.67)	$1.4x10^{-14}$	594	164
CF02	940	666.3 (154.45)	70.0 (7.12)	188.7 (62.83)	$2.1x10^{-14}$	951	353
CF03	962	668.3 (75.02)	70.0 (7.12)	188.7 (62.83)	0	954	354
CF04	1093	205.67(20.95)	34.00 (6.89)	123.33 (87.67)	0	605	166
CF05	1089	91.00 (12.03)	65.67 (20.47)	282.50 (11.50)	0.12	138	32
CF06	1025	261.00 (23.54)	65.67 (20.47)	282.50 (11.50)	$1.6x10^{-12}$	379	92
CF07	1085	141.33 (16.11)	34.00 (6.89)	123.33 (87.67)	$1.1x10^{-11}$	415	115
CF08	1063	231.67 (46.44)	65.67 (20.47)	282.50 (11.50)	$3.5x10^{-10}$	352	82
CF09	1069	23.3 (16.58)	19.0 (6.38)	162.3 (18.35)	0.6	122	14
CF10	1081	540.7 (88.58)	70.0 (7.12)	188.7 (62.83)	0	774	287
CF11	1075	181.7 (13.77)	19.0 (6.38)	162.3 (18.35)	$3.3x10^{-16}$	956	111

Stage2: Neutrophil migration studies with fractions of faecal supernatant

Migrated neutrophil counts for three experiments are shown in tables 6.3 to 6.5, and are illustrated in the corresponding figures. ANOVA for the fractions of aliquot 1114 (Patient CF01, figure 6.3) showed significant differences amongst the fractions ($F=8.02$ $p=1.2 \times 10^{-5}$). In these fractions there were significant peaks of neutrophil chemotactic activity in fractions 8 and 12. (Fraction migrated neutrophil count - 2SEM > control + 2SEM)

Similarly ANOVA for fractions of aliquot 1092 (Patient CF04, figure 6.4) showed significant differences amongst the fractions ($F=10.34$, $p=1.2 \times 10^{-6}$). All fractions of this aliquot except 4 ($t=2.29$, $p= 0.08$) had a mean migrated neutrophil count significantly greater than the HBSS negative control. There was an unexpectedly intense neutrophil migration in fraction 3, and after this there were bimodal peaks with one in fraction 6 and a further peak in fractions 9 to 10. All fractions had significantly lower migrated neutrophil counts than the unfractionated aliquot control. These results are consistent with the aliquot having chemotactic activity in both the formyl-methionyl peptides fractions and in the void volume, but also suggest that the migrated neutrophil count in the control wells has been reduced.

Both fractions 7 and 8 for aliquots from patients CF03 (aliquot 961) and CF02 (aliquot 940) had a significantly larger mean migrated neutrophil count than the control samples ($p<0.015$, table 6.5). This is in contrast to the corresponding samples from patients CF07 (Aliquot 1084) and CF10 (Aliquot 1080). Therefore four of the six tested fractionated aliquots had neutrophil chemotactic activity in the void volume, a fraction not expected

to contain formyl peptides.

Figure 6.3 Patient CF01

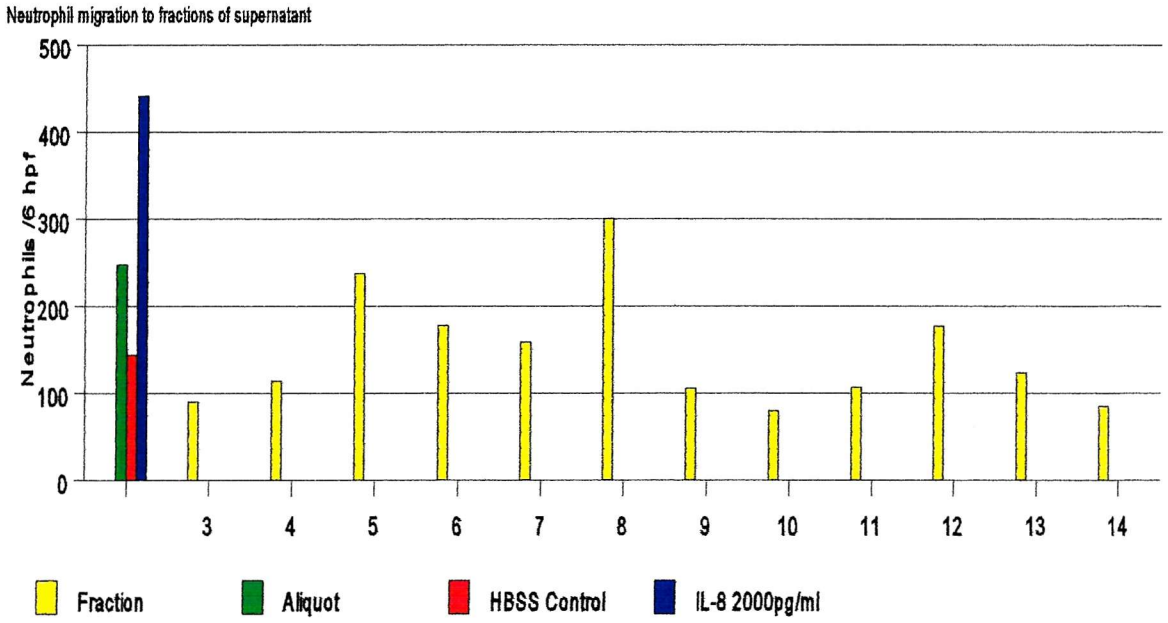


Figure 6.2 Patient CF04

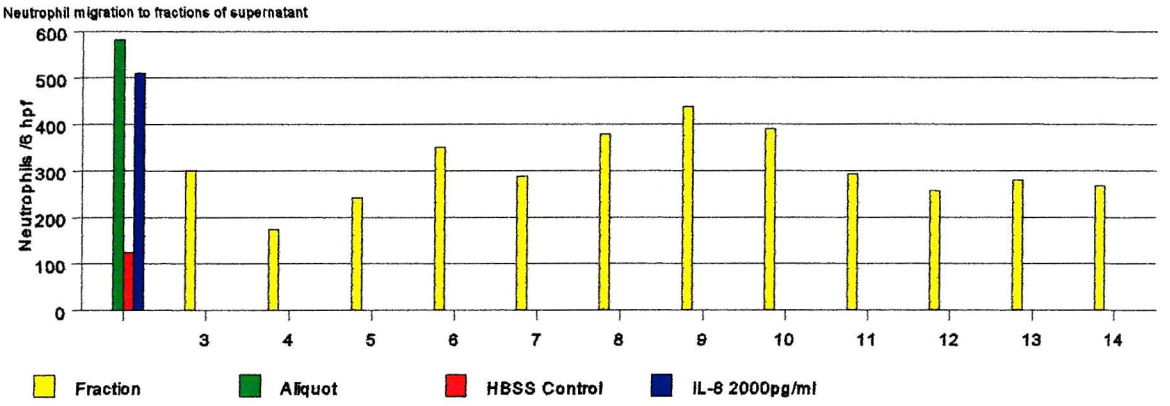


Table 6.3 Patient CF01 (Aliquot 1114)

Sample	Neutrophil count /6hpfs	SEM
Standards & Controls		
IL-8 2000pg/ml	441.0	47.08
fMLP	124.3	36.54
Neat Aliquot	247.5	22.50
HBSS	143.7	6.94
Fractions		
3	89.7	16.58
4	114.3	33.73
5	237.3	73.62
6	177.3	17.46
7	158.3	10.66
8	301.0	66.97
9	105.7	31.14
10	79.7	11.61
11	106.3	22.29
12	177.0	2.94
13	123.3	11.09
14	84.3	16.28

Table 6.4 Patient CF04 (Aliquot 1092)

Sample	Neutrophil count /6hpfs	SEM
Standards & Controls		
IL-8 2000pg/ml	510.7	25.04
fMLP	458.7	18.66
Aliquot diluted x 2	582.3	51.62
HBSS	125.0	27.53
Fractions		
3	301.0	38.29
4	174.0	12.36
5	242.0	23.37
6	351.0	29.63
7	288.0	10.23
8	379.0	19.20
9	438.7	28.57
10	390.3	26.61
11	293.3	29.89
12	257.3	45.43
13	280.3	60.54
14	267.3	26.39

Table 6.5 Patients CF 02, 03, 07, &10

(Aliquots 1080, 1084, 961 & 940)

Sample	Neutrophil count /6hpfs	SEM	t test results sample vs HBSS	
			t	p
Standards & Controls				
IL-8 2000pg/ml	235.0	70.28		
HBSS	73.0	37.18	N/A	N/A
Fractions of aliquot 1080 (Patient CF10)				
7	77.67	8.32	0.47	0.66
8	116.0	17.45	2.11	0.10
Fractions of aliquot 1084 (Patient CF07)				
7	88.3	12.76	0.95	0.39
8	104.3	9.81	1.78	0.14
Fractions of aliquot 961 (Patient CF03)				
7	224.3	45.26	4.19	0.013
8	267.7	12.12	9.58	0.00066
Fractions of aliquot 940 (Patient CF02)				
7	140.0	17.96	3.14	0.034
8	162.3	11.90	4.54	0.01

Stage3: Polymyxin B coincubation

In this experiment (table 6.6 & figure 6.6) one aliquot (patient CF06) had a significant increase in mean migrated neutrophil count with polymyxin B co-incubation, seven were not significantly different and three (patients CF 01, 03, & 07) had a significant reduction.

Figure 6.6 Effect of Polymyxinb on Neutrophil migration

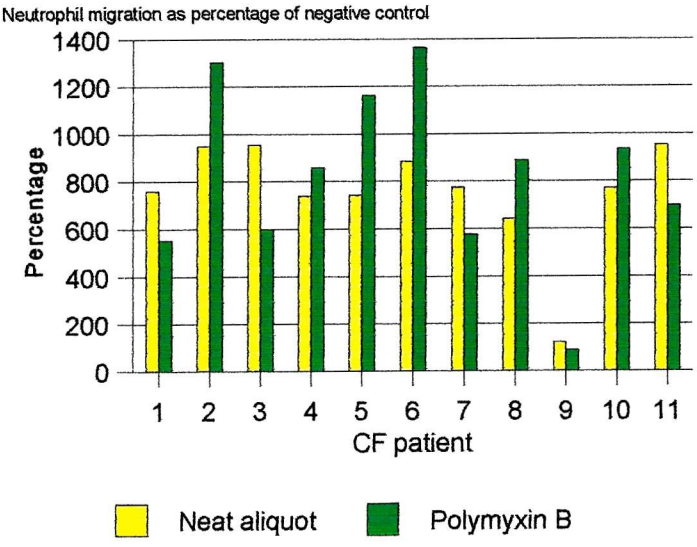


Table 6.6 Polymyxin B experiment

Sample	Mean (SEM) Migrated Neutrophil count				t value (p)
	2000pg/ml IL-8	HBSS Control	Native Aliquot	Aliquot plus Polymyxin B	
CF01 1114	188.7 (62.83)	70.0 (7.12)	532.3 (28.34)	387.0 (63.41)	3.14 (0.034)*
CF02 940	188.7 (62.83)	70.0 (7.12)	666.3 (154.45)	912.3 (94.41)	1.92 (0.120)
CF03 961	188.7 (62.83)	70.0 (7.12)	668.3 (75.02)	419.3 (86.68)	3.07 (0.037)*
CF041092	188.7 (62.83)	70.0 (7.12)	517.3 (143.15)	603.3 (22.45)	0.84 (0.448)
CF05 1089	162.3 (18.35)	19.0 (6.38)	141.0 (26.55)	221.7 (33.41)	2.67 (0.055)
CF06 1025	162.3 (18.35)	19.0 (6.38)	168.3 (13.91)	259.7 (13.52)	6.65 (0.002)*
CF07 1084	188.7 (62.83)	70.0 (7.12)	543.7 (53.58)	404.0 (41.96)	2.90 (0.044)*
CF08 1063	162.3 (18.35)	19.0 (6.38)	122.3 (15.17)	169.7 (41.33)	1.52 (0.203)
CF09 1069	162.3 (18.35)	19.0 (6.38)	23.3 (16.58)	17.3 (18.26)	0.34 (0.748)
CF10 1081	188.7 (62.83)	70.0 (7.12)	540.7 (88.58)	655.0 (108.47)	1.15 (0.312)
CF11 1075	162.3 (18.35)	19.0 (6.38)	181.7 (13.77)	133.7 (11.09)	3.84 (0.018)

Table 6.7 Cystic Fibrosis patients (neutrophil migration summary)

Pt	M F	Age	NIH Score	Neutrophil migration expressed as % of that in negative control or native aliquot						
				Aliquot	Fraction 7		Fraction 8		Polymyxin B treated	
				% CTRL	% CTRL	p (t-test)	% CTRL	p (t-test)	% aliquot	p (t-test)
CF01	F	17.18	57	594	110	0.26	209	1.3×10^{-7}	72.7	0.034*
CF02	F	9.40	61	951	192	0.034	222	0.01	136.9	0.120
CF03	F	6.46	95	954	307	0.013	366	0.0007	62.7	0.037*
CF04	F	14.11	73	605	230	5.4×10^{-12}	303	3.3×10^{-16}	116.0	0.448
CF05	M	12.24	90	138	Insufficient sample				157.0	0.055
CF06	F	8.06	88	397	Insufficient sample				154.0	0.002*
CF07	M	15.07	86	415	121	0.39	143	0.14	74.4	0.044*
CF08	M	13.68	69	352	Insufficient sample				138.5	0.203
CF09	F	16.81	58	122	Insufficient sample				75.2	0.748
CF10	M	15.55	79	814	106	0.66	159	0.10	121.2	0.312
CF11	F	13.37	80	952	Insufficient sample				73.4	0.018

Discussion

In contrast to the study conducted in Southampton, there was no difference in faecal interleukin-8 concentration between cystic fibrosis patients and healthy controls, in that neither had measurable chemokine. Similarly none was detectable in any of the disease controls except those patients with inflammatory bowel disease, and possibly one girl with bronchiectasis. The persisitence of high concentrations of the chemokine in the faeces of patients with colitis, serves to defend the validity of using this assay protocol in this study. It suggests that if as we asserted in the original study, faecal IL-8 concentrations in cystic fibrosis and inflammatory bowel disease were similar, this assay protocol should have been able to measure chemokine concentrations in these CF faeces. The absence of elevated IL-8 concentration in cystic fibrosis, bronchiectasis, asthma and pneumonia patients supports the conclusion that faecal IL-8 concentration does not reflect severity of pulmonary inflammation in these diseases.

Conclusions drawn from the undetectable TNFα concentrations in CF, healthy control and respiratory disease control subjects are not as solidly supported by my data as those drawn from undetectable IL-8 concentrations, because samples from inflammatory bowel disease patients who clearly had active colitis at the time of the faecal sample collection also had an undetectable faecal TNFα concentration. It is therefore possible that Faecal TNFα concentration is not a good marker of either intestinal or pulmonary inflammation. The TNFα spiking experiments in the validation studies do however lend support to the notion that increasing faecal TNFα concentration will result in an increased measurement

of TNFα concentration by this assay.

The neutrophil migration studies have been summarised in table 6.7. There was a degree of variability in the neutrophil counts per 6 high power fields. Such variation is to be expected in biological experiments. Repetition of the experiments would reduce the impact of such variation on the analysis of the data. In particular it might have clarified the relevance of the chemotactic activity in the large number of fractions in patient CF04. Unfortunately the limited supplies of these samples prevented these repetitions from being performed. The results show that nine of the eleven CF patients had marked neutrophil chemotacic activity despite the lack of ELISA measurable IL-8. Six of these nine patients had the faecal supernatant fractionated and retested, and in four of these patients chemotactic activity resided in fractions which would have harboured molecules of molecular weight equal to or greater than that of the chemokines. Two of these four samples had their chemotactic activity significantly reduced by treatment with polymyxin B, implying that endotoxin had played a role in mediating the neutrophil chemotaxis in these samples. Therefore in four of the eleven samples there was no evidence of IL-8 chemotactic activity, in a further two there was positive evidence of the influence of other biological molecules in mediating the chemotaxis.

The design of the neutrophil migration studies was optimised to isolate neutrophil chemotactic activity due to formyl peptides with molecular weights below 700 Da, from that due to IL-8. It does not allow the chemotactic activity of IL-8 to be separated from other chemokines or higher molecular weight chemotaxins. Taken together with the

results of the ELISAs these results are consistent with, the absence of IL-8 from the faecal supernatants of four of the eleven patients in the study, and suggest, but do not prove that neutrophil chemotactic activity in the remaining seven patients' samples may be due to non IL-8 chemotaxins of over 700 Da molecular weight.

These studies have not refuted the hypothesis that some IL-8 passes through the gut intact resulting in some chemotactic activity and a small amount of IL-8 immunoreactivity. However the fact that this immunoreactivity has not allowed quantification of the faecal IL-8 concentration in this population of CF patients, many of whom had established lung disease and some of whom had advanced lung disease, renders this assay impotent to detect presymptomatic pulmonary inflammation in patients too young to expectorate sputum.

It seems that these cytokines are significantly degraded by their passage through the gut, presumably by digestive enzymes or bacterial activity. These issues will be explored in chapter 7.

