INVESTIGATION OF ACUTE INFLAMMATION IN CROHN'S DISEASE AND CHRONIC GRANULOMATOUS DISEASE

By

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A thesis submitted to the University of London for the degree of

Doctor of Philosophy

Department of Medicine

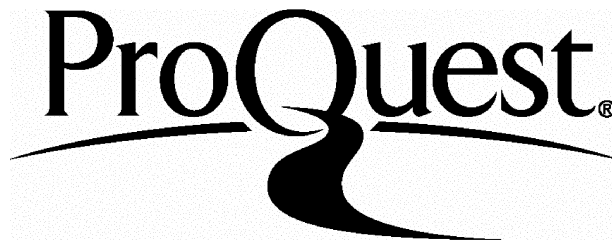University College London

June 2002

ProQuest Number: U643165

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ABSTRACT

Neutrophil function is defective in Crohn's disease and chronic granulomatous disease (CGD). Patients with CGD develop a colitis that is indistinguishable from Crohn's disease.

In order to develop a model to assess treatment strategies, colitis was studied in CGD mice. CGD mice did not develop colitis spontaneously. Colitis induced by dextran sodium sulphate augmented with human faecal flora produced self-limiting chronic inflammation. Chronic colitis did develop after oral administration of Diclofenac. Crystals developed in the lungs of CGD mice. They were shown to be composed of the protein Ym1. This was located in neutrophil granules and gastric epithelial cells and demonstrated to have weak β-hexosaminidase activity.

In order to investigate inflammation in Crohn's disease, a method was developed to induce acute inflammation in humans using cantharidin blisters. Neutrophil migration into blisters was reduced in Crohn's disease, independent of blister fluid chemokines and cytokines. The respiratory burst of blister fluid macrophages was increased in Crohn's disease after stimulation with opsonised bacteria. Neutrophils isolated from venous blood of Crohn's disease patients demonstrated normal oxygen consumption. Digestion of radiolabelled bacteria by neutrophils was assessed in a small number of Crohn's disease patients and found to be normal.

Experimental evidence suggested haploinsufficiency of $p47^{phox}$ as a possible cause of Crohn's disease. DNA from patients with inflammatory bowel disease was screened for this mutation. No excess in the $p47^{phox}$ haplotype was found. However, reduction in the $p47^{phox}$ gene to $p47^{phox}$-pseudogene ratio was associated with Crohn's disease.

Augmentation of neutrophil function by Granulocyte Colony Stimulating Factor (G-CSF) was assessed in Crohn's disease. Serum G-CSF was raised slightly in Crohn's disease. Two days G-CSF administration *in vivo* caused a reduction in venous neutrophil oxygen consumption and enhanced neutrophil penetration of cantharidin blisters, equally in control and Crohn's disease subjects. Therapy with G-CSF for one month may have improved the clinical condition of a minority of patients with Crohn's disease.

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ABBREVIATIONS

ANCOVA, analysis of co-variance; β-hex, β-N-acetylhexosaminidase; BP, basepair; BSA, bovine serum albumin; C, complement; CD, Crohn's disease; CDAI, crohn's disease activity index; CGD, Chronic Granulomatous Disease; CPM, counts per minute; CRP, C-reactive protein; ΔGT, GT deletion; DHR, dihydro rhodamine; DSS, dextran sodium sulphate;

EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; ENA-78, epithelial neutrophil activating peptide 78; ESR, erythrocyte sedimentation rate; FACs, flow cytometry; FAD, flavine adenine dinucleotide; FAE, follicle associated epithelia; FBC, full blood count; FISH, fluorescent in-situ hybridisation; FITC, fluorescein isothyocyanate;

fMLP, f-MET-LEU-PHE; G-CSF, granulocyte-colony stimulating factor; [GlcNAc], N-acetyl glucosamine; GROα, growth related oncogene alpha; GSD-Ib, glycogen storage disease 1b; IBD, inflammatory bowel disease; IBDQ, inflammatory bowel disease questionnaire; Ig, immunoglobulin; IL, interleukin;

LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MHC, major histocompatibility complex; MIP-1α, macrophage inflammatory protein-1α; MPO, myeloperoxidase; MU, 4-methyl umbelliferone; MU-[GlcNAc]n, (n=1,2,3) β1-4 linked mono/di/trisaccharides of [GlcNAc] linked to MU;

NSAID, non-steroidal-anti-inflammatory-drug; ν-NCF1, product following ΨNCF1 to NCF1 conversion; ν-p47^{phox}, protein derived from ν-NCF1; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PG-PS, peptidoglycan polysaccharide; PMA, phorbol myristate acetate;

ΨNCF1, NCF1-pseudogene; SCID, severe combined immuno-deficient; SDS, sodium dodecyl sulphate; SPF, specific pathogen free; SH3, Src-homology 3 domain; TBS, TRIS-buffered saline; TCA, tri-chloro acetic acid; TCR, T-cell receptor; TNBS, trinitrobenzenesulfonic acid; TNFα, tumour necrosis factor alpha; UC, ulcerative colitis.

INTRODUCTION

OVERVIEW

"If a lot of cures are suggested for a disease, it means that the disease is incurable". So wrote Anton Chekhov in The Cherry Orchard in 1904. Although not referring to Crohn's disease (CD), a form of inflammatory bowel disease first described 28 years later (Crohn et al. 1932), the quotation is apt for the disease. For, although there are many treatments for CD, none is curative. This is in part due to the fact that the causes of CD are (largely) unknown.

The genetic causes of a much rarer disease with some similar manifestations to CD have been elucidated. Both chronic granulomatous disease (CGD) and CD have a similar intestinal phenotype, with patchy granulomatous inflammation, perianal abscess formation, small bowel involvement and oral ulceration (Werlin et al. 1982).

CGD arises due to germline mutations affecting the function of a myeloid cell protein complex called the NADPH oxidase. This results in absent O_2^- production thereby preventing adequate digestion of microbes by cellular granule proteases (Reeves et al. 2002). The similarity between CGD enteritis and CD suggests that genetic defects that impair myeloid cell function could also predispose to CD. However, despite considerable understanding of the molecular pathology of CGD, a cure can be achieved only by related bone marrow transplantation. At present, "A desperate disease requires a dangerous remedy" (Guy Fawkes, 1605).

Most diseases occur due to the interaction between an adverse environment and a susceptible genotype. In both CGD and CD the environmental factors that predispose to disease are largely unknown. If they were known, susceptible patients could be advised to avoid them. In this way, the disease could be prevented whether or not the causative germline mutations were defined.

The theme for the work has been to investigate factors that are aetiopathogenic for CGD or CD, in particular concentrating on the neutrophil. Studies in CGD have been conducted in knockout (-/-) mice whereas human studies have been performed in CD.

CROHN'S DISEASE

Clinical features of CD

CD is characterised by chronic inflammation of the human alimentary tract, particularly the terminal ileum and colon (Figure 1). The disease causes abdominal pain, diarrhoea, intestinal bleeding and malnutrition. Disease incidence has increased during the latter part of the twentieth century and is highest in northern Europe and North America where prevalence approaches 100/100,000 (Calkins & Mendeloff 1986). Although the mortality rate has fallen during the past twenty years the disease causes considerable morbidity (Sandler & Eisen 2000a).

Figure 1: Crohn's disease of the colon. This is characterised by inflammatory skip lesions and aphthous ulceration, due to transmural chronic inflammation.

The diagnosis is suspected at the time of endoscopy when "snail track" and aphthous ulcers are seen in the colon and/or terminal ileum, although more proximal regions of the small intestine are affected in a minority of patients. Often areas of relatively normal mucosa separate the affected areas and classically the rectum is an uncommon site for the disease.

Multiple biopsies should be reviewed by an experienced histopathologist since the microscopic features are non-specific and include transmural inflammation, lymphoid hyperplasia, and in half the patients granuloma formation. A granuloma comprises a collection of macrophages, formed in response to the presence of non-digestible material. In some diseases, for example tuberculosis, the source of the offending antigen is known; however, often this is not the case, for example in sarcoid, Wegener's granulomatosis and CD.

The mainstay of treatment for CD is to suppress the immune response. This can be done effectively with steroids, mesalazine derivatives, azathioprine or with "biological therapies", such as a monoclonal antibody directed against the inflammatory cytokine TNFα. However, none of these treatments are curative; all have side effects; and only a few prevent disease relapse.

Aetiology of CD

Two very important principals should always be remembered when considering the causes of CD

Firstly, factors that initiate the inflammatory response may be distinct from those that cause it to persist. "It is incident to physicians, I am afraid, beyond all other men, to mistake subsequence for consequence" (Dr. Johnson). The aetiological factors responsible for disease onset, disease relapse and disease persistence are largely speculative. It is generally accepted that CD is caused by an abnormal interaction between the intestinal flora and the mucosal surface or the sub-mucosal immune system.

Secondly, that CD is probably caused by several genetic and environmental factors that differ between individuals, which present as a common phenotype. The number of genetic and environmental abnormalities within the population and the number that is required in any one individual is unknown. Genetic, phenotypic, environmental and population heterogeneity mean that it is very difficult to determine the causes of CD. Consequently, it is likely that disease subtypes will first be defined genetically followed by determination of the effect of each mutation at the cellular and then environmental level.