Chapter 7

Fate of Swallowed cytokines

Introduction

The failure to find faecal cytokines in CF patients with significant lung disease implies that any cytokines swallowed are degraded in the course of their passage through the CF gut. This is either the result of host biological activity or the activity of comensal GI tract organisms. In sections 1 and 2 of this chapter the abilities of pancreatic enzyme preparations and colonic comensal organisms to digest IL-8 and TNFα will be tested in vitro under conditions simulating the CF gut. In the third section the ability of these cytokines to pass through a mamalian gut intact will be tested in vivo in the Wistar rat.

It is self evident that any potent biological effector mechanism must have some process for ending the action of the effector. The subsequent recycling of chemokine amino acids and peptides must involve enzymatic digestion, because chemokines are not stored in sub-cellular organelles. It is equally self evident that if micro-organisms are capable of completely recycling biological matter from human remains then this group of organisms must posess the means to digest all human proteins. However given the imperfect intra-lumenal digestion occuring in patients with CF and the paucity of data on the normal enteric flora of CF patients,[144] there is significant doubt that these mechanisms could account for the apparent lack of these swallowed cytokines in CF faeces.

Interleukin-8 is relatively resistant to proteases. Attempts to digest the 72 amino acid molecule with laboratory proteases have not produced smaller active fragments.[145] However there are no descriptions of the effects of porcine pancreatic preparations on IL-8, and studies to explore this digestive capacity of these enzymes are required. These studies can easily be conducted in the laboratory and this will be described in section 1.

In the faecal flora experiment, in section 2, it is necessary to separate the effect of any enzymes present in faeces from that of the faecal flora. Therefore it will be necessary to incubate faecal material on primary plate cultures and take representative mixed subcultures from these plates, and use these subcultures for an experiment in a broth environment where cytokines and organisms are coincubated.

In section 3, the fate and effect of IL-8 and TNFα administered to the upper gut of the Wistar rat will be described. This will test both the ability of digestive enzymes and enteric organisms to degrade cytokine in vivo. This experiment has been conducted in animals because of ethical and design concerns in using human volunteers.

The cytokines of interest are human TNF alpha and IL-8 and these might be produced by intestinal cells at several points in the gut, so there can be no gaurantee that cytokine detected in the faeces, following administration of hrTNFα and hrIL-8 to the human upper gut, had originated from the administered dose. Secondly, there is no data available on the safety of oral ingestion of these cytokines, and therefore it is not justified to administer these cytokines to humans.

The Wistar rat is a logical laboratory animal to use. Wistar rats have been shown to respond to human recombinant Interleukin-8 and Tumour Necrosis Factor alpha,[146,147] with responses that paralell the human response to these cytokines. Additionally they have been used in experimental colitis models, and faecal material from rats with experimental colitis can be analysed for cross immunoreactivity to rhTNFα and rhIL-8, as a disease control in the rat.

In order to make this animal model representative of a CF patient with optimal enzyme replacement therapy, dosages of administered cytokine should paralell the exposure of the human cystic fibrosis gut to cytokines. Measurements of sputum concentrations of IL-8 and TNF together with estimates of daily sputum production allow calculation of an experimental dose.

Section 1: Invitro enzyme digestion

Study design

In this experiment concentrated IL-8 and TNFα solutions are to be coincubated with a range of porcine pancreatic enzyme preparations and control buffer at an alkaline pH and at a pH which might be encountered in the duodenum of a pancreatic insufficient patient. At the end of the incubation period the solutions will be subjected to ELISA for the two cytokines, additionally a second aliquot will be assayed from each sample early in the incubation period in an attempt to show that any changes are progressive. Finally CF sputum rather than aqueous cytokine solution will be similarly treated.

Method

Outline

Solutions of Creon Forte (Solvay), Pancrease (Cilag) & Cotazym S (Organon), were made up in an alkaline buffer solution to a standard protease activity/ml of solution. Aliquots of these solutions were analysed for trypic activity in the hospital laboratory. Triplicate samples of the enzyme solution were inoculated with Interleukin 8 to a concentration of 1000pg/ml, and incubated at 37°C for four hours. The enzyme activity was inhibited at four hours by the addition of the protease inhibitors "Soy bean Trypsin inhibitor" and "Phenylmethylsulphonlylflouride", the chelating agent "EDTA", and newborn calf serum was used to act as a substrate for any remaining protease. The samples were frozen at -70°C until analysis.

Detail

Enzyme solutions were prepared by adding 1 Creon 25,000 capsule, 3 Cotazym-S capsules or 5 Pancrease capsules in 100mls buffer. Solutions of IL-8 (2000pg/ml) and TNFα (4,545pg/ml) were prepared by diluting a 10μg/ml solution tenfold in Newborn calf serum and further diluting this in phosphate buffered saline.

The remaining reagents were prepared; 0.3M EDTA in deionised water, 1.74g% PMSF in 95% alcohol, SBTI 0.1g% in PBS, and calf serum as supplied by Sigma (Cat no N4762).

1ml of enzyme suspension or control diluent was placed in each test tube. To this 1ml of warmed buffer, IL-8 (2000pg/ml) or TNF-α (4545pg/ml) was added. Tubes were incubated for 1 hour at 37°C, tubes were mixed and a 500μl sample removed. Tubes were reincubated for a further three hours at 37°C. Meanwhile enzyme activity in the removed sample was inhibited by the addition of 50μl SBTI, 28μl EDTA and 12μl PMSF. After two minutes 30μl NBCS was added and the samples frozen. After a total of four hours incubation the remaining tubes were mixed, and to each tube 150μl SBTI, 84μl EDTA and 36μl PMSF were added and two minutes later 90μl NBCS. These were divided into 500μl aliquots and frozen.

Without any cytokine digestion, the sample dilution in this protocol would lead to concentrations of cytokine of 806.45pg/ml IL-8, and 1832.84pg/ml TNFα.

Assay Validation

Triplicate samples from digestion of IL-8 were assayed spiked to a concentration of 1000 pg/ml IL-8.

Sputum

Sputum from a CF patient was mixed with an equal weight of either pH 8.0 buffer or pH 8.0 buffer with dissolved Creon Forte. It was incubated in a waterbath at 37°C for four hours, then centrifuged at 20,000g for 15 minutes and the supernatant was assayed neat and at dilutions of x3 and x9.

Results

Interleukin-8 assay results

No IL-8 immunoreactivity was detected in any of the triplicate tubes loaded with both enzyme and cytokine, at either four or one hour's incubation, at either pH 8 or pH 4.5. Mean IL-8 concentrations in the spiked buffer samples were 603pg/ml at 1 hour, and 853 pg/ml at four hours at pH 8.0 and 587 pg/ml at 1 hour and 514 pg/ml at four hours at pH 4.5. There was no significant difference in spiked buffer IL-8 concentration at 1 and 4 hours at pH 4.5 ($t=2.31$, $p=0.08$), but the mean measured 4 hour IL-8 concentration was significantly greater than the 1 hour concentration at pH 8 ($t=6.05$, $p=0.004$).

Spiked IL-8 digestion samples

The mean (sd) measured IL-8 concentration for the nine samples from the IL-8 digestion at pH 8, that were spiked to 1000pg/ml using the assay 2000pg/ml IL-8 standard as spiking solution was 856.5 (17.4)pg/ml.

Mean (sd) IL-8 concentrations were 873.68 (12.2), 857.87 (9.1) & 838.09 (5.8)pg/ml for Creon Forte, Pancrease and Cotazym respectively. The triplicate samples for the three different enzyme preparations were statistically significantly different (Anova $F=5.368$ $p=0.046$). Cotazym S spiking recovered significantly less than Creon Forte ($t=4.5$, $p=0.01$) and Pancrease ($t=3.16$, $p=0.03$). The measured pH of the three enzyme preparations before addition of cytokine was 4.49 (Creon Forte), 4.39 (Cotazym-S) and 4.48 (Pancrease).

Sputum sample

Interleukin-8 recovered from the CF sputum incubated with buffer was 10,013pg/ml whilst 3,052pg/ml was recovered from the sample incubated with Creon Forte at pH 8.0.

TNFα assay results

In contrast TNFα was at measurable concentrations in all samples where the cytokine was coincubated with enzyme after incubation at pH 4.5 and pH 8.0. All control samples which had not been innoculated with TNFα had no TNFα immunoreactivity. Results are shown in table 7.0 and figure 7.0 Measured TNFα concentration in all of the control

samples was well in excess of the expected 1830pg/ml. In contrast all enzyme coincubated samples show a markedly reduced concentration at both pH4.5 and pH8, which progressed between 1 and 4 hour of incubation.

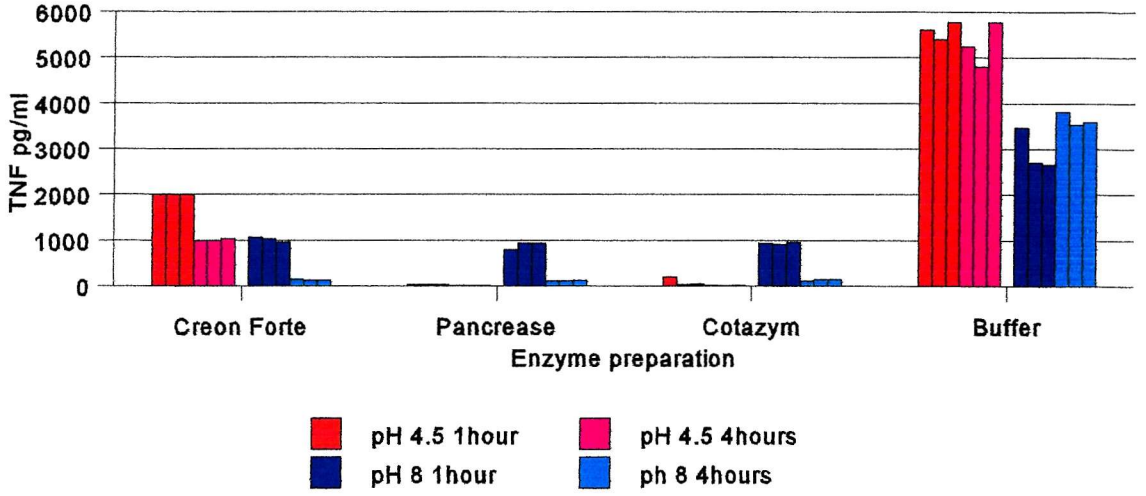
Table 7.0

Preparation & (Tryptic activity)	TNFα concentration pg/ml			
	pH 8		pH 4.5	
	4 Hours	1 Hour	4 Hours	1 Hour
Creon Forte	134.82	1066.20	986.36	>2000
(4.3 kU/l)	124.14	1027.67	1000.54	>2000
	125.67	955.16	1024.05	>2000
Pancrease	118.56	797.61	7.38*	31.59
(17.7 kU/l)	115.26	933.60	5.12*	29.93
	128.72	929.73	8.31*	35.00
Cotazym S	117.29	934.71	16.22	197.67
(15.1 kU/l)	140.43	908.74	12.77*	35.00
	136.61	961.80	13.64*	39.56
Buffer	3822.90	3470.90	5244.66	5614.91
(<0.05 kU/l)	3542.54	2711.32	4807.61	5405.56
	3592.49	2659.56	5779.02	5779.02

* Calculated by extrapolation of the calibration curve.

Figure 7.0 TNF alpha concentration

After 1 & 4 hours enzyme coincubation



Sputum samples

Neither the digested nor the undigested sputum samples contained assayable concentrations of TNFα with the dilution that occurred in the experiment.

Discussion

The presence of assayable quantities of TNFα in the samples for all three enzyme preparations at both time points, allows me to conclude that there has been a progressive fall in TNFα concentration with time in these samples. That this fall was reduced in the preparation with the lowest measured tryptic activity strongly suggests that enzymatic activity is responsible for this at least in the case of TNFα. One might argue that the failure to detect IL-8 was the result of enzymatic interference with the assay rather than cytokine digestion, but the recovery of an average of 85% of spiked IL-8 when such

samples are spiked after the addition of protease inhibitors makes this untenable. I am lead to conclude that both IL-8 and TNFα are efficiently digested by all three tested pancreatic enzyme preparations both at pH4.5 and pH8. The four fold reduction in measured Sputum IL-8 concentration on incubation with Creon Forte under circumstances which have abolished IL-8 immunoreactivity in aqueous samples suggests that sputum may provide a degree of protection for the chemokine against pancreatic enzymes.

Two technical points in these assays deserve comment, firstly I have shown a significant reduction in IL-8 recovery in buffer incubation at 1 hour and not at four hours, and secondly the calculated concentrations of TNFα following buffer incubation at both pH4.5 and pH8 show concentrations, over twice the administered concentration. These illustrate the sensitivity of the ELISA assays to minor changes in the chemical composition of the assay wells. Despite these variations from ideal behaviour in the control samples, the contrast with the changes seen in the enzyme incubated samples is marked, and there remains convincing evidence of enzyme digestion in those wells.

Section 2: In vitro CF colonic flora: effect on cytokines in solution

Study Design

To identify the influence of CF faecal flora on IL-8 and TNFα, representative colonies from anearobic and aerobic plate cultures of CF faecaes were coincubated in broth with IL-8 and TNFα and the resulting cytokine concentration measured by ELISA.

Design Constraints

Laboratory culture techniques for clinical samples have been developed to isolate pathogens in stool by providing an environment which is selective for pathogens. In this study, however the objective requires culture of flora representative of the CF colon. In primary plate cultures an element of selection may inadvertently occur. To minimise this, broad spectrum media (Brain heart infusion agar and Horse blood agar) were selected for primary plate culture and tryptone soy broth for the broth culture phase. This broth culture was conducted in a closed system, so that anaerobes and aerobes could grow in paralell, simulating the colonic environment.

Culture in broth was expected to provide ample substrate for a wide spectrum of organisms, but this supply of nutrients may have blunted the ability of the experiment to detect bacterial cytokine digestion. Some organisms are capable of swiching substrate and whilst they ordinarily synthesise enzymes for digesting simple substrate, they may

synthesize the enzymes required for more complex substrate only under conditions of low substrate availability.[148] This study design might fail to detect such facultative cytokine digestion. This limitation and the limitation imposed by not specifically culturing fungi must be acknowledged at the outset.

There are additional technical contraints. Because it is not routine to perform cytokine ELISAs in the tryptone Soy broth environment, it is possible that the broth itself or the high glucose or amino acid concentration may bring about a chemical reaction with the cytokines, and interfere with antibody binding of cytokines in the ELISA, or that the glass bijou, necessary for the closed system might also interact with the cytokines. A supplementary experiment to address these issues is therfore required.

Bacteriology experiments

The broth culture phase of the study was conducted in Tryptone Soy broth (TSB), as this supports the growth of a wide range of bacteria. It was conducted in non stirred bijous so that aerobic conditions would persist near the surface of the broth, but anaerobic conditions could develop at the base. The plate cultures were to be conducted on separate aerobic and anaerobic culture plates.

Patients

Patients were recruited from the Cystic Fibrosis clinic at the Royal Children's Hospital, Brisbane. The only selection criterion being their willingness to provide a faecal sample. Patient details are shown in table 7.1.

Table 7.1

Stool Number	Age (yrs)	sex	NIH Score	Height z-Score	Weight z-score	% Predicted		
						FEV1	FVC	FEF 25-75
67	8.06	F	88	-0.34	0.25	60	82	38
69	6.46	F	95	0.81	-0.17	70	73	62
81	15.44	M	79	-1.84	-2.13	66	78	40
84	2.42	F	98	-0.25	0.29	-	-	-
86	5.41	M	97	-0.72	-0.91	-	-	-

Method

Outline

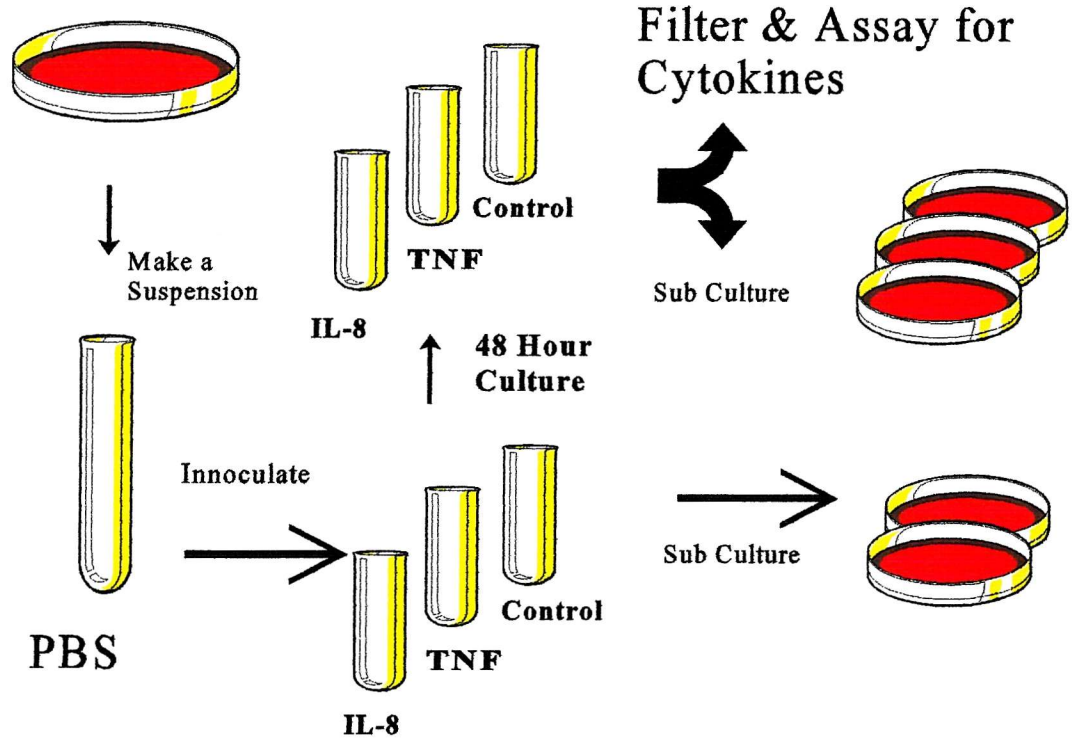
The design is summarised in the cartoon in figure 7.2.