Several pathogenic mechanisms have been implicated (Figure 2).
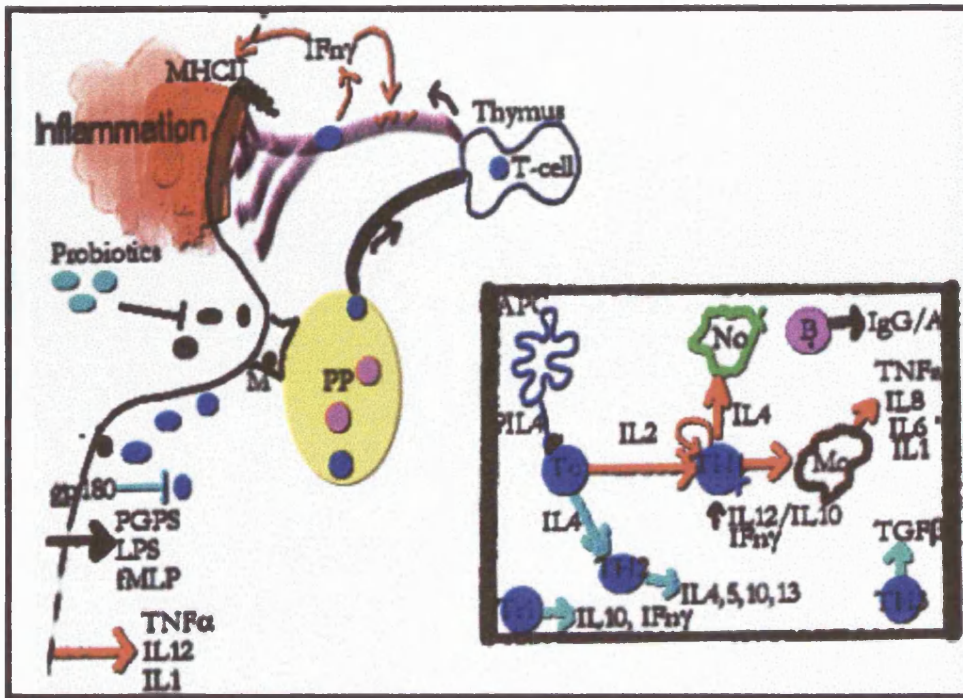
Figure 2. The interface between gut bacteria and the mucosal immune system.

The normal response: M cells sample luminal contents by pinocytosis. They pass bacteria to antigen presenting cells, including dendritic cells and macrophages, which present antigen to naïve T - and B-cells in Peyer's patches (PP). In addition, the epithelial cell, in association with MHC II expression can pass bacterial antigens to the antigen-presenting cell. B- & T-cells migrate to regional lymph nodes and T-cells to the thymus before returning to the mucosa, mediated by increased expression of addressins. They then release IgA, and pro-inflammatory (TH1) cytokines that recruit macrophages (Mo) and neutrophils (No), again mediated by addressins (e.g. E-selectin; ICAM1) and leukocyte integrins (e.g.CD11b/CD18). The response is normally controlled by suppresser T-cells which release counter-inflammatory cytokines (TH2; Tr1; TH3).

Implicated pathogenic mechanisms in CD: Certain micro-organisms (e.g. adherent *E. Coli*) may have increased pathogenic potential and overcome host defence mechanisms. Secretary IgA may be reduced facilitating their invasion. Permeability of the mucosa, particularly adjacent to "M" cells may be increased, perhaps exacerbated in those that take NSAIDs. An excessive lamina propria T-cell / antigen presenting cell / epithelial cell TH1 response, with up-regulation of antigen presenting cells' co-stimulatory molecules & MHC II, may occur in response to microbe invasion. Reduced suppresser responses, for example a reduced TH2 cytokine profile (e.g. reduced IL-4) or failure of (possibly perinatally derived) oral tolerance, may occur. The latter may be mediated by absence of epithelial gp180. The innate immune response may be deficient, for example reduced neutrophil migration into the inflamed area (perhaps due to abnormal integrins), or reduced macrophage sensing of bacterial antigens e.g. LPS, PG-PS or fMLP (perhaps through abnormal NOD2 protein). This could provoke an excessive acquired immune response. Finally, wound healing may be aberrant in CD, particularly in those with stricturing disease.

(Adapted from Inflammatory Bowel Disease (5th edition), Kirsner J.B. (ed.), WB Saunders (2000), London).

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Genetic factors implicated in CD

Monozygotic twins have approximately a 50% concordance for CD (Breslin et al. 1997), however, patterns of inheritance are more typical for a polygenic disease in dizygotic twins and siblings (Binder 1998). This demonstrates two things. Firstly, a susceptible genotype still requires environmental events to trigger and perhaps maintain the CD phenotype. Secondly, multiple gene loci predispose to CD. There is also an increased incidence of ulcerative colitis (UC) in relatives of patients with CD, although less than the increased risk of CD itself (Yang et al. 1993). Therefore it is likely that there are both susceptibility genes for inflammatory bowel disease (IBD) and disease specific genes for CD and UC. This may explain why some patients can not be classified or are misclassified as having CD or UC.

The genes that predispose to CD are largely unknown. Associations have been found with various loci on chromosome 6 in the HLA DR/DQ region (Yang & Rotter 2000) and independently with the TNF microsatellite haplotypes a2b1c2d4e1 (Plevy et al. 1996). These loci increase the relative risk for CD three-fold, only a small component of the incremental genetic risk (λ) for siblings of CD patients, estimated to be between 50-100 (Yang & Rotter 2000).

The strongest association with CD is found on chromosome 16. Last year, the gene that accounts for the majority of this association was discovered, which encodes the protein NOD2 (Hugot et al. 2001; Hampe et al. 2001; Ogura et al. 2001a). Three mis-sense mutations (P268S; R702W; G908R) and one frameshift insertion (3020insC) in the C-terminal, leucine-rich region of the protein are the commonest mutations. They are found in 15% to 20% of patients with CD but also in about 5% of normal subjects. Interestingly, they are associated with ileal CD (Cuthbert et al. 2002; Ahmad et al. 2002). NOD2 is a component of a monocyte intracellular pathway that results in increased NF-κB expression (Ogura et al. 2001b) and therefore the transcription of inflammatory genes. In the original report associating CD and *NOD2* (Ogura et al. 2001a), cells transfected with mutant NOD2 appeared to not recognise lipopolysaccharide (LPS). Therefore it is possible that LPS-sensing is a major host defence mechanism in the ileum. This is corroborated by the fact that a related protein, NOD1, is also a component of a pathway that recognises LPS (Inohara et al. 2001).

In addition to chromosome 6 and 16, there are several chromosomal regions in linkage dysequilibrium with CD, in particular 3p21, 5q31 and 7 (Satsangi et al. 1996; van-Heel et al. 2000; Rioux et al. 2001). It is likely that other CD susceptibility genes contained within these regions will be identified soon by a combination of family based genome wide scanning, refinement by association studies and identification using a positional candidate gene approach. It is likely that some mutations will include other components of the innate inflammatory response.

Environmental factors implicated in CD

The environmental risk factors for CD are ill defined. However, importantly, patients can be advised to avoid two factors associated with CD. The first is cigarette smoking, which increases disease onset (Calkins 1989) and relapse rates (Timmer et al. 1998). The mechanism of action is unknown but may include a reduction in visceral blood flow and therefore impair the integrity of the epithelium (Sandler & Eisen 2000a).

The second is the use of non-steroidal-anti-inflammatory-drugs (NSAID's), which are believed to be associated with increased CD relapse rates. They increase gastrointestinal permeability (Bjarnason 1994; Soderholm et al. 1999), enhancing translocation of bowel flora into the gut mucosa, which can be attenuated by metronidazole (Yamada et al. 1993). It has been estimated that NSAID use increases the relative risk of non-CD colitis five-fold (Tanner & Raghunath 1988). Surprisingly, there are no trials comparing NSAID use and CD onset or relapse, although many case reports support an association. Hopefully, with an increased understanding of the aetiopathogenesis of CD, further controllable environmental factors will be defined.

Bacteria and Crohn's Disease

There is strong evidence that bacteria and food products are critical environmental factors in CD.

➢ Enteric inflammation occurs in the terminal ileum and cecum where there is a high bacterial load (Onderdonk 2000).

➢ Effective treatments for CD include antibacterials (for example metronidazole (Achkar & Hanauer 2000)); and elemental diets (O'Morain et al. 1984), amino acid feeds that reduce dietary antigens, bacteria and their fermentation products (e.g. butyrate which is raised in CD (Treem et al. 1994)).

> Surgical bypass of CD-affected bowel is a recognised treatment for CD although rarely used now (Farmer et al. 1976).

> Relapse occurs when ileal effluent is introduced into the defunctioned rectum (Harper et al. 1985) or ileum (D'Haens et al. 1998); but does not occur if the effluent is passed through a 0.2 µM filter which removes large particulate matter and bacteria (Harper et al. 1985).

> Finally, in germfree animals there is minimal lymphoid tissue or soluble IgA (Crabbe et al. 1968). Moreover, the implicit requirement for bacteria during intestinal inflammation is reinforced by animal models of colitis (Fedorak & Madsen 2000a), which all require the presence of intraluminal bacteria..

So are specific bacteria known to cause CD? There are over 400 different bacterial species in the human gastrointestinal tract & possibly many more that have never been cultured. Anaerobes, mainly *Bacteroides* and *Bifidobacterium* species are a 1000-fold more plentiful than aerobes within the colon (Duchmann et al. 1999a) but their numbers are equal at the mucosal surface. In the terminal ileum, a common site for CD, *Bacteroides* species predominate, whereas *Lactobacilli* dominate in the proximal small bowel. The plethora of species with sub-populations adjacent to the mucosa, difficulties in organism culture (Onderdonk et al. 1992) and identification, and changes in bacterial composition consequential to disease all make investigation of the aetiopathogenic effect of the bowel flora in colitis very difficult.

Animal models have implicated *Bacteroides vulgatus* (Rath et al. 1999) as a requisite pathogen in the colitis of the HLA-B27 transgenic rat but species susceptibility varies since this organism does not cause colitis on colonisation of the gnotobiotic IL (interleukin)-10 -/- mouse (Sellon et al. 1998). In active CD, greater numbers of aerobes and *E. coli* and lower numbers of *Bifidobacteria* and *Lactobacilli* species have been found (Giaffer et al. 1991). A separate study found *B. vulgatus* to be increased (Krook et al. 1981). However, these changes may occur as a consequence of the disease and therefore not relate to the primary pathogenic process. In addition, the composition of the mucosal associated flora in CD is unknown.

Recently a possible pathogenic DNA sequence, termed I2 and unique to CD has been discovered by subtraction hybridisation of bacterial DNA from intestinal biopsies

(Sutton et al. 2000). Quantitative polymerase chain reaction (PCR) demonstrated this sequence in ileal biopsies of all subjects but preferentially in CD colonic biopsies, independent of disease activity and associated with serum immune reactivity. This may represent a primary aetiopathogenic process since I2 has recently been shown to strongly stimulate CD4+ T-cells (Dalwadi et al. 2001). The identification of organisms that contain this sequence are awaited.