Bacterial preparation

Five thawed stools from patients with cystic fibrosis, and a phial of sterile PBS, were plated onto Horse Blood Agar and Brain heart infusion agar (Oxoid) and incubated at 37°C for 48 hours under aerobic and anaerobic conditions respectively.

At 48 hours a representative mixture of colonies from aerobic and anaerobic plates was suspended in each of 5 phials containing 10 mls of sterile Phosphate Buffered Saline. The

Figure 7.2



organism concentration was adjusted by adding sterile saline to achieve a McFarlane's standard by eye. Fifteen mls of sterile saline was inoculated into the 6th 20 ml test tube.

TNF-α and IL-8 stock solution preparation

Lyophilised TNF-α and IL-8 (R&D Illanois) was reconstituted by adding 1 ml of sterile PBS with 0.1% bovine serum albumin to 10μg of cytokine. From this solutions of 6000pg/ml Il-8 and 3000pg/ml TNF alpha were produced by dilution in PBS.

Preparation of culture media

Sterile 6000pg/ml IL-8 (15.6mls) was added to 50 mls of sterile tryptone soy broth. Three 2.8ml aliquots of the spiked broth were placed in triplicate bijous for each supernatant and the sterile PBS. Then 1ml of the appropriate bacterial suspension was added to each bijou. Similarly 15.6mls of 3000pg/ml TNF-α was added to 50mls of TSB, placed in bijous and inoculated with 1ml of each bacterial suspension, and finally 2.8 mls of TSB alone was similarly placed in further triplicate bijous and inoculated.

From each bijou one 10μl loop was taken for aerobic plate culture (Horse blood Agar) and one for anaerobic plate culture (Brain Heart Infusion Agar).

Incubation

All bottles were tightly capped and incubated at 37°C for 48 hours without shaking. Each bijou was labelled with a stool number (0 being a PBS control), and a suffix. Suffixes 1-3 indicating that culture medium is spiked with IL-8, 4-6 with TNF alpha, and 7-9 indicating that unspiked tryptone soy broth has been used. The plates were incubated for 48 hours, and then examined and photographed. Pre and post broth culture plates were examined to determine which organisms were present. Plate colonies were classified according to colony appearances, and growth was classified as being scanty (+), moderate (++) or heavy (+++). After 48 hours each bijou was gently mixed, and 10µl was taken for repeat aerobic and anaerobic culture. These were cultured for a further 48 hours before being examined and photographed. The photography was intended to provide a permanent record of the plates. Pre broth incubation plates were retained and refrigerated so that they could be compared directly with the post broth incubation plates.

When an organism was cultured on a post broth incubation plate, which had not been cultured on any of the pre broth incubation plates from the same stool, it was classified as a contaminant. When an organism which had been cultured on the pre-broth incubation plate was not cultured on the corresponding post-broth incubation plate it was classified as having undergone flora modification.

ELISA sample preparation

Each bijou was filtered through a 0.22μ filter and filtrate was decanted into cryovials and frozen at -70°C until analysis. These samples were assayed using IL-8 and TNF alpha Quantikine kits according to manufacturers instructions for culture supernatants.

Supplementary experiment

To test the possibility that Tryptone Soy Broth, its sugar content or amino acid content could influence measured IL-8 concentrations, a supplementary experiment was conducted where spiked samples were assayed after a short incubation.

Polypropylene test tubes were loaded with 500μl of either 50% Dextrose, 20% Synthamen (Nestlé Clinical, Rickmansworth, Hertfordshire) and Tryptone Soy Broth in duplicate. To one of each pair of test tubes 500μl of newborn calf serum was added, whilst 500μl of phosphate buffered saline was added to the other. All test tubes were then spiked with IL-8 to 2000pg/ml by adding 500μl of 6000pg/ml stock IL-8. Two control tubes, one containing 1000μl of newborn calf serum and the other containing 1000μl of phosphate buffered saline were similarly spiked.

These test tubes were incubated at 37°C in a waterbath for two hours, and were then aliquoted and stored at -70°C until assayed in a Quatikine ELISA for IL-8 according to manufacturers instructions for tissue culture supernatants.

Two aliquots per test tube were assayed, and differences in cytokine concentration

between the media (tryptone soy broth, glucose, amino acids and nothing) and between the two diluents (phosphate buffered saline and newborn calf serum) were sought using two way analysis of variance with 1 repetition.

Results

Bacterial Flora

Table 7.2 shows the results of plate subculture of the broth bijous before and after the 48 hour broth incubation period. No control plate from the pre broth incubation series showed growth. At the end of the 48 hour broth culture period three control bijous had become cloudy, these were 0/2 & 0/3 which were IL-8 spiked media and 0/7 which was unspiked medium. All three were contaminated with Staphylococci. Bijous 67/2 and 67/3 were contaminated with Streptococci. Seventeen cytokine containing bijous underwent flora change in the course of the incubation. These bijous are highlighted in green in table 7.3.

Photographs of the culture plates are shown in plates 1-18, (after page 173) and these images are annotated with the filename of the corresponding file on the accompanying CDrom.

Table 7.2		IL-8						TNFα						Control					
	Bijou	1		2		3		4		5		6		7		8		9	
	Incubation	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
	Organism																		
67	Bacteroides	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Coliforms	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Staphylococci									+++									
	Streptococci				+++		+++												
69	Clostridia	+++		+++	++			+++		+++		+++		+++		+++		+++	
	Bacteroides		+++		+++	+++	+++	+++	+++		+++		+++	+++	+++	+++	+++	+++	+++
	Coliforms	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Staphylococci	+++		+++		+++		+++		+++		+++		+++		+++		+++	
	Streptococci	+++		+++		+++		+++		+++		+++		+++		+++		+++	

Table 7.2		IL-8						TNFα						Control					
	Bijou	1		2		3		4		5		6		7		8		9	
	Incubation	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
	Organism																		
81	Clostridia	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++		++	+++
	Bacteroides	+++				+++		+++		+++		+++	+++	+++		+++		++	+++
	Coliforms																+++		+++
	Staphylococci						++							+					
	Streptococci	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	++	+++

Table 7.2		IL-8						TNFα						Control					
	Bijou	1		2		3		4		5		6		7		8		9	
	Incubation	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
	Organism																		
84	Clostridia	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Bacteroides	+++		+++		+++		+++	+++	+++		+++	+++	+++	+++	+++	+++	+++	+++
	Coliforms	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Streptococci					+++		+++	++	++	++	++	++	+++	++	+++	+++	++	+++
86	Clostridia	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Coliforms			+				++						+				+	
	Streptococci	+++	+++	+++	+++	++	+++	+++	+++	++	+++	++	+++	+++	+++	+++	+++	++	+++
0	Staphylococci				+++		+++												
	Streptococci														+++				

Key + = Scanty growth, ++ = Moderate growth, +++ = Heavy growth

Plate 1

Stool 0

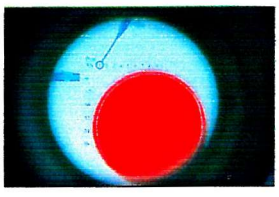
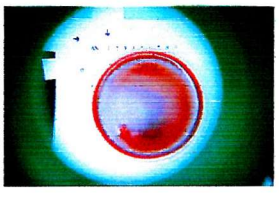
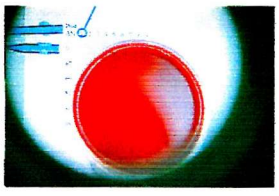
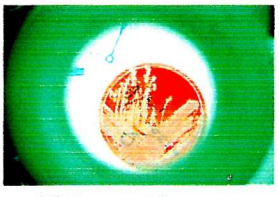
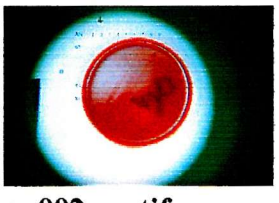
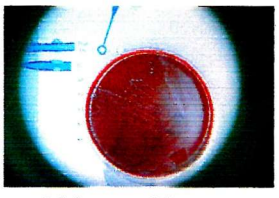
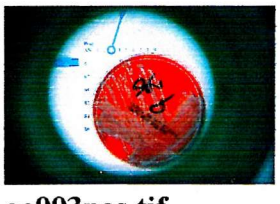
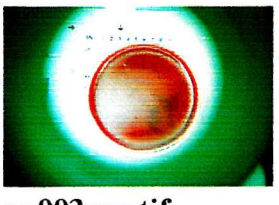
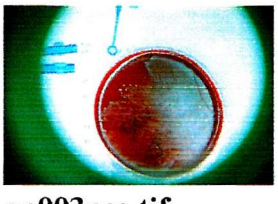
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 0 Bijou 1 IL-8 Co-culture			
 ae001pre.tif	 ae001pos.tif	 an001pre.tif	 an001pos.tif
Stool 0 Bijou 2 IL-8 Co-culture			
 ae002pre.tif	 ae002pos.tif	 an002pre.tif	 an002pos.tif
Stool 0 Bijou 3 IL-8 Co-culture			
 ae003pre.tif	 ae003pos.tif	 an003pre.tif	 an003pos.tif

Plate 2

Stool 0

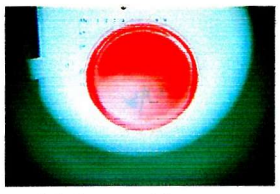
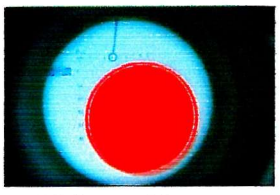
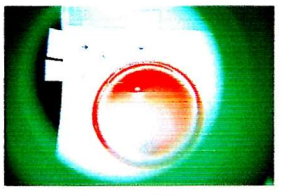
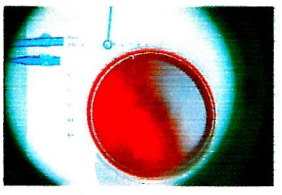
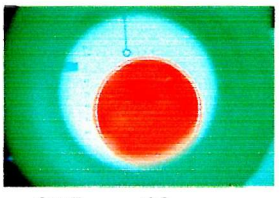
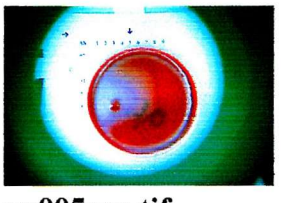
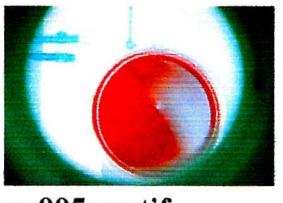
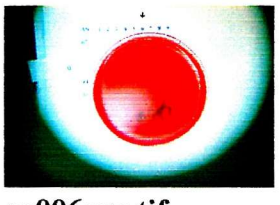
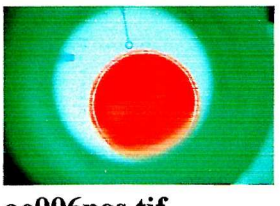
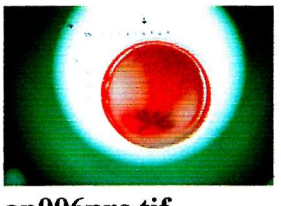
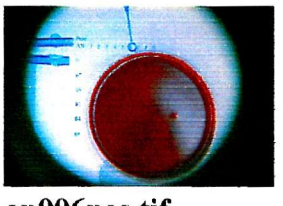
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 0 Bijou 4 TNF Co-culture			
 **ae004pre.tif**	 **ae004pos.tif**	 **an004pre.tif**	 **an004pos.tif**
Stool 0 Bijou 5 TNF Co-culture			
 **ae005pre.tif**	 **ae005pos.tif**	 **an005pre.tif**	 **an005pos.tif**
Stool 0 Bijou 6 TNF Co-culture			
 **ae006pre.tif**	 **ae006pos.tif**	 **an006pre.tif**	 **an006pos.tif**

Plate 3

Stool 0

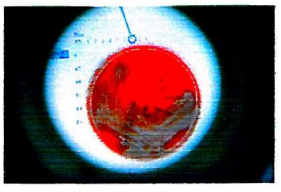
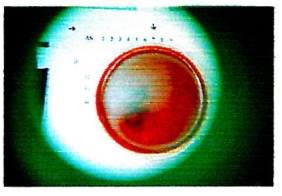
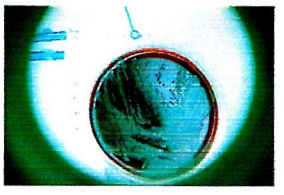
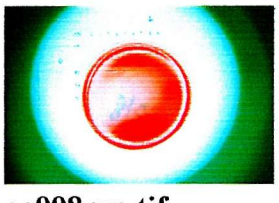
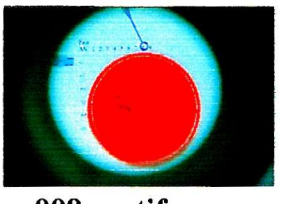
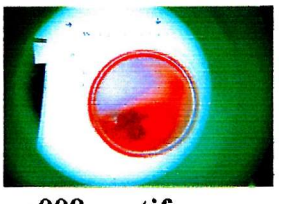
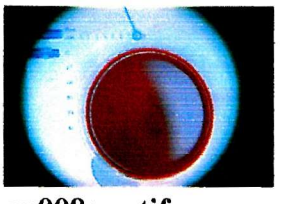
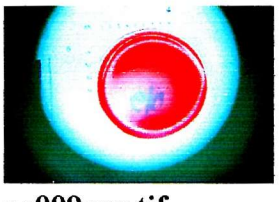
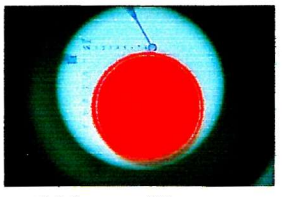
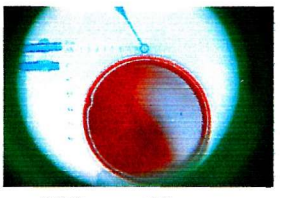
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 0 Bijou 7 Control			
			
ae007pre.tif	**ae007pos.tif**	**an007pre.tif**	**an007pos.tif**
Stool 0 Bijou 8 Control			
			
ae008pre.tif	**ae008pos.tif**	**an008pre.tif**	**an008pos.tif**
Stool 0 Bijou 9 Control			
			
ae009pre.tif	**ae009pos.tif**	**an009pre.tif**	**an009pos.tif**

Plate 4

Stool 67

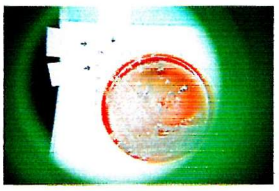
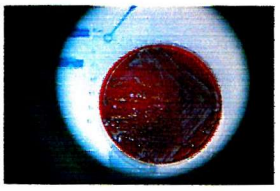
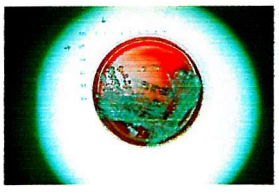
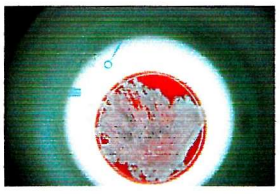
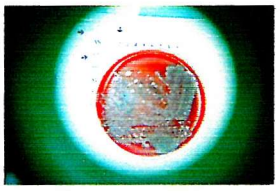
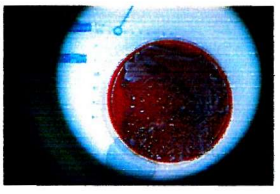
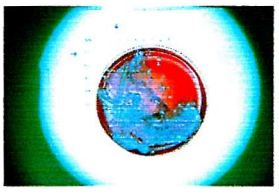
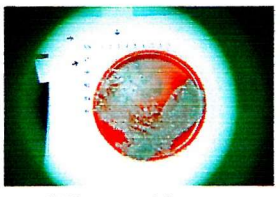
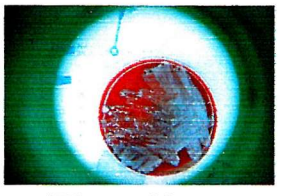
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 67 Bijou 1 IL-8 Co-culture			
 ae671pre.tif	 ae671pos.tif	 an671pre.tif	 an671pos.tif
Stool 67 Bijou 2 IL-8 Co-culture			
 ae672pre.tif	 ae672pos.tif	 an672pre.tif	 an672pos.tif
Stool 67 Bijou 3 IL-8 Co-culture			
 ae673pre.tif	 ae673pos.tif	 an673pre.tif	 an673pos.tif

Plate 5

Stool 67

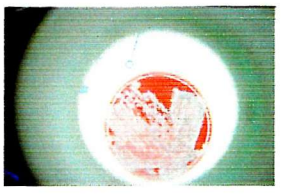
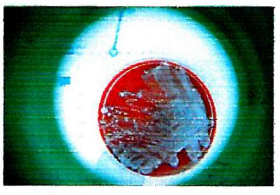
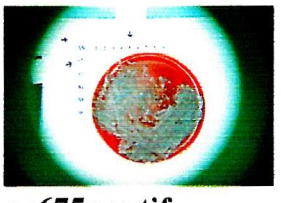
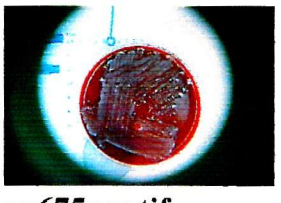
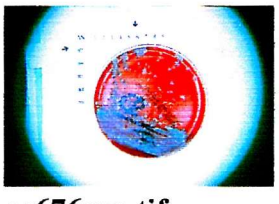
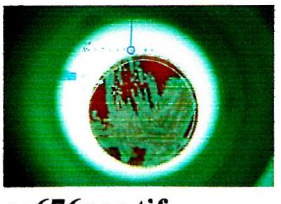
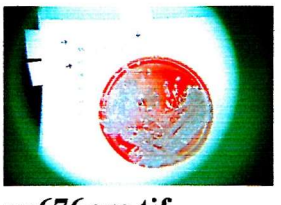
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 67 Bijou 4 TNF Co-culture			
 ae674pre.tif	 ae674pos.tif	 an674pre.tif	 an674pos.tif
Stool 67 Bijou 5 TNF Co-culture			
 ae675pre.tif	 ae675pos.tif	 an675pre.tif	 an675pos.tif
Stool 67 Bijou 6 TNF Co-culture			
 ae676pre.tif	 ae676pos.tif	 an676pre.tif	 an676pos.tif

Plate 6

Stool 67

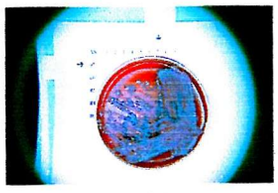
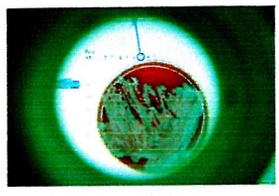
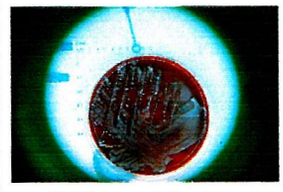
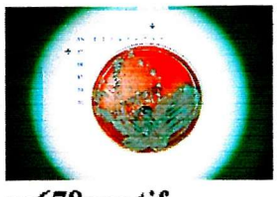
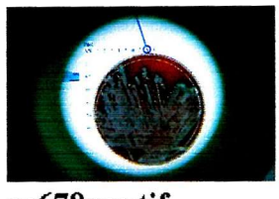
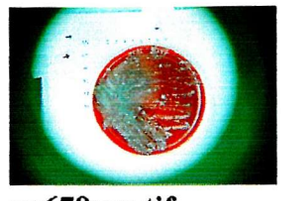
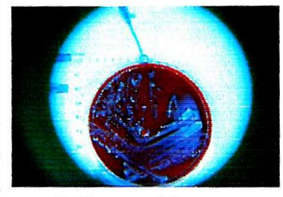
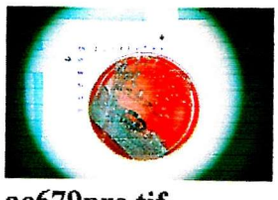
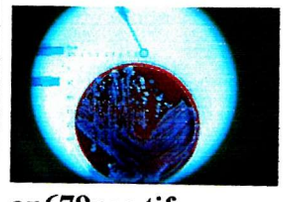
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 67 Bijou 7 Control			
 **ae677pre.tif**	 **ae677pos.tif**	 **an677pre.tif**	 **an677pos.tif**
Stool 67 Bijou 8 Control			
 **ae678pre.tif**	 **ae678pos.tif**	 **an678pre.tif**	 **an678pos.tif**
Stool 67 Bijou 9 Control			
 **ae679pre.tif**	 **ae679pos.tif**	 **an679pre.tif**	 **an679pos.tif**

Plate 7

Stool 69

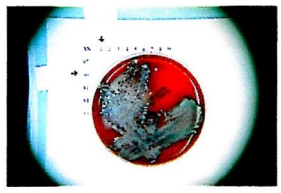
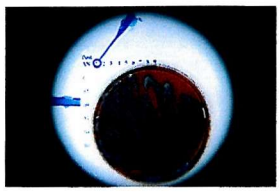
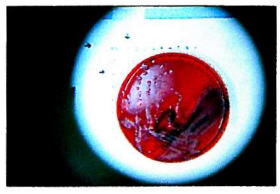
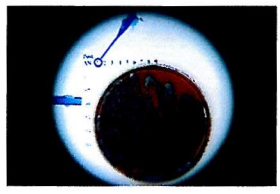
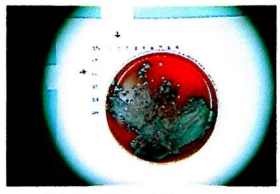
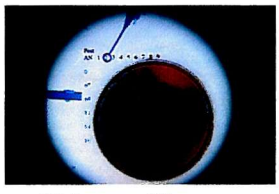
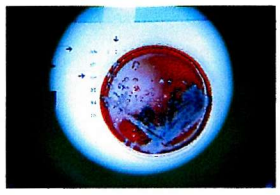
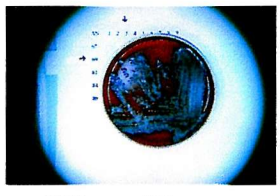
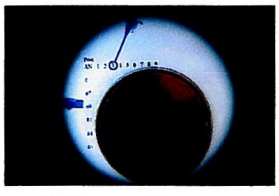
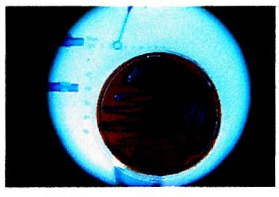
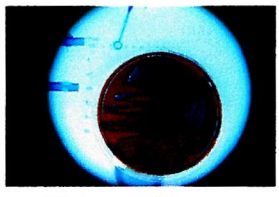
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 69 Bijou 1 IL-8 Co-culture			
 **ae691pre.tif**	 **ae691pos.tif**	 **an691pre.tif**	 **an691pos.tif**
Stool 69 Bijou 2 IL-8 Co-culture			
 **ae692pre.tif**	 **ae692pos.tif**	 **an692pre.tif**	 **an692pos.tif**
Stool 69 Bijou 3 IL-8 Co-culture			
 **ae693pre.tif**	 **ae693pos.tif**	 **an693pre.tif**	 **an693pos.tif**

Plate 8

Stool 69

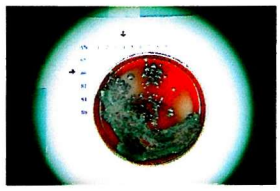
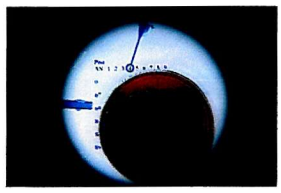
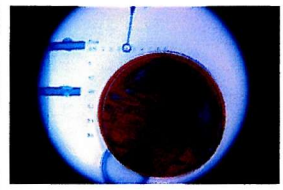
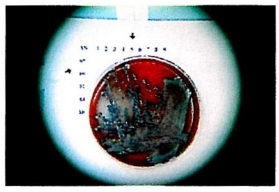
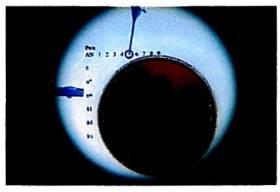
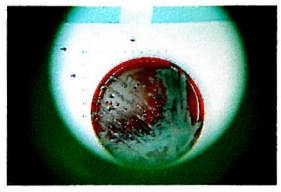
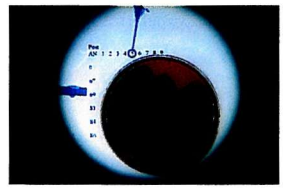
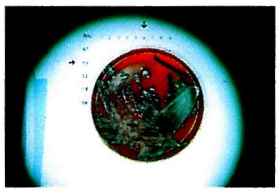
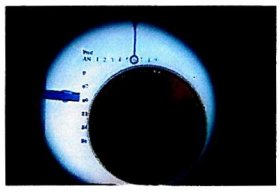
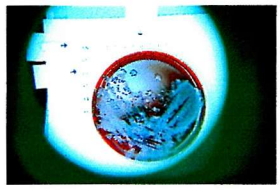
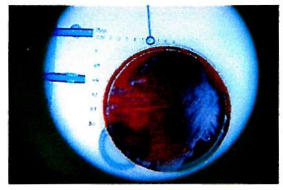
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 69 Bijou 4 TNF Co-culture			
 ae694pre.tif	 ae694pos.tif	 an694pre.tif	 an694pos.tif
Stool 69 Bijou 5 TNF Co-culture			
 ae695pre.tif	 ae695pos.tif	 an695pre.tif	 ae695pos.tif
Stool 69 Bijou 6 TNF Co-culture			
 ae696pre.tif	 ae696pos.tif	 an696pre.tif	 an696pos.tif

Plate 9

Stool 69

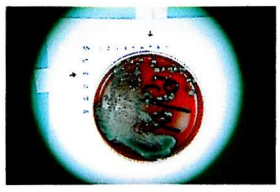
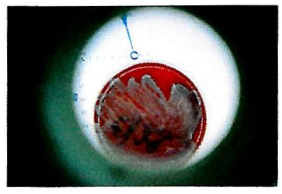
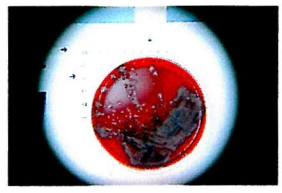
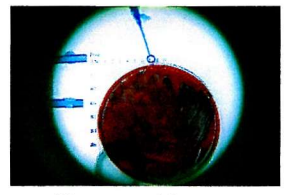
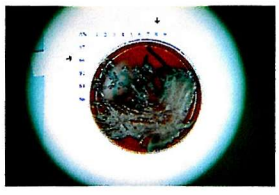
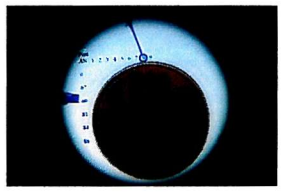
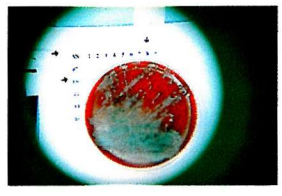
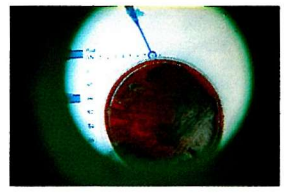
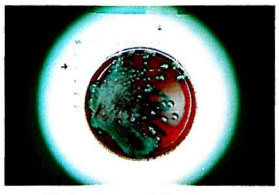
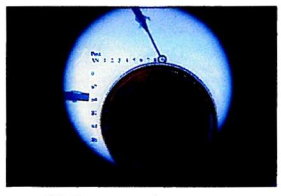
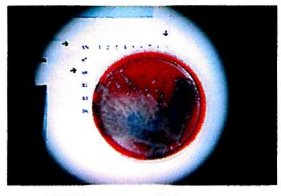
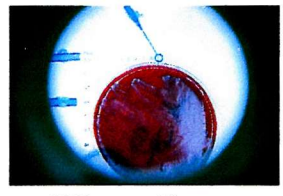
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 69 Bijou 7 Control			
			
ae697pre.tif	**ae697pos.tif**	**an697pre.tif**	**an697pos.tif**
Stool 69 Bijou 8 Control			
			
ae698pre.tif	**ae698pos.tif**	**an698pre.tif**	**an698pos.tif**
Stool 69 Bijou 9 Control			
			
ae699pre.tif	**ae699pos.tif**	**an699pre.tif**	**an699pos.tif**

Plate 10

Stool 81

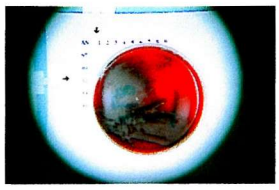
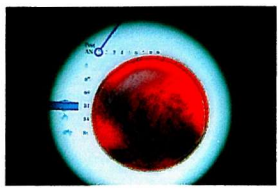
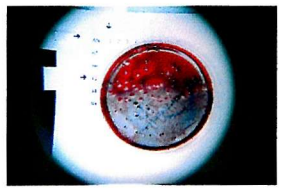
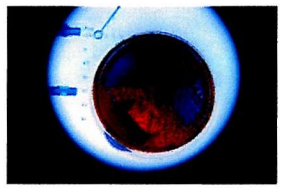
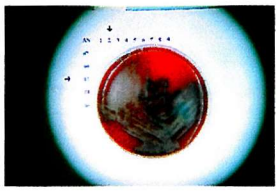
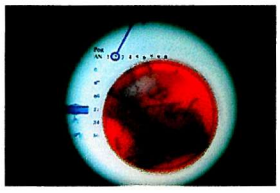
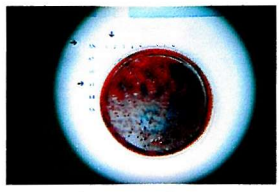
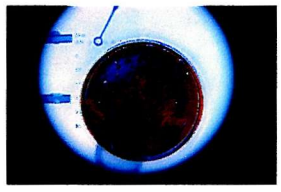
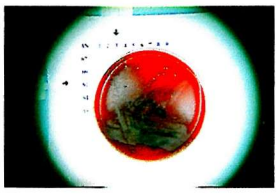
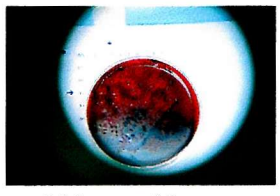
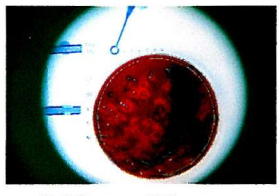
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 81 Bijou 1 IL-8 Co-culture			
 **ae811pre.tif**	 **ae811pos.tif**	 **an811pre.tif**	 **an811pos.tif**
Stool 81 Bijou 2 IL-8 Co-culture			
 **ae812pre.tif**	 **ae812pos.tif**	 **an812pre.tif**	 **an812pos.tif**
Stool 81 Bijou 3 IL-8 Co-culture			
 **ae813pre.tif**	 **ae813pos.tif**	 **an813pre.tif**	 **an813pos.tif**

Plate 11

Stool 81

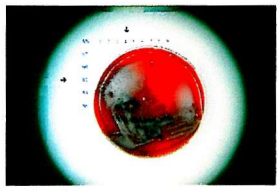
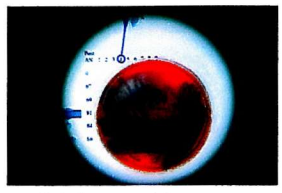
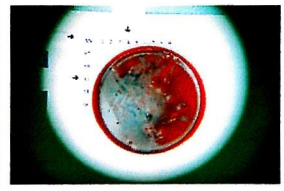
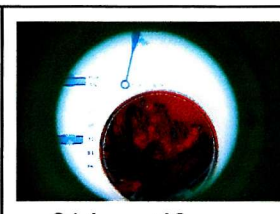
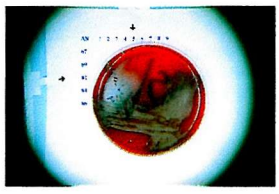
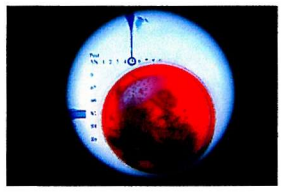
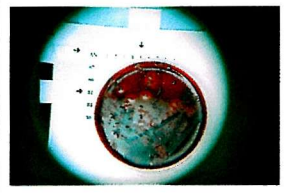
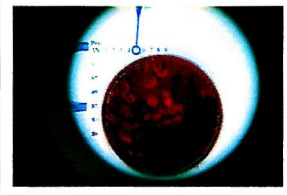
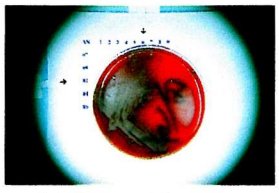
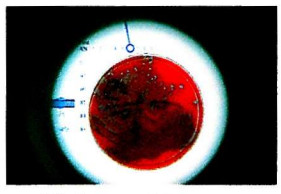
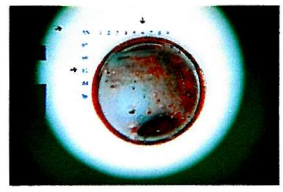
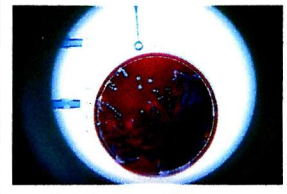
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 81 Bijou 4 TNF Co-culture			
 ae814pre.tif	 ae814pos.tif	 an814pre.tif	 an814pos.tif
Stool 81 Bijou 5 TNF Co-culture			
 ae815pre.tif	 ae815pos.tif	 an815pre.tif	 an815pos.tif
Stool 81 Bijou 6 TNF Co-culture			
 ae816pre.tif	 ae816pos.tif	 an816pre.tif	 an816pos.tif