There has been controversial debate as to whether the increased prevalence of a single microbial species could account for the increased CD incidence, for example *Mycobacterium paratuberculosis* (Chiodini et al. 1984) (Thayer, Jr. et al. 1984) or the measles virus (Wakefield et al. 1995). Certainly, there are bacteria which are more invasive than others, for example adherent invasive *E. Coli* (Glasser et al. 2001). However, redundancy within host defence pathways makes it unlikely that a single pathogen will evade recognition and removal. It is more likely that CD occurs due to defective host inflammatory responses that result in an inability to recognise or completely digest different components of micro-organisms.

The main microbial components recognised by the mammalian immune system are LPS, a component of the outer membrane of gram negative bacteria; peptidoglycan polysaccharide (PG-PS), the primary structural polymer of bacterial cell walls; and formylated oligopeptides such as f-methyl--leucyl-diphenylalanine (fMLP), the n-terminal sequence of an *E. coli* SOS operon (Broom et al. 1993). Structure and quantity of these molecules differ between bacterial species, which probably accounts for their variable antigenicity. In addition, antigenicity may also be host-determined in IBD. For example, peptidases that degrade luminal fMLP are decreased in some patients with IBD (Chadwick et al. 1990); intracellular recognition of LPS may be deficient in CD patients with mutations in NOD2 (Ogura et al. 2001a); and certain PG-PS polymers are resistant to degradation (Schwab 1993) and therefore could mediate granulomatous inflammation.

Probiotics, a novel bacterial therapy for IBD

In most studies of the bacterial flora composition in IBD, there is a reduction in *Lactobacillus* and *Bifidobacter* species (Giaffer et al. 1991; Fabia et al. 1993). These organisms are thought to be beneficial to the host and therefore are described as "probiotics", defined as "living organisms, which upon ingestion in certain numbers,

organisms are thought to be beneficial to the host and therefore are described as "probiotics", defined as "living organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition" (Faubion & Sandborn 2000). First alluded to by Metchnikoff (Metchnikoff 1907), their importance for normal gut function has been appreciated only recently. For example, a Medline™ search between 1991 and 1995 revealed 37 original articles published on probiotics, but 309 articles between 1996 and 2000. Large numbers of harmless, commensal microbes may function as probiotics, many as yet undiscovered, but *Lactobacillus* and *Bifidobacter* species are major components (Campieri & Gionchetti 2001). Although their mechanism of action has not been defined (Campieri & Gionchetti 1999), preliminary evidence is available to support their therapeutic use in intestinal infection and inflammation.

Lactobacillus species prevent spontaneous colitis (Madsen et al. 1999) and abrogate established colitis (Schultz et al. 1998) in IL-10 -/- mice. In human studies, *Lactobacillus* GG has successfully been used to prevent travellers' diarrhoea (Oksanen et al. 1990), to treat *C. difficile* colitis (Biller et al. 1995) and increased (protective) IgA in children with CD (Malin et al. 1996). Another "probiotic", *E. coli* (strain Nissle 1917) may be as effective as mesalazine in preventing relapses of UC (Kruis et al. 1997; Rembacken et al. 1999).

However, the most convincing data to date are reported by Gionchetti et al. They used a high dose cocktail of 8 probiotics (4 strains of Lactobacilli, 3 strains of *Bifidobacteria* and 1 strain of *Streptococcus salivarius* subspecies *Thermophilus*) (VSL#3, Yovis; Sigma-Tau, Pomezia, Italy). Following antibiotic-induced remission, ileal reservoir inflammation (pouchitis) relapse was prevented in 17 of 20 patients, whereas all 20 patients in the placebo group relapsed (Gionchetti et al. 2000a). In addition, using the same preparation, they successfully prevented the development of UC pouchitis (Gionchetti et al. 2000b), maintained UC remission (Venturi et al. 1999) and treated patients with active UC (Schultz & Sartor 2000).

A very interesting recent report from Madsen *et al.* demonstrated that as yet unidentified proteins secreted by VSL#3 reduced colitis in the IL-10 -/- mouse, improved intestinal epithelial integrity and prevented intestinal mono-layer invasion by Salmonella (Madsen et al. 2001). Clearly it will be important to establish the identity of these proteins.

exaggerated by genetic manipulation of probiotic bacteria to secrete "healing" cytokines, such as IL-10 (Steidler et al. 2000). It is likely that probiotics will also be of benefit in CD and the results of clinical trials are awaited.

Helminths in experimental IBD

IBD is rare in Asia (Teh et al. 1987), Africa and South America (Sandler & Eisen 2000b). However, migrants to countries with a high prevalence of IBD develop the disease rate of their adopted country (Fellows et al. 1988) (Calkins et al. 1984). Although this may represent the gain of a risk factor (Table I), it is an attractive hypothesis that improved intestinal hygiene predisposes to CD through a reduction in helminth-derived TH2 bystander immune suppression and through a reduction in intestinal tolerance to the commensal flora.