Plate 12

Stool 81

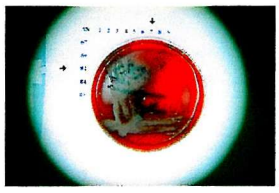
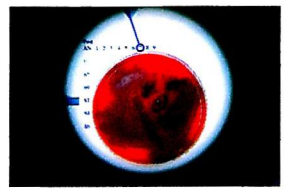
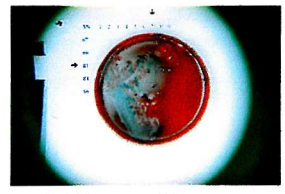
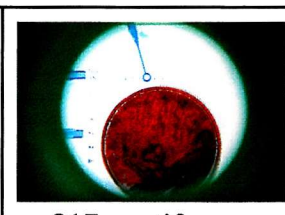
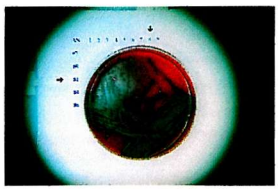
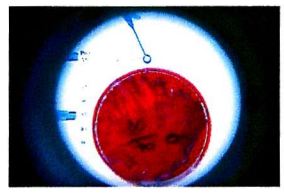
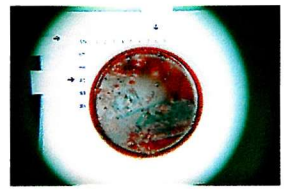
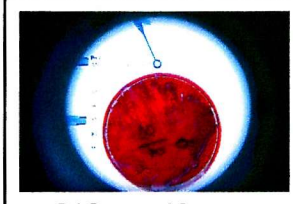
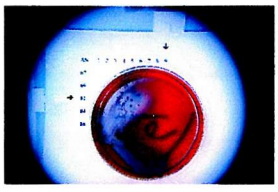
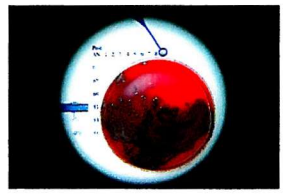
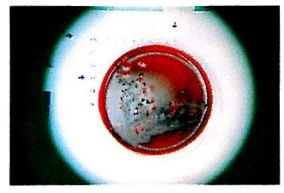
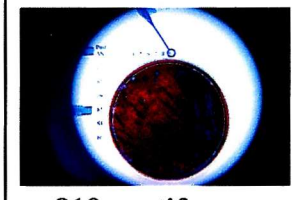
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 81 Bijou 7 Control			
 ae817pre.tif	 ae817pos.tif	 an817pre.tif	 an817pos.tif
Stool 81 Bijou 8 Control			
 ae818pre.tif	 an818pos.tif	 an818pre.tif	 an818pos.tif
Stool 81 Bijou 9 Control			
 ae819pre.tif	 ae819pos.tif	 an819pre.tif	 an819pos.tif

Plate 13

Stool 84

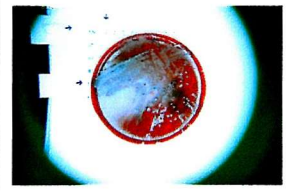
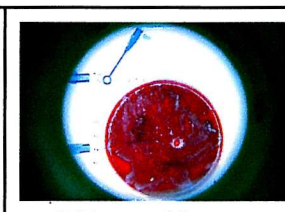
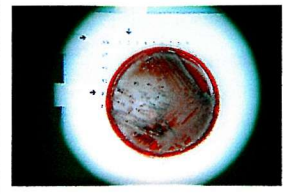
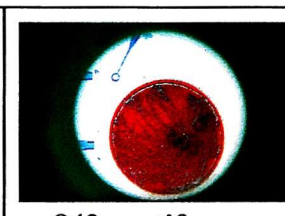
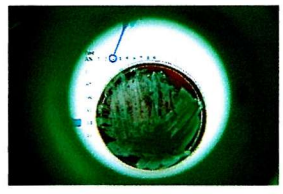
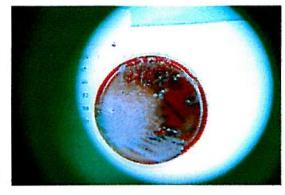
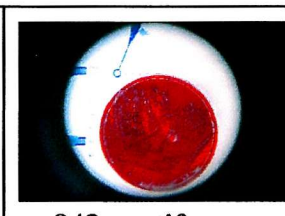
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 84 Bijou 1 IL-8 Co-culture			
 ae841pre.tif	 ae841pos.tif	 an841pre.tif	 an841pos.tif
Stool 84 Bijou 2 IL-8 Co-culture			
 ae842pre.tif	 ae842pos.tif	 an842pre.tif	 an842pos.tif
Stool 84 Bijou 3 IL-8 Co-culture			
 ae843pre.tif	 ae843pos.tif	 an843pre.tif	 an843pos.tif

Plate 14

Stool 84

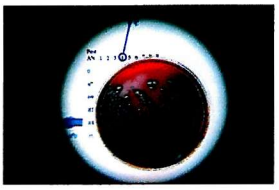
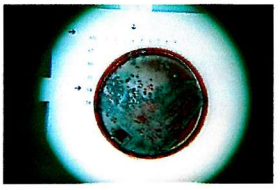
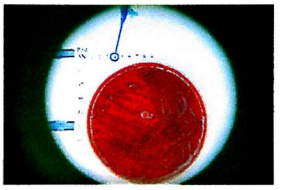
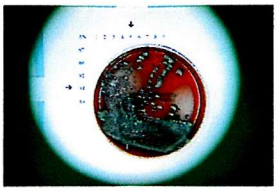
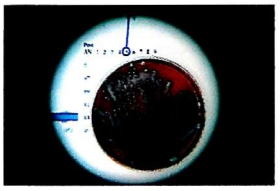
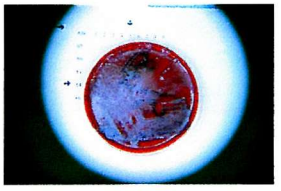
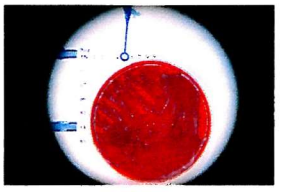
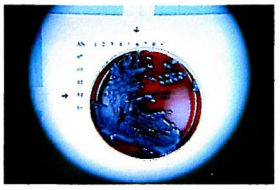
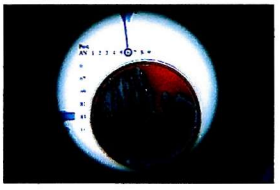
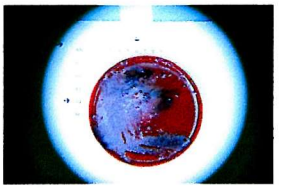
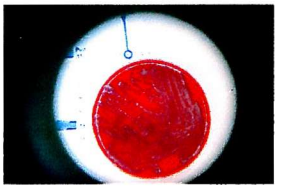
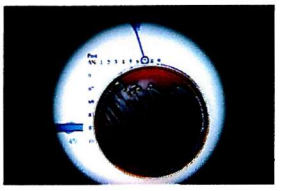
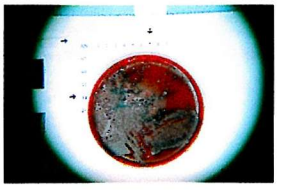
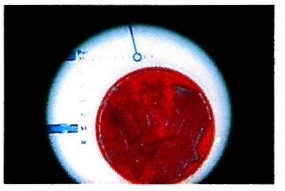
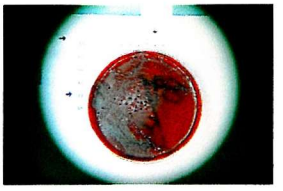
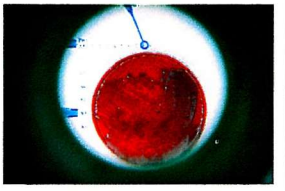
Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 84 Bijou 4 TNF Co-culture			
 ae844pre.tif	 ae844pos.tif	 an844pre.tif	 an844pos.tif
Stool 84 Bijou 5 TNF Co-culture			
 ae845pre.tif	 ae845pos.tif	 an845pre.tif	 an845pos.tif
Stool 84 Bijou 6 TNF Co-culture			
 ae846pre.tif	 ae846pos.tif	 an846pre.tif	 an846pos.tif

Plate 15

Stool 84

Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 84 Bijou 7 Control			
 ae847pre.tif	 ae847pos.tif	 an847pre.tif	 an847pos.tif
Stool 84 Bijou 8 Control			
 ae847pre.tif	 ae848pos.tif	 an848pre.tif	 an848pos.tif
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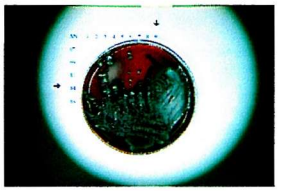
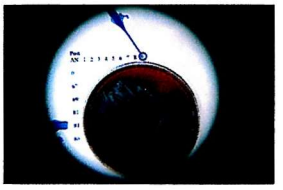
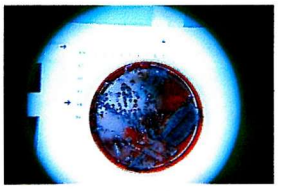
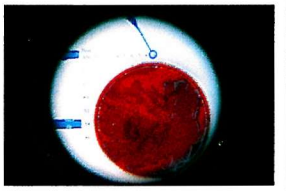
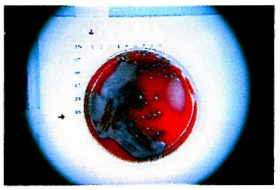
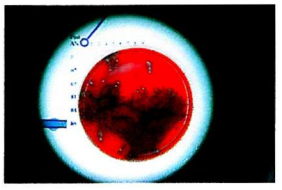
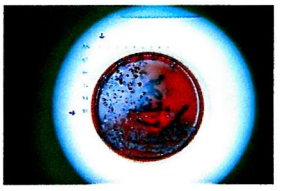
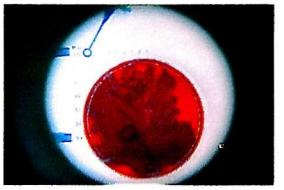
 ae849pre.tif	 ae849pos.tif	 an849pre.tif	 an849pos.tif

Plate 16

Stool 86

Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 86 Bijou 1 IL-8 Co-culture			
 ae861pre.tif	 ae861pos.tif	 an861pre.tif	 an861pos.tif
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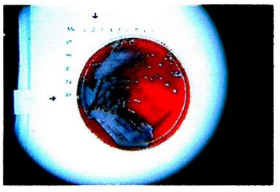
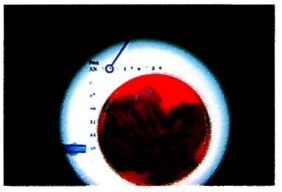
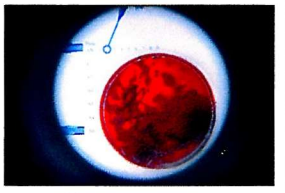
 an861pos.tif	 ae862pos.tif	 an862pre.tif	 an862pos.tif
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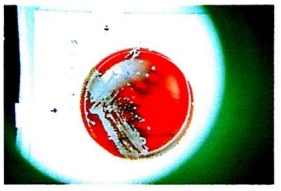
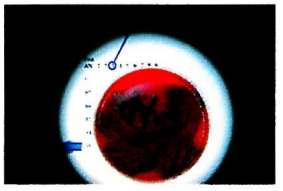
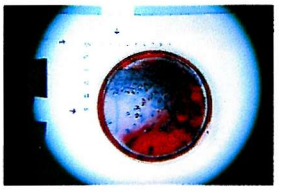
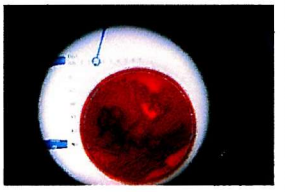
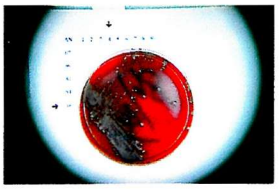
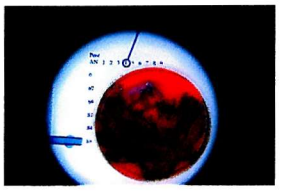
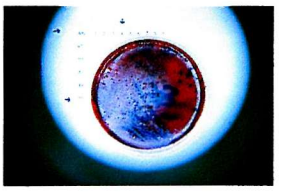
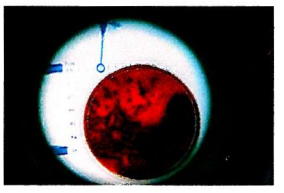
 ae863pre.tif	 ae863pos.tif	 an863pre.tif	 an863pos.tif

Plate 17

Stool 86

Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 86 Bijou 4 TNF Co-culture			
 ae864pre.tif	 ae864pos.tif	 an864pre.tif	 an864pos.tif
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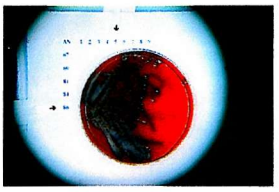
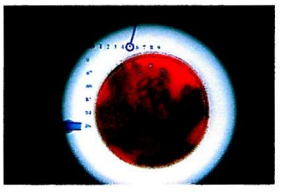
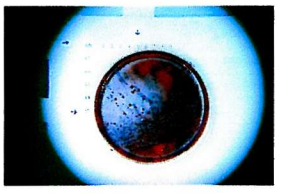
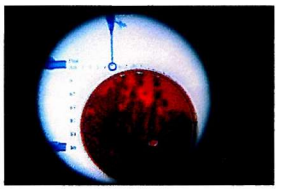
 ae865pre.tif	 ae865pos.tif	 an865pre.tif	 an865pos.tif
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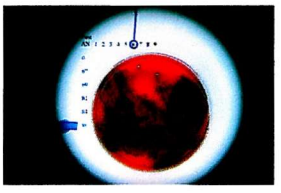
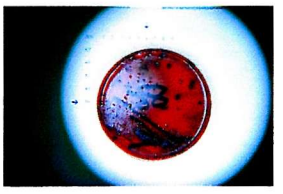
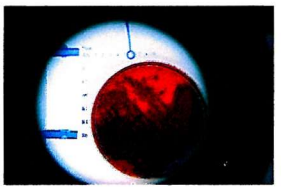
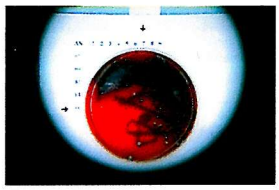
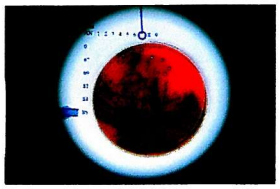
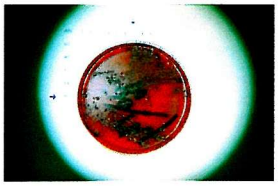
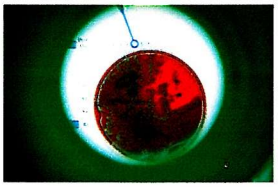
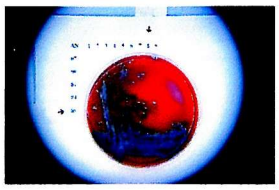
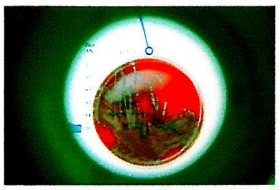
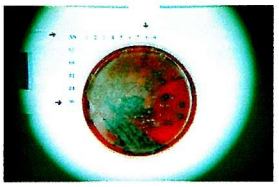
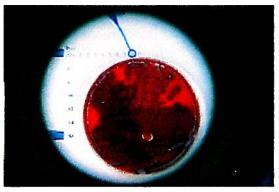
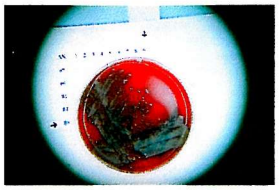
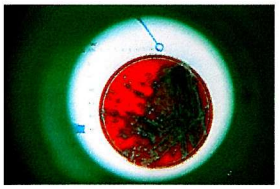
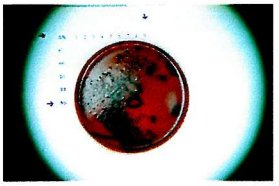
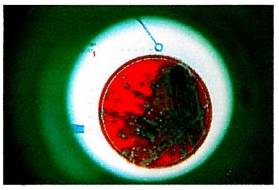
 ae866pre.tif	 ae866pos.tif	 an866pre.tif	 an866pos.tif

Plate 18

Stool 86

Aerobic		Anaerobic	
Pre culture	**Post Culture**	**Pre culture**	**Post Culture**
Stool 86 Bijou 7 Control			
 ae867pre.tif	 ae867pos.tif	 an867pre.tif	 an867pos.tif
Stool 86 Bijou 8 Control			
 ae868pre.tif	 ae868pos.tif	 an868pre.tif	 an868pos.tif
Stool 86 Bijou 9 Control			
 ae869pre.tif	 an869pos.tif	 an869pre.tif	 an869pos.tif

Assays

None of the control bijous containing organisms and tryptone soy broth had IL-8 or TNFα immunoreactivity. Cytokine concentrations for the bijous where IL-8 and TNFα were coincubated with organisms are shown in table 7.3.