Table 1: Well-defined epidemiological factors in CD
Northern latitude
Refined sugar intake
Lack of breast feeding
Perinatal maternal infection
Perinatal childhood infection
Infant gastroenteritis
High living standard
Oral contraceptive use
Smoking cigarettes
(in Sandler and Eisen, 2000)
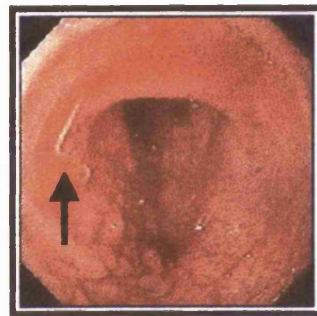
Figure 3: Terminal ileal pinworm

Preliminary evidence supports an aetiologic, immune-suppressive role for intestinal helminths in CD (Figure 3) (Elliott et al. 2000). Intraperitoneal administration of *Schistosomiasis mansoni* abrogated colitis in the trinitrobenzenesulfonic acid (TNBS) murine model (Elliott DE et al. 1999) and elicited a strong TH2 cytokine response. The beneficial effect was less impressive following tapeworm infection, which reduced ion transport abnormalities in the murine dextran sodium sulphate (DSS) model of colitis but had no effect on other outcome measures (Reardon et al. 2001). No trials have been reported yet in IBD.

Oral Gastrointestinal Tolerance

The human intestinal tract contains between 10^{14} and 10^{16} organisms comprising at least 350 different species (Onderdonk 2000). It is remarkable therefore that the intestinal immune reaction is not more pronounced. Down regulation of intestinal

immunity is mediated by oral tolerance which results in "chronic, physiologic inflammation". Oral tolerance, immune inhibition following oral feeding, was first described in 1911 by H.G.Wells (Wells 1911). Antigen specific suppression of the immune response by oral feeding later became known as the Sulzberger-Chase phenomenon (Spahn & Weiner 2000).

Low doses of oral antigen stimulate the murine gut associated lymphoid tissue to generate an immune suppressive response mediated by TH2 (immunoglobulin (Ig)G1 & IgE; Il-4 & IL-10), Tr1 (IL-10) (Groux et al. 1997) and TH3 (TGFβ) (Fukaura et al. 1996) cytokine profiles. These suppresse the TH1 response (IgG2; IL-2 & interferon-γ). High doses of oral antigen cause clonal T-cell apoptosis within Peyer's patches (Chen et al. 1995). In general, just as oral tolerance is mediated by a TH2 milieu, conversely, it is abrogated by a TH1 stimulus (Zhang & Michael 1990; Marinaro et al. 1997).

Murine models demonstrate a role for tolerance in the pathogenesis of colitis. Low dose tolerance following oral TNBS successfully abrogated murine colitis in the TNBS model (Neurath et al. 1996). Similarly, following rectal TNBS administration, tolerance to autologous intestinal flora was lost, reversed by IL-10 or anti-IL-12 administration (Duchmann et al. 1996). Interestingly, oral LPS administration potentiates tolerance induced by oral myelin basic protein in experimental autoimmune encephalomyelitis (Khoury et al. 1990) and tolerance to subsequent systemic LPS (Erroi et al. 1993). Whether this is defective in humans deficient in NOD2 deserves attention.

The TH1 / TH2 paradigm and the role of tolerance are less clear in (human) IBD. It is noteworthy that cleanliness is a risk factor for CD (Duggan et al. 1998) as perhaps is appendectomy (Russel et al. 1997) but speculative that this is due to a loss of antigen that would normally induce tolerant T-cell production in the appendix. Loss of tolerance to autologous bacterial antigens has been demonstrated in isolated IBD lamina propria mononuclear cells, but only from sites of active disease (Duchmann et al. 1995), which suggests that this is consequent and not causal to the disease process. Similarly, the lack of response of IBD mononuclear cells to the down-regulatory cytokine IL-4 is related to disease activity (Schreiber et al. 1995).

Low dose tolerance is thought to be dependent on the antigen presenting cell ligand B7-2 co-stimulating the T-cell through CTLA-4 (Liu et al. 1999). B7-2 is found on resident mucosal macrophages in the intestine (Rugtveit et al. 1997). Epithelial cells lack these molecules (Sanderson et al. 1993; Bloom et al. 1995) but express a surface glycoprotein, gp180 (Yio & Mayer 1997), that binds to CD8 molecules on T-cells and induces the suppresser phenotype. There is one report of deficiency of gp180 in IBD epithelial cells (Toy et al. 1997). It will be important to further investigate this and other mechanisms of oral tolerance in IBD.

Mucosal integrity in Crohn's disease

The integrity of the intestinal mucosa is interrupted regularly by follicle associated epithelia (FAE) (Bockman & Cooper 1973) containing specialised "M" cells (Owen & Jones 1974) that sample the luminal contents by pinocytosis. M cells present antigen to a collection of dendritic cells, T-cells and naïve B-cells, contained within sub-mucosal lymphoid follicles. This region is known as the Peyer's patch, after Johann Conrad Peyer (1653-1712) who noted these structures in patients that had died from typhoid (Fujimura & Owen 2000). Therefore, within the normal mammalian intestine the FAE forms an incomplete barrier to luminal antigens (Warshaw et al. 1977), which enables bacterial entry into the sub-mucosa (Vazquez & Fang 2000).