TNF alpha results

The mean bijou TNFα concentration was 159.25, 9.58, 270.13, 133.38, 66.71 and 10.27pg/ml respectively for stool samples 0, 67, 69, 81, 84 and 86. One way ANOVA shows that there were significant differences between these means ($F=19.008$ $p=2.5x10^{-5}$). At 9.58 & 10.27pg/ml, the mean bijou TNFα concentration for two stools (67 and 86) were significantly lower than controls ($t=3.346$, $p=0.028$, stool 67 and $t=3.33$, $p=0.0291$, stool 86). Mean bijou TNFα concentration for the remaining stools (69, 81 and 84) were not significantly different from control values.

Interleukin-8 results

The mean bijou IL-8 concentration for the spiked stool aliquots of stools 0, 67, 69, 81, 84 and 86 were 158, 106, 261, 304, 277 and 154pg/ml. There were significant differences between the Bijou IL-8 concentrations (ANOVA $F=7.64$, $p=0.0019$). However these differences were not between control and stool 67 ($t=0.22$, $p=0.44$) nor between control and stool 86 ($t=0.05$, $p=0.96$). No experimental bijou having a significantly lower IL-8 concentration than the broth control.

Contamination and Flora modification

Mean IL-8 concentration in the two Staphylococcus contaminated controls (0/2 & 0/3) was 209pg/ml in contrast to 54.7pg/ml in the uncontaminated control (0/1). The two streptococcus contaminated stool bijous (67/2 &67/3) had a mean IL-8 concentration of 96.6pg/ml compared with 124.7 pg/ml in the corresponding uncontaminated sample (67/1).

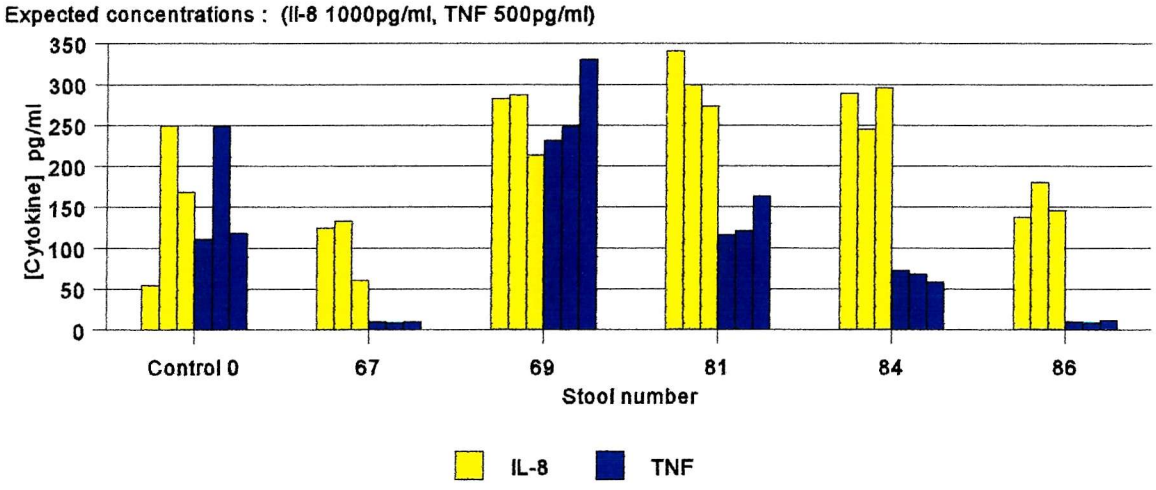
Mean (SD) IL-8 concentration in 9 bijous which were subject to flora change 267.7 (47.8)pg/ml was significantly greater than that of the remaining 9 bijous 152.8 (79.7)pg/ml ($p=0.002$). There was no significant difference in mean (SD) TNFα concentration between the 8 flora change bijous 141.8 (117)pg/ml and the 10 remaining bijous 81.3 (80.1)pg/ml ($p=0.2$).

Table 7.3

Stool	IL-8 pg/ml			TNFα pg/ml		
	Bijou					
	1	2	3	4	5	6
0	54.7	250.12	168.63	110.9	248.6	118.25
67	124.72	132.86	60.37	10.01	8.99	9.75
69	282.74	287.12	213.63	231.37	248.31	330.71
81	340.83	299.55	273.28	115.99	120.81	163.34
84	289.31	245.08	296.62	73.07	68.64	58.43
86	137.63	180.49	146.53	10.01	8.74	12.06

Bijous that underwent flora modification are highlighted in green

Figure 7.3 Cytokine concentration after incubation with faecal organisms



Supplementary experiment results

Mean IL-8 concentration for two aliquots of each test tube are shown in the table. There were no significant differences between samples with newborn calf serum or with phosphate buffered saline as diluent ($F=1.29$, $p=0.29$). Neither were there significant differences between samples containing Dextrose, Synthamen, tryptone soy broth or diluent ($F=1.58$, $p=0.27$). Finally the interaction between the samples and the diluent types showed no significant differences ($F=0.37$, $p=0.78$).

Table 7.4

Solution	**Diluent**	
	Newborn calf serum	Phosphate buffered saline
	IL-8 concentration (pg/ml)	
Dextrose	1813	1732
Synthamin	1663	1688
Tryptone soy broth	1991	1873
Newborn calf serum	2021	
Phosphate buffered saline		1771

Discussion

The study protocol for sample spiking should have lead to bijou IL-8 concentrations close to 1051pg/ml, and TNF alpha concentrations close to 525pg/ml, however the maximum spike recovery from bijous shown to remain sterile throughout the incubation period was 5.2% for IL-8 and 47.3% for TNF alpha. Such a reduction is most likely to have resulted from adhesion of the cytokine onto the glass wall of the incubation bijou, because no reduction was seen when tryptone soy broth, dextrose and aminos acids were coincubated with cytokines in polypropylene bottles. This makes chemical modification of the cytokine an unlikely cause, although adhession to the 22μ filter is not excluded.

In spite of this reduction in cytokine concentration in the control samples, there was a significantly greater reduction in TNFα concentration in two of the five stool samples. The lack of such significant change for the IL-8 samples and for three of the five TNFα samples implies that under these close to ideal conditions, these bacterial faecal flora do not digest IL-8 and are not efficient in digesting TNFα. It might even be the case that the significant reduction in the two reduced TNFα concentration stools was not the result of bacterial degradation, but an increase in adhesion to the glass bijou, possibly mediated by an altered pH.

These data leave open the possibility that fungi might digest these cytokines, and the possibility that in an environment of substrate scarcity even the bacterial flora might

upregulate appropriate enzymes and acquire cytokine digestion activity. Such substrate scarcity is not likely to occur in faecal matter either in the intestine or the specimen pot. Particularly in patients with a normal gut transit time and in specimens that are promptly frozen.

Whilst flora change was monitored to detect competitive selection of organisms which might not digest cytokine, mean TNFα concentration of bijous undergoing flora change was not different to those mainatining their flora. The IL-8 bijous undergoing flora change did have a significantly higher IL-8 concentration than those that maintained their flora, however the lower IL-8 concentration in these latter bijous was still not significantly lower than the controls. Flora modification has had no demonstrable effect on the cytokine concentration in this experiment.

It is safe to conclude that CF bacterial feacal flora do not significantly digest IL-8 and may not digest TNFα.

Section 3: Upper intestinal administration of human recombinant IL-8 and TNFα in the Wistar rat: fate and effect of cytokines.

This experiment tests the hypothesis that cytokines in swallowed sputum traverse the gastrointestinal tract intact. It also investigates whether these swallowed cytokines induce intestinal inflammation. It relies on the cytokine ELISA protocol validated for use in human faecal samples. In outline cytokine and marker were gavaged into the rats and the corresponding faecal pellets were assayed for cytokine, and then the rat guts were examined histologically for inflammatory changes.

In the initial study design carmine was to be used as marker, but this proved unsuitable because it was not visible in the dry faecal pellets. Some data from an initial carmine pilot study will be presented because it is pertinent to the discussion later.

Indian ink does not have significant toxicity and human ingestions are treated entirely on a symptomatic basis. It has been injected into rats brains to investigate the ability of neural tissue to clear particulate foreign bodies, and has been left in situ for up to two years without evidence of distress in the animal.[149] It was therefore selected as the faecal marker.

Because of the possibility of cytokines being adsorbed onto the carbon paticles, the rats were dosed with cytokine in PBS or PBS alone, faecal collection commenced and six

hours later, they were given a further gavage of Indian ink alone.

Method

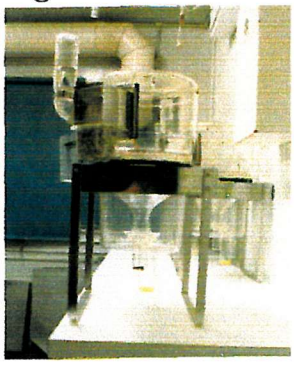
Cytokine Transit

Animals were obtained from the University of Queensland animal breeding facility, and housed prior to and during the experiment at the Medical School Animal House. They were caged in metabolic cages (figures 7.4a & 7.4b) which incorporate a wire mesh allowing the separation and collection of liquid and solid waste, and reducing the rat's normal coprophagic activity to a minimum.

Figure 7.4a

Fifteen male Wistar rats (200g) were divided into three groups of 5 rats. All 15 rats were be given an initial 1ml gavage of either phosphate buffered saline (PBS), interleukin-8 (3000pg) in PBS or interleukin-8 (3000pg) and tumour necrosis factor alpha (200pg) in PBS. Stool collection was commenced, and six hours later the rats received a 1ml gavage of Indian ink (Pelikan), and stool collection continued until the faecal pellets became black. The appearance of black coloured stools was abrupt, often occuring in the middle of a long faecal pellet. Faecal pellets containing ink were not included in the collection for homogenisation.

Figure 7.4b



The cytokine dosages are eqivalent to a 60kg patient swallowing 30g of sputum containing typical amounts of IL-8[150] and TNF-α.[151] This is not an unusual occurrance for a teenager with established cystic fibrosis lung disease. Faecal samples were collected at six hourly intervals and frozen immediately. Frozen faecal samples were powdered using mortar and pestle, homogenised in five times their weight of phosphate buffered saline, centrifuged at 20,000g for fifteen minutes. Supernatant was frozen at -70°C and subsequently analysed for IL-8 and TNF concentrations, using the protocol previously validated for use of the Quantikine kits with human samples.

Validation

Supernatants from faeces of four of the five rats dosed with only phosphate buffered saline were spiked with interleukin-8 to 500pg/ml to check that the assay protocol was valid in Wistar rats. Additionally faeces from a rat with experimental colitis (kindly donated by Dr G Davidson, University of Adelaide) were assayed for IL-8 and TNFα, to investigate any cross reactivity between antibodies to these human cytokines, and equivalent rat cytokine which might be excreted in colitic faeces.

Histology

When faecal specimen collection was complete, the rats were euthanased with a lethal (1-3mls) intra-peritoneal dose of Phenobarbitone sodium (325mg/ml)(Lethobarb,Virbac, Sydney, NSW). Their gastrointestinal tracts were dissected and representative samples of stomach, duodenum, ileum and colon taken and processed for histological examination. These were examined blindly for the presence of intestinal inflammation.

Blinding was achieved in the histology proceesing laboratory by technical staff who played no part in examination of the histology. Cassettes containing the rat tissue were labelled with the rat's serial number and a suffix to denote the site of origin of the tissue. Sections were cut from the wax blocks, they were stained with haematoxylin and eosin and labled with a random serial number. Rat serial numbers, suffixes and randomised slide numbers were recorded on a log sheet which was placed in a sealed envelope, which was not opened until after the slides had been examined.

Each slide was examined for the presence of neutrophil polymorphs. In order to identify all possible foci of inflammation, irrespective of morphology changes with processing and handling, any cell on the slide whose morphology resembled a neutrophil was recorded. Cells with clearly Eosinophilic granules were interpreted as Eosinophils, but it remains possible that some cells recorded as Neutrophils may have been Eosinophils. To allow for this, a focus of inflammation was defined as "at least three neutrophils in one high powered field."

Carmine pilot

Three rats were gavaged with 1ml of 0.5% Carmine (Sigma) in phosphate buffered saline, and three with phosphate buffered saline. Faeces were collected for 24 hours, and no perceptible reddening of the faecal pellets was observed by this time. The rats were then killed and dissected. In no rat was there any red intestinal contents. Intestinal contents and faecal pellets were removed, stored, and weighed.

Ethical approval

The study was approved by the University of Queensland animal experimentation ethics committee.

Results

Stool weights

Carmine pilot

The total weight of faeces plus intestinal conents for the total study period is shown in table 7.5 and figure 7.5 for both carmine and PBS rats. Mean faecal weight for the carmine dosed rats 10.43g was greater than the PBS dosed rats 5.39g, by a factor appoaching 2.

Figure 7.5 Faecal weights for carmine pilot

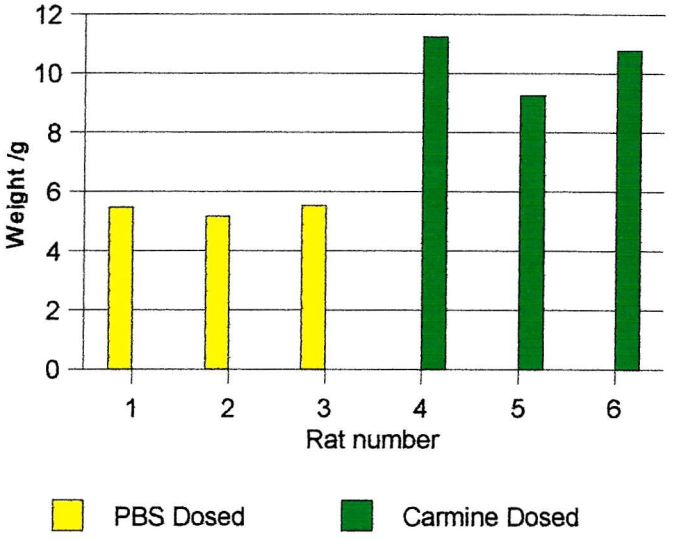


Table 7.5

Rat	Faecal Weight	Dosing
1	5.48	PBS
2	5.17*	PBS
3	5.53	PBS
4	11.24	Carmine
5	9.26	Carmine
6	10.79	Carmine

*Killed at 18 hrs because of apparent distress.

Main study

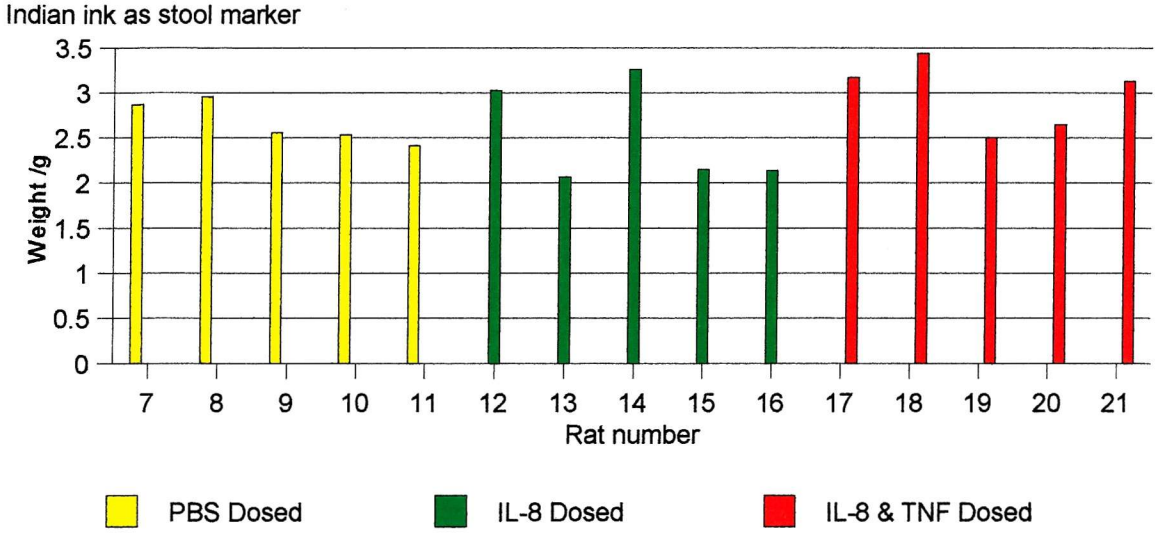
Mean stool weight collected was 2.67g for rats dosed with PBS alone, 2.53g for rats dosed with 3000pg IL-8 in PBS, and 2.98g for rats dosed with 3000pg IL-8 and 200pg TNF alpha in PBS. These stool weights were not significantly different ($F=1.51$, $p=0.26$ by ANOVA).

The measured stool weight for each rat is shown in table7.6.

Table 7.6

Rat number	Faecal weight/g
PBS gavaged rats	
7	2.87
8	2.96
9	2.56
10	2.54
11	2.42
IL-8 gavaged rats	
12	3.03
13	2.07
14	3.26
15	2.15
16	2.14
TNF alpha & IL-8 gavaged rats	
17	3.17
18	3.44
19	2.51
20	2.65
21	3.13

Figure 7.6 Faecal weights (main experiment)



TNFα Assay

No TNFα immunoreactivity was detected in the faecal samples of the five rats that were given TNFα and IL-8, nor in the five control rats that received phosphate buffered saline. No aliquots were assayed after spiking with TNF alpha for validation. Faeces from the rat with a severe experimental colitis contained no TNFα immunoreactivity.

Il-8 Assay

No significant IL-8 immunoreactivity was detected in any experimental or control rats, nor in the rat with experimental colitis. Of the four faecal samples that were spiked to 500pg/ml, all had assayable quantities of IL-8. Their mean measured IL-8 concentration was 773pg/ml. The measured IL-8 concentrations are shown in table 7.7. These concentrations are the mean of two values calculated from two dilutions. The percentage variation of the individual results from that mean is shown in the table's second column

to illustrate the assay protocol's linearity in this environment.

Table 7.7

IL-8 concentration in aliquots spiked to 500pg/ml

Rat number	IL-8 concentration pg/ml	Dilution difference/%
7	745	4.2
8	809	3.5
9	733	5.5
10	807	2.1

Histology

Of the four sampling sites for the 15 rats, a single slide demonstrated a focus of inflammation. This occurred in stomach tissue just distal to the gastro-oesophageal junction, in a rat who had been dosed with 3000pg of IL-8. This rat also had 2 isolated neutrophils in his distal small bowel, and two isolated neutrophils in his colon. Two other rats dosed with Il-8 alone had tissue neutrophils identified, one with three isolated neutrophils in his colon and the other with a single neutrophil in his proximal small bowel.

A single neutrophil was seen in the colon of one rat dosed with only Phospate Buffered saline. Whilst four of five rats dosed with both IL-8 and TNF were noted to have solitary tissue neutrophils that did not satisfy the study criteria for an inflammatory response. One

of these rats had solitary neutrophils in Stomach, distal small bowel and colon, one in distal small bowel and colon, and one rat each in proximal and distal small bowel.

Discussion

In this rat gut cytokine transit experiment it was necessary to have a significant interval between cytokine administration and Indian ink administration so that the cytokines were not adsorbed onto the ink's carbon particles. The inevitable dilution of cytokines occurring as a result of this interval did limit the ability of the study to be confident that failure to detect cytokine was not the result of cytokine dilution below the detection limit of the assay. This effect can be estimated by calculating the individual sample dilutions. The weight of faecal pellets recovered from the rats ranged between 2.07 and 3.44g. If all of the 3000pg IL-8 and 200pg TNFα, administered to each rat, passed through the gut, cytokine concentration in the aliquots would be above145pg/ml for IL-8 and 9.6pg/ml for TNF alpha in all rats. This concentration of IL-8 is assayable, and at a tenfold reduction in concentration, it should still be detectable. The dose of TNFα would be detectable in this assay system even if reduced by 50%. It is safe to conclude that the Wistar rat destroys at least half of the administered dose of TNF alpha and at least 90% of the administered dose of IL-8, with passage through its intestine. This is consistent with the efficient digestion of these cytokines in the pancreatic sufficient mammal.

This efficient digestion is consistent with the lack of an intestinal inflammatory response. The sole instance of intestinal inflammation in one rat stomach, just distal to the oesophago-gastric junction, could be a result of the administration of Interleukin-8 to the stomach, however the fact that only one rat is so affected is more consistent with inflammation having been caused by the process of passing the gavage needle through the

gastro-oesophageal junction. This study was designed not to miss a subtle acute inflammatory focus, and the observed tissue neutrophils that were not sufficiently numerous to satisfy the study criteria for a focus of inflammation could signify mild acute inflammation. All but one of the slides containing isolated neutrophils were in rats who had been gavaged with a dose of IL-8, despite the blinding of the histological assessor to the origin of the slides. Because of the possibility of misclassifying the morphology of an individual polymorph the study required three neutrophils per high power field to conclude that a focus of inflammation was present. Whilst this criterion was arbitrary it was set at the stage of study design and should be adhered to. In making a post-hoc assessment of these data, one would have to consider the proportion of the rat's gut that had been examined. I estimate that a total of 8Cm x 5μ of tissue was examined for each rat, and that the length of each rat gut was in excess of 40cms. With this proportion of tissue examination I do not believe that the data support the existence of even the most subtle of inflammatory responses to gavaged cytokine.

Repetition of the experiment using higher cytokine doses might provoke an inflammatory response. The current dosages were selected to reflect the estimated cytokine load placed on CF gut at times of a chest exacerbation, and the relevance of an inflammatory response in a higher dose model would not be clear.

I conclude that the bulk of swallowed IL-8 and TNFα is digested in pancreatic sufficient animals.

Chapter 8

Intestinal inflammation and high lipase microsphere enzymes (Creon Forte) in cystic fibrosis

Introduction

Following the initial report of fibrosing colonopathy in cystic fibrosis patients taking high strength pancreatic enzyme preparations, [152] seventy confirmed cases have been recorded worldwide. These have been associated with the minitab (Pancrease HL, Panzytrat & Nutrizym 22), rather than microsphere (Creon) preparations.[153] The distinction between these preparations is that the former have a methacrylic acid copolymer coating [154], a monomer contaminant of which (ethyl acrylate) has been shown to be toxic to rat intestine, causing mucosal ulceration and submucosal oedema and fibrosis.[155] Whilst the mechanism by which pancreatin preparations cause strictures remains obscure, there have been suggestions that intestinal generation of proinflammatory cytokines including TNF-α may be involved.[156]

In the original CF faecal cytokine assays in Southampton, I reported elevated faecal Interleukin-8 and TNF-α concentrations in nine children with cystic fibrosis. All but one of these patients were then taking high lipase enzyme preparations.[157] Seven were taking Creon 25000, now marketed in Australia as Creon Forte. The eighth who was taking Nutrizym 22 subsequently developed fibrosing colonopathy.[158] Whilst the validity of the assays used is now highly questionable, the possibility that high lipase pancreatic enzyme

preparations might generate IL-8 and TNFα from the intestinal mucosa merits specific investigation

When On 1st February 1996 the pancreatic enzyme preparations Creon and Creon Forte (marketed elsewhere as Creon 25000) received a licence for use in Australia in patients with pancreatic insufficiency, this presented an opportunity to measure, faecal cytokine concentrations and markers of intestinal inflammation in a population of CF children who commenced Creon Forte therapy for the first time. Extension of the study to twelve months of serial measurements should be sufficient to detect any intestinal inflammation which might have been a precursor to fibrosing colonopathy, because the initial cases reported from the Liverpool CF clinic occurred approximately 12 months after the introduction of high lipase enzyme preparations to the clinic.

The intra-mural fibrosis of fibrosing colonopathy increases bowel wall thickness. Increases in ultrasound measurements of colonic wall thickness, have been reported following the introduction of high strength enzymes, including microsphere preparations.[159, 160,161] Superficially these data could be interpreted to suggest that the microsphere preparations, which are widely used in children with cystic fibrosis, produce a forme fruste of fibrosing colonopathy. Therefore the addition of serial ultrasound colon wall thickness measurements to the study protocol provides the opportunity to explore this possibility. Furthermore, the concordance or otherwise of faecal cytokine measurements and ultrsound changes in the colon wall thickness will serve to clarify the interpretation of the study's results.

Patients

Patients

Ten CF patients who were starting Creon Forte therapy and 10 CF controls who remained on low lipase preparations were to be recruited and faeces specimens collected at 0,1,3,6,9 and 12 months after recruitment for patients on Creon forte, and at least at 0,3 & 6 months for controls.. Randomisation was not performed as patients were recruited to the study after a clinical decision had been made to commence high strength enzyme therapy. Controls were recruited from patients attending the CF clinic who were taking low strength enzyme preparations.

Methods

Patient Assessment

Pancreatic enzyme dosage was recorded and NIH scoring[162] performed as a measure of disease severity at routine clinic visits.

Ultrasound assessment

Measurement of colonic wall thickness according to the ultrasonographic methods of MacSweeney et al.[163] was made at study enrolment and at six months.

The inherent variability of the measurement technique was assessed by making 12 serial measurements of bowel wall thickness in the right iliac fossa in a single patient over 15 minutes. The operator was blinded to the measurement value, by occlusion of part of the

display by a third party. The ultrasound probe was removed from the abdomen between each measurement to simulate the variation inherent in repeated scans.

Specimen collection and processing & Cytokine Assays

These are described in chapter 4

Mucosal integrity

Intestinal mucosal integrity was assessed by measurement of alpha-1-antitrypsin concentration in all serial faeces samples, by radial immuno-diffusion[164], and screening for faecal occult blood.

Ethical approval

Informed parental consent was obtained for participation in the study, and where they were deemed competent to give consent this was also obtained from the paticipating children. The study was approved by the ethics committee of The Royal Children's Hospital Brisbane.

Results

Longitudinal study

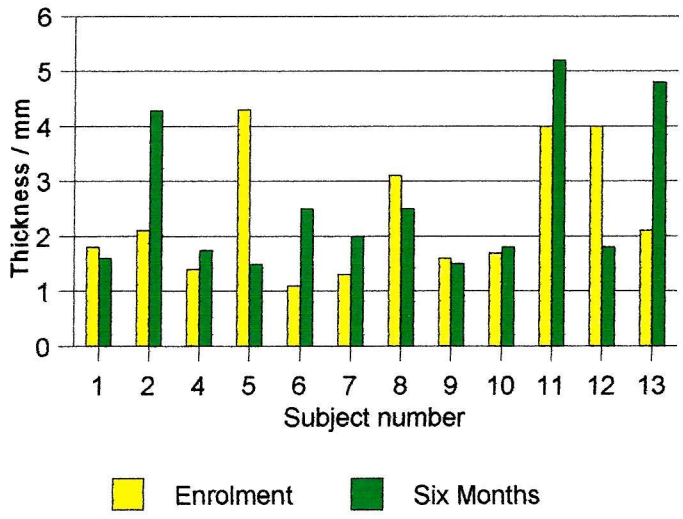
Nine patients and nine controls were invited to participate in the study. All patients and 6/9 controls gave informed consent, two controls were withdrawn from the study one was

unwilling to provide faecal samples and the other provided only a control sample. Individual patient's results are shown in table 8.1. The mean (SD) age, NIH score and enzyme consumption for the patients on Creon Forte were 12.48 (3.81)years, 78.22 (10.67), and 6310 (4020) units of lipase per kilogram per day respectively. The corresponding figures for the controls were 10.67 (4.68) years, 83.80 (14.06) and 8030 (6620) units of lipase per kg/day.

Bowel wall thickness measurements

The coefficient of variation for repeated measurements at the same examination was 16% (n=12, mean measurement 3.4mm, sd 0.55mm). All patients on Creon Forte and controls had duplicate right sided measurements.These are shown in table 8.1 and figure 8.1. The mean (SD) increase in bowel wall thickness for controls was 0.77 (1.24)mm, and for Creon Forte patients 0.04 (1.74)mm. There was no significant difference between the changes in bowel wall thickness for study and control groups (t=0.66, p=0.52).

Figure 8.1 Bowel wall thickness



Faecal cytokine assays

Not all patients and controls were able to provide faecal samples at the specified protocol times. Some had difficulty in producing a sample in the few days preceding their protocol sample date, others were unable to travel to the study centre at the time of the protocol sample. Serial faecal samples from 9 patients on Creon Forte were analysed for cytokine concentration. The mean (SD) number of specimens collected per patient was 4.11 (1.2) over a mean (SD) period of 0.89 (0.27) years. Similarly serial samples from four controls were assayed for cytokine concentration. The mean (SD) number of specimens collected per control subject was 3.75 (1.3) over a mean (SD) period of 0.90 (0.70) years.

Measurable quantities of interleukin-8 were detected in only three samples. The first, a sample taken four months after starting Creon Forte, from subject 6 had an IL-8 concentration of 698pg/g. The second, a sample taken three months after starting Creon Forte from subject 11, had an IL-8 concentration of 928 pg\g. The final IL-8 containing sample was taken from subject 13 before he commenced Creon Forte. It had an IL-8 concentration of 311pg/g. No sample had a measurable TNFα concentration.

Table 8.1. Cystic fibrosis patient's characteristics

Number	Sex	Age (Years)	NIH Score	Years interval between		Initial Bowel wall thickness (increase) /mm	Enzyme Preparation	Capsules /day	Wt /Kg	Lipase units /kg /day /1000
				Bowel wall thickness measurements	First and last study stool samples					
Control subjects										
1	F	14.37	85	0.58	0.36	1.8 (-0.2)	Cotazym	20	60	3.33
2	F	17.18	57	0.72	0.72	2.1 (2.18)	Cotazym	15	50.2	2.99
3	M	7.15	87	n/a	0.32	n/a	Pancrease	25	19	6.58
4	M	4.28	97	0.47	2.18	1.4 (0.34)	Pancrease	30	15	10

Table 8.1. Cystic fibrosis patient's characteristics

Number	Sex	Age (Years)	NIH Score	Years interval between		Initial Bowel wall thickness (increase) /mm	Enzyme Preparation	Capsules /day	Wt /Kg	Lipase units /kg /day /1000
				Bowel wall thickness measurements	First and last study stool samples					
Creon Forte subjects										
5	F	8.76	72	0.31	0.31	4.3 (-2.81)	Creon Forte	11	22.5	12.22
6	F	3.51	91	0.75	2.12	1.1 (1.4)	Cotazym*	23	13.7	16.79
7	F	14.10	73	0.38	0.90	1.3 (0.7)	Creon Forte	10	41.5	6.02
8	M	12.23	93	0.55	0.99	3.1 (-0.6)	Creon Forte	12	38.5	7.79
9	M	15.06	86	0.55	0.82	1.6 (-0.1)	Creon Forte	6	51.5	2.91
10	M	13.59	69	0.42	0.87	1.7 (0.1)	Creon Forte	2	31	2.94
							Creon	3		
11	F	16.56	58	0.80	1.20	4.0 (1.2)	Creon Forte	9	51	4.41
12	F	13.06	83	0.76	1.32	4.0 (-2.2)	Creon Forte	30	47.8	16.38
13	M	15.44	79	0.77	0.77	2.1 (2.7)	Creon Forte	10	41	6.1

*Took Creon Forte for 3 months then reverted to Cotazym-S-Forte

Intestinal mucosal integrity

Faecal alpha-1 antitrypsin concentrations are shown in table 8.2. The scatter of these measurements with time is shown in figures 8.2 a&b. Two faecal samples had a faecal alpha-1 antitrypsin concentration above the laboratory reference range (<1.5 mg/g). Both samples were from patients recruited to take Creon Forte, and both were also Haemoccult positive. The first with a concentration of 2.4mg/g was from subject 7 prior to her commencement of Creon Forte, and the second, with a concentration of 3.0mg/g was from subject 8 taken after three months on Creon Forte. Between the first and last collected samples there was a mean (sd) decrease in alpha 1 antitrypsin concentration of 0.25 (0.47)mg/g for patients on Creon Forte and a mean (sd) increase of 0.025 (0.09) mg/g for low lipase controls.

Table 8.2

Patient Number	**Faecal Alpha 1 antitrypsin concentration mg/g (Interval since recruitment / years)**					
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
Low lipase controls						
1	0.6 (0.00)	1.0 (0.32)	0.6 (0.37)			
2	0.4 (0.00)	0.3 (0.50)	0.3 (0.73)			
3	0.5 (0.00)	1.1 (0.17)	0.6 (0.33)			
4	0.5 (0.00)	0.6 (0.84)	0.7 (1.00)	0.4 (1.23)	0.6 (1.23)	
Creon Forte patients						
5	0.7 (0.00)	0.7 (0.32)				

Table 8.2

Patient Number	Faecal Alpha 1 antitrypsin concentration mg/g (Interval since recruitment / years)					
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
6	0.9 (0.00)	1.0 (0.08)	0.7 (0.63)	1.0 (0.75)		
7	2.4 (0.00)	0.5 (0.25)	1.1 (0.48)	0.4 (0.67)	1.0 (0.90)	
8	1.0 (0.00)	0.9 (0.12)	3.0 (0.25)	1.4 (0.55)	0.6 (0.69)	1.1 (0.99)
9	0.9 (0.00)	1.1 (0.13)	0.6 (0.61)	0.8 (0.82)		
10	0.6 (0.00)	0.7 (0.09)	0.8 (0.43)	0.5 (0.88)		
11	0.8 (0.00)	0.4 (0.24)	0.5 (0.80)	0.3 (1.21)		
12	0.6 (0.00)	0.6 (0.47)	0.5 (1.02)			
13	0.9 (0.00)	0.9 (0.11)	0.6 (0.77)			

Seven patients and one control had at least one stool sample with a trace of blood or more on the haemoccult test. This control and two of these haemoccult positive patients were female and post pubertal, but none were known to have been menstruating at the time of specimen collection. Eight of eighteen stool samples with a trace or more of blood were taken from patients who had not at that time been exposed to Creon Forte, compared with 16 of 32 haemoccult negative samples. There was no significant difference between Creon Forte exposed and non exposed samples in their rates of haemoccult positivity (Chi squared 0.142 df=1 p=0.71).