Normally, bacteria do not invade epithelial cells adjacent to the FAE (Boedeker 1994), due in part to a stable, complex bacterial microenvironment (Hentges 1983); mucosal mucus (Cepinskas et al. 1993); cellular integrity, maintained by intercellular tight junctions (Madara 1989); secretion of antibacterial compounds such as trefoil peptides (Playford 1997); the secretion of heat shock proteins (molecular chaperones that may inhibit the unfolding of host defence proteins during times of stress) (Beck et al. 1995); soluble IgA (Mestecky et al. 1991); and Paneth cell cryptdins (bactericidal cationic pore forming proteins) (Lencer et al. 1997). However, bacterial invasion of the colon epithelial cell has been demonstrated in UC (Ohkusa et al. 1993) and in normal individuals at times of severe stress (Hendrickson et al. 1999).

It is likely that incomplete mucosal integrity is aetiopathogenic in CD. Studies of intestinal permeability have demonstrated an increase in CD patients and also their apparently unaffected relatives (Hollander et al. 1986), which is exaggerated after aspirin ingestion (Hilsden et al. 1996). This suggests that intestinal integrity is determined

partly by genotype, although this does not occur in a Mendelian pattern (Peeters et al. 1997).

The mechanism may be due in part to loosening of the epithelial cell tight junction, which has been demonstrated in both inflamed and non-inflamed CD mucosa (Marin et al. 1983). However, it is more likely that the primary defect in mucosal integrity is associated with the FAE: aphthoid ulcers, one of the earliest CD lesions, are located adjacent to the Peyer's patch (Rickert & Carter 1980); aphthoid ulcers are temporally (Van Kruiningen et al. 1997) and spatially correlated with CD; and electron microscopy has demonstrated ulceration adjacent to the FAE in CD (Fujimura et al. 1996), possibly because the acute inflammatory response is unable to contain bacterial invasion with subsequent ulcer formation and further antigen intrusion.

The FAE is found in high concentration in the rectum (O'Leary & Sweeney 1986) and therefore it is unlikely to account for the rectal sparring of CD. In fact, rectal sparring may be caused by a lack of migration of effector T-cells to the rectum (Mayer 2000), in contrast to the rest of the mucosa associated lymphoid tissue.

Acquired immunity in Crohn's disease

Following antigen presentation, T cell clonal proliferation and differentiation occurs, causing the release of pro-inflammatory cytokines, resulting in neutrophil and macrophage recruitment. Naïve B-cells migrate to regional lymph nodes before returning to the lamina propria where they secrete soluble IgA into the gut lumen. This dimerizes and coats invading micro-organisms, rendering them harmless.

It is very hard to determine whether the immune responses observed in IBD are primary and therefore aetiopathogenic or merely secondary to dysregulated inflammation. Certainly, case reports of CD remission following bone marrow transplant (Lopez-Cubero et al. 1998) and CD onset in normal small bowel, transplanted into a CD recipient (Sustento-Reodica et al. 1997) implicate immune dysregulation as a causative factor. The temporal association between CD relapses and respiratory tract infections (Mee & Jewell 1978) suggests that generalised immune stimulation could promote enteritis in susceptible individuals or may represent epithelial damage by virus particles.

However, immunologic abnormalities in CD such as an increase in the IgG/IgA ratio (Macpherson et al. 1996); an increase in serum anti-*Saccharomyces cerevisiae* (Giaffer et al. 1992), which is found only in those monozygotic twins concordant for the disease (Lindberg et al. 1992); clonal expansion of CD4$^+$ cells in aphthoid lesions (Nakajima et al. 1996); enhanced T-cell proliferation to *S. aureus* and LPS (Fiocchi et al. 1981) and perhaps to antigens of the resident flora (Duchmann et al. 1999b); increased lamina propria CD4$^+$ lymphocyte production of interferon-γ (Fuss et al. 1996) and IL-12 (Parronchi et al. 1997); T-cell resistance to apoptosis associated with an increased Bcl-2/Bax ratio (Ina et al. 1999); and reduced mucosal IL-4 (West et al. 1996) all could reflect secondary chronic active inflammation. Further evidence is required to establish whether one or more of these factors is aetiopathogenic for CD.

It is more likely that some of the extra-intestinal manifestations of IBD are secondary to induced auto-antibodies (Bhagat & Das 1994; Mandal et al. 1994) either directed against previously sequestered antigens, e.g. tropomyosin (Geng et al. 1998), neo-antigens created by a microbial hapten, or self-antigens cross reacting with microbial epitopes (Tiwana et al. 2001). The enterohepatic circulation of PG-PS (Lichtman et al. 1992) may contribute to IBD-associated sclerosing cholangitis.

Innate immunity in Crohn's disease

The ability to mount an acute inflammatory response and clear antigenic material that has crossed the mucosal barrier is essential in order to maintain immunoregulation within the gut. Neutrophils are an early defence after debris or bacteria or fungi have breached the mucosal barrier (Horstedt et al. 1989). Normally, neutrophils are rare in the intestinal lamina propria. Bacterial invasion provokes the release of cytokines and vasoactive factors by activated lymphocytes and resident macrophages, with neutrophil and macrophage influx, and phagocytosis and digestion of bacteria.

If this process is deficient, bacterial products will remain and stimulate an enhanced influx of chronic inflammatory cells, predominantly macrophages, which damage the local environment and allow further bacterial invasion. For example, in CGD and Glycogen storage disease 1b (GSD-1b), both characterised by neutrophil dysfunction, a chronic colitis occurs (Couper et al. 1991; Mitomi et al. 1999).

Colitis is commonly found in other innate immune-deficiencies, such as common variable immune deficiency and X-linked agammaglobulinaemia (Washington et al. 1996); leukocyte adhesion deficiency (D'Agata et al. 1996) in which colitis resolved following bone marrow transplant; auto-immune (Stevens et al. 1991) and congenital (Vannier et al. 1982) neutropenia. In addition, the colitis in the Hermansky-Pudlak syndrome is thought to occur due to a toxic accumulation of ceroid-like pigment in macrophages (Shanahan et al. 1988). Indeed, it is likely that any major failure of the immune response can cause intestinal inflammation, since the intestinal tract is far from sterile (Onderdonk 2000) and the mucosa subject to bacterial invasion.

Is there evidence for a primary abnormality in the neutrophil in CD? Neutophil migration into small skin abrasions ("skin windows") has been shown to be reduced in CD, independent of disease activity (Segal & Loewi 1976; Morain et al. 1981). This suggests that the migration of neutrophils to an inflammatory site within the gut wall may also be deficient and predispose to chronic inflammation. Several investigators have suggested that O_2^- production by CD neutrophils is reduced, although no clear consensus can be drawn from the results published to date (see chapter 5).

And what of macrophages? There is evidence that suggests macrophages are activated in CD, however, many of the results may represent secondary immune activation since the studies have been undertaken in patients with active disease. For example, lysozyme was increased in CD monocytes (Mee & Jewell 1980) but correlated with disease activity; antigen presentation to T-cells by lamina propria macrophages from inflamed IBD mucosa was enhanced (Mahida et al. 1988); serum CD monocytes from patients with active CD produced a greater respiratory burst following LPS priming (Baldassano et al. 1993); and CD macrophages recruited to sites of active disease up-regulated CD80 and CD86, indicating their activated state (Hara et al. 1997). In one study the findings were independent of disease activity and showed that phagocytosis but not killing of *C. albicans* and opsonised *S. aureus* was increased in IBD (Tanner et al. 1984). It is possible that a hyperactive innate immune response causes inappropriate tissue destruction and therefore chronic inflammation. The recent discovery that mutated NOD2 is associated with ileal CD conclusively demonstrates that abnormalities in monocytes can predispose to CD. Although the physiological significance of these mutations is still to be defined, it does underscore the requirement for a fully functional innate immune response in order to prevent CD.

Is digestion deficient in CD? There is no direct evidence that non-digested enteric antigens (probably of bacterial origin) cause the granulomatous inflammatory response in CD. However, electron microscopy has demonstrated particles with a diameter of 30-70 nm in CD macrophages near ulcerative lesions (Riemann 1977); and the granuloma, a hallmark histological lesion in CD found in 50% of resection specimens (Schmitz et al. 1984), is thought to be produced due to the presence of undigested material within the bowel wall (Luijendijk et al. 1996).

Inflammation resolution in Crohn's disease

It is important to heal the ulcerated surface following a mucosal insult. Recruited macrophages secrete matrix metalloproteinases, which re-model the extra cellular matrix allowing restitution, that is adjacent epithelial cell migration into the defect in order to re-establish an effective barrier, promoted by growth factors, for example epidermal growth factor (Dieckgraefe et al. 1997). TGFβ, secreted by neutrophils (Grotendorst et al. 1989), attracts myofibroblasts, which secrete collagen (Graham et al. 1990), fibronectin and other extracellular matrix components. Capillary endothelial cells then migrate into the region to provide a new blood supply.

Little is known about tissue healing in IBD. Fibroblasts from CD strictures preferentially secrete TGFβ_2, which is inefficient in promoting wound repair (McKaig et al. 2002), perhaps by producing excess type III collagen (Stallmach et al. 1992). The significance of this and the increased numbers of mast cells found in CD strictures (Gelbmann et al. 1999) is unknown.

ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE

Germline models

Animal models of enteritis can be divided into those produced by a specific environmental factor (e.g. DSS administration (Cooper et al. 1993)) and those occurring in a genetically modified host (e.g. IL-10 -/- mice (Kuhn et al. 1993b)). The majority of models need specific conditions in both host and environment for the development of chronic enteritis. For example, the effect of DSS in mice is strain specific (Mahler et al. 1998a) and an intestinal bacterial flora is required for IL-10 -/- mice colitis to occur (Madsen et al. 2000).

At least 20 germline animal models have been described in which chronic bowel inflammation occurs. This illustrates that the inflammatory response has many potential causes but a limited phenotypic response. However, none of the many animal models described reproduces the CD phenotype of "skip" lesions; granulomata; perianal disease and transmural inflammation. However, they do provide clues to the pathogenesis of bowel inflammation and illustrate its complexity. Some examples are given below.

Colitis occurs in the IL-2 -/- mouse (Sadlack et al. 1993), also in the IL-2 , B-cell double knock-out mouse, but not in the IL