Figure 8.2a **Faecal Alpha 1 antitrypsin Creon Forte patients**

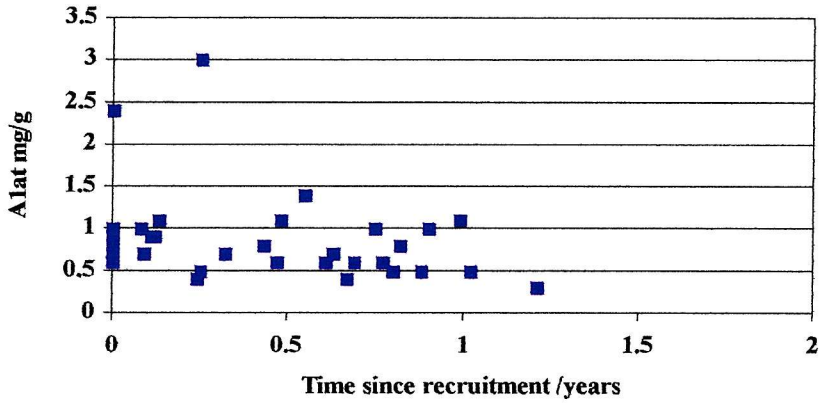
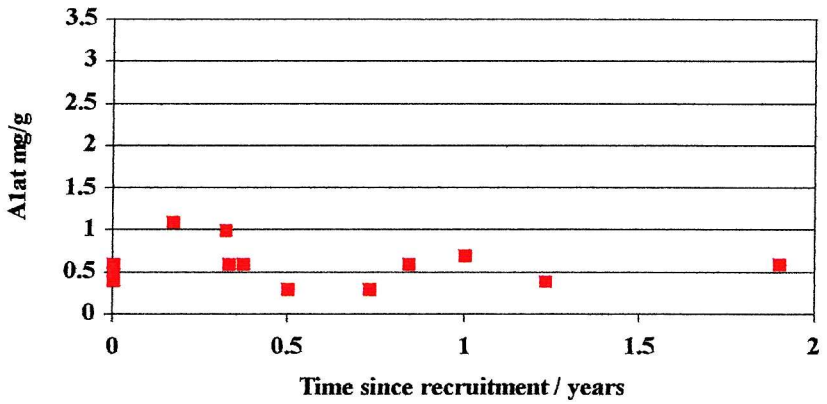


Figure 8.2b **Faecal Alpha 1 antitrypsin control patients**



Discussion

In this prospective non randomised study there was no increase in faecal IL-8 or TNFα concentration over a mean follow up period of 0.89 years, following the introduction of high lipase microsphere pancreatin preparations in cystic fibrosis. In contrast to others, the assay protocol used has been demonstrated to be linear and accurate. Furthermore it has been shown to be capable of detecting elevation of faecal cytokine concentration in acute intestinal inflammation and reduction with therapy (Chapter 6). Secondly, whilst faecal alpha-1-antitrypsin concentration was elevated in two isolated faecal samples, one of these was taken before exposure to Creon Forte. Finally, haemoccult positivity was no more common in stools taken after exposure to Creon Forte than in stools taken prior to exposure. Taken together these data suggest that either this microsphere enzyme preparation does not provoke intestinal inflammation or these tests are not sufficiently sensitive to detect it.

In contrast to the work of MacSweeney et al. I found no significant change in the mean maximum right colon wall thickness in the patients who went on Creon Forte. The 16% coefficient of variation for repeated measurements of the same area of bowel at the same sitting, implies that an increase in bowel wall thickness of greater than 32% is required before one can be confident that a real increase in bowel wall thickness has occurred in an individual patient. Whilst these data suggest that bowel wall thickening is not a result of high lipase microsphrere enzyme preparations the insensitivity of the ultrasound measurement technique prevents the exclusion of a small effect. Measurement of bowel

wall thickness by ultrasound is hampered by the motile nature of the colon. In this study, employing real time abdominal ultrasound images, variations in bowel wall thickness were evident over short intervals as segments of colon contracted and extended.

Chapter 9

Discussion

The objective of this thesis was to find out whether measurement of faecal IL-8 and TNFα concentrations in CF, using commercial ELISAs, could be used as an index of pulmonary inflammation. The validity of this approach rests on the hypothesis that these cytokines may pass through the gut of the pancreatic insufficient CF patient in a form which will be recognisable to the cytokine ELISAs. It is now clear from this body of work that measurement of faecal cytokine concentrations with these techniques does not have utility in the estimation of severity of pulmonary inflammation.

To establish a relationship between pulmonary inflammation and faecal cytokine concentration, it was necessary to show that faecal cytokine concentration could be accurately and reproducibly be measured, that the concentrations reflected the severity of pulmonary inflammation in a cross sectional study, and that they mirrored changes in severity of pulmonary inflammation occurring with time. Additionally it was necessary to show that mixing of sputum cytokines and their passage through the gut was biologically plausible. This study has negotiated the first hurdle of measurement, but has found no evidence to support any of the other criteria.

The initial work which suggested there may be a relationship between pulmonary disease severity and faecal cytokine concentration was conducted speculatively using an assay technique which had not been validated for use in faecally derived samples. When the

validity of this assay was tested, it was found wanting. It was not linear, and indeed gave paradoxical results, such that when samples were diluted, the measured cytokine concentration in the sample increased. Similar results occurred when alternative ELISAs were trialed. One of which managed to detect cytokine in a sample which contained none according to the validated assay.

I discovered that by using newborn calf serum as the sample diluent, that linearity of the assays for IL-8 and TNFα could be achieved, for one manufacturers commercial assay kits.

Using these validated assay protocols, I was unable to reproduce the elevated faecal cytokine concentrations in Australian patients which had been seen in the UK patients using the unvalidated assays. I could therefore not confirm any relationship, either in cross sectional or longitudinal studies between faecal cytokine concentration and pulmonary disease severity. The assays however were able to detect faecal IL-8 and TNFα in some faecal samples from patients with colitis, and I found evidence to suggest that faecal IL-8 concentration reflected the severity of this colonic inflammation in that faecal concentration fell with therapy and remission.

This observation is particularly pertinent, as I have clearly shown a difference in cytokine content, between faecal samples from colitis and CF patients. The original work by Nicolls et al.[35] and Braeger et al.[34] showing elevated faecal TNFα concentrations in inflammatory bowel disease patients was conducted using an unvalidated assay

technique, and my use of that technique on stools from CF patients resulted in my suggestion that CF patients had faecal cytokine concentrations similar to IBD patients. Thus the work presented in this thesis is the first report of faecal cytokine concentrations in IBD using a validated assay. It establishes the principle that faecal IL-8 and TNFα concentrations can be quantified in individual in vivo derived human samples. Unlike the original work of Braegger, I did not demonstrate consistently elevated faecal TNFα concentrations in inflammatory bowel disease. This brings into question the conclusions of those authors that faecal TNFα concentration is elevated in inflammatory bowel disease. Whether my techniques give representative results from repeated processing of the same sample or from consecutive samples, or whether they can be taken as an objective index of the severity of colonic inflammation has yet to be established.

It seems likely that if unaltered IL-8 and TNFα were present in CF faeces as a result of their being swallowed in sputum they should have been detected by these techniques. The frailty of the ELISA technique lies in it's dependence on the visibility to the capture antibody, of the peptide antigen within the molecule of interest. It therefore remained possible that active cytokine which had been antigenically modified by passage through the gut might still be present within the faecal samples. The supposition that these cytokines are absent from the faecal samples was strengthened by data from cytokine bioassays.

My neutrophil chemotaxis studies were performed as a bioassay for IL-8 on a selection of the faecal sample homogenates. Nine of the eleven samples were chemotactic for

neutrophils, but it was anticipated that these studies would at the very least be complicated by the presence in the samples of other chemotaxins, most predictably by bacterial formyl methyl peptides. When steps were taken to remove formyl methyl peptides by column chromatography, and other techniques for other chemotaxins, there was no support for the presence of IL-8 mediated neutrophil chemotaxis in the majority of samples. This has served to reinforce my belief that the ELISA failed to detect faecal cytokines because they were not present in the samples, rather than because they had been altered beyond recognition by the ELISA capture antibody in the course of their passage through the GI tract.

Biological plausibility

In my initial report, I argued that IL-8 and TNFα may pass through the intestine unaltered in CF by virtue of the fact that the majority of CF patients are pancreatic insufficient. Whist they are encouraged to take exogenous pancreatic enzymes with their food, to facilitate its digestion and absorption, they can not be expected to have enzymes in close association with sputum swallowed between meals.

I have shown in initial experiments that pancreatic enzyme supplements available in Australia efficiently digest IL-8 and TNFα both at pH8 and pH4.5 in 1-4 hours. I conclude that cytokines swallowed with pancreatic enzymes will be efficiently digested in the CF gut.

In contrast faecal flora from patients with CF had no clear degradative action on these

cytokines in laboratory experiments, but it must be acknowledged that the experiments will not have identified such activity facultatively produced in times of substrate paucity.

Non Pulmonary sources of cytokines

If IL-8 and TNFα are destroyed by pancreatic enzymes but not by faecal organisms, there remains the question of whether faecally derived cytokines in CF patients might originate from intestinal inflammation rather than pulmonary inflammation. Separating these two potential sources of cytokine in an in vivo experiment is difficult. However the use of recombinant human IL-8 as an upper GI bolus in a laboaratory animal which can not produce human IL-8 in its gut has allowed me to explore this question. I tested the ability of IL-8 and TNFα to transit the gut of a pancreatic sufficient mammal using the Wistar rat as an animal model. These studies revealed no evidence of significant faecal cytokines nor indeed of intestinal inflammation in these rats.

This series of experiments suggests that IL-8 and TNFα delivered into the upper gut from the lung are likely to be digested by pancreatic enzymes and will not appear in the faecal residue. This makes it likely that the high faecal cytokine concentrations seen in the Southampton study are either solely the result of artefact or that they may be an indication of some intestinal inflammatory process within the CF gut. If it were the latter, then the disparity between the samples from Southampton and Brisbane CF patients would suggest that some factor that the Southampton patients had been exposed to might be responsible for any such intestinal inflammation.

High lipase pancreatic enzymes as a potential cause of intestinal inflammation

The UK patients were similar in many respects to the Australian patients, sharing a common genetic stock and a developed world standard of nutrition and hygiene. Overt differences were in environmental temperature and the use of high strength pancreatic enzymes and of carmine markers in sample collection.

I have shown carmine to increase faecal weight in Wistar rats, and this implies that it is not an inert stool marker and might generate inflammation in human subjects, however in the one Australian patient who had been exposed to carmine I did not detect any faecal cytokines.

High lipase enzymes had been in use in the UK patients at the time of the study (7/9 patients were using Creon 25,000) but not in the Australian patients. Fortuitously at the time of the Australian study, Solvay gained a licence for the use of Creon Forte, marketed elsewhere as Creon 25,000, in patients with pancreatic insufficiency. This gave the opportunity to prospectively study the effects of these enzymes on faecal cytokine concentrations, and these studies failed to show elevation in faecal cytokine concentrations, or any other manifestation of fibrosing colonopathy, despite continued therapy for one year, a period of high strength enzyme therapy believed to have precipitated the initial epidemic of fibrosing colonopathy in Liverpool.

These data strengthen the view that the initial high faecal cytokine concentrations were largely the result of artefact, and supports the notion that Creon Forte does not cause

fibrosing colonopathy.

Intestinal inflammation in cystic fibosis

This conclusion contrasts with data from Croft et al who demonstrated elevated cytokine concentration in whole gut lavage fluid in patients with cystic fibrosis[165]. More recently the same group have published further data on measured excretion of inflammatory markers in whole gut lavage fluid, and have interpereted their data as confirming that non idiopathic intestinal inflammation occurs constituitivly in CF patients, as a consequence of a proinflammatory effect of the patient's CFTR mutations.[166]

There are several factors which make their conclusion unsafe. Firstly, in the latest study using whole gut lavage fluid, they report marginally elevated excretion of IgG, IgM, IL-1β, neutrophil elastase and eosinophil cationic protein, and much more dramatic increases in excretion of IL-8 and albumin, but no increase in excretion of α1-antitrypsin or IgA. In their study they administered polyethylene glycol at a constant rate and collected clear gut lavage aliquots. With this discontinuous collection, rather than collecting several hours lavage fluid and then aliquoting it, small differences in excretion of inflammatory markers could result from collection artefact. In their results the sole inflammatory marker where they had no overlap in the range of excretion rates between CF patients and healthy controls was interleukin-8. Their evidence of intestinal inflammation therfore relies heavily on their IL-8 assay. They used the Quantikine assay which I have shown here to give spuriously high cytokine concentrations at dilutions of faecal material down to 1:120,000, and they do not report any validation of their assay

protocols. Secondly, as I have demonstrated digestion of sputum cytokines with pancreatic enzymes over 1 to 4 hours, it remains a distinct possibility that any cytokine they may have been detected in whole gut lavage fluid could be lung derived, because on the one hand they will have markedly reduced intestinal transit time, and on the other will not have stimulated any rump of remaining pancreatic enzyme secretion in their pancreatic insufficient patients, such as might occur after a meal, neither have they administered exogenous pancreatic enzymes. Therefore cytokines arriving in the stomach by the function of the respiratory muco ciliary escalator have every prospect of appearing in the whole gut lavage fluid.

My studies and those of Croft et al. have left areas of uncertainty. They believe they have found evidence of constitutional intstinal inflammation in cystic fibrosis, whilst I believe they have detected inflammatory mediators which have passed through the gut. Something that my studies have been unable to do. It is now clear that when IL-8 and TNFα are swallowed in CF patients taking a normal diet, they do not usually appear in the faeces in an assayable form. There is not an immediate prospect of using faecal cytokine concentration as a proxy measure of the severity of pulmonary inflammation in patients too immature to expectorate sputum.

Potential uses of faecal cytokine assays

It is a disappointment that this technique can not be used to assess the severity of pulmonary inflammation in cystic fibrosis, but it might present an opportunity for measuring the severity of colonic inflammation. Further work will be necessary in

patients with established inflammatory bowel disease to explore the reproducibility and sensitivity and specificity in colitis. There might be the potential to detect or exclude colitis with the assay of a single frozen stool.

Furthermore there may be a role in the detection of relapses of colitis or the confirmation of pathological remission and this would have implications for the drug treatment of these patients. In paediatric inflammatory bowel disease clinics there frequently occurs a situation where inflammatory markers are normal but a patient clearly has colitic symptoms. If biopsy positive disease were reliably associated with a particular level of cytokine concentration this would be of clinical usefulness.

With the commercial ELISA kit required for my validated IL-8 assay protocol having been withdrawn from sale by the manufacturers, and the TNFα protocol failing to register any TNFα immunoreactivity in some patients with colitis, the prospects are not good for developing an intestinal inflammation marker directly from this work.

It is not beyond doubt that the ELISAs used by others in their IBD studies, were linear and accurate in Inflammatory bowel disease patients. It is possible that they were linear and accurate in these patients by virtue of leakage of serum proteins into the intestinal lumen, which is a feature of active colitis. Leakage of albumin might have reproduced conditions in the faecal homogenates that I have produced in the ELISA samples by the use of newborn calf serum.

If faecal cytokine measurements were to have utility in the assessment of colonic inflammation, then this expensive ELISA assay would have to have significant advantages over the cheaper measurement of various faecal protein concentrations. There is a plethora of other indices of intestinal inflammation that have been suggested. These have included polymeric IgA[167], haemoglobin[168], lysozyme and lactoferrin [169], neutrophil elastase[170] myeloperoxidase[171], leukocyte esterase[172] and calprotectin[173,174]. Calprotectin has been developed as a clinical test and a commercial test kit for measuring faecal calprotectin concentration will shortly be available. These other potential indicators have been studied in patients with active and inactive intestinal inflammation and in controls, but have not had tests developed to the stage of assessing their ability to predict the severity of inflammation in individual patients. With the limitations of faecal TNFα and IL-8 ELISAs that I have identified in this thesis it seems unlikely that these cytokines will usurp calprotectin as the next inicator of intestinal inflammatory bowel disease activity.

Potential for future studies

If patients with cystic fibrosis do not exhibit intestinal inflammation, then there is potential to repeat the in vivo and invitro studies that are desribed in this thesis using calprotectin or other faecal "markers of colonic inflammation" to assess their utility as a non-invasive marker of pulmonary inflammation in cystic fibrosis.

Resolution of the problems in confirming or refuting the existence of a non idiopathic intestinal inflammatory reaction in cystic fibrosis is pivotal to future developments. If as I assert Smyth at al are reporting pulmonary derived inflammatory markers in whole gut

lavage fluid, then they have demonstrated an invasive technique for assaying pulmonary cytokines that have transitted the bowel. The way would thus be open to develop less invasive techniques to repeat their feat. One area which could logically be explored would be the effect on faecal cytokine concentrations of omitting the pancreatic enzymes that cause their destruction.

There may still be a prospect of a non invasive, reliable screening test for pulmonary inflammtion in cytic fibrosis patients who are too young to expectorate sputum.

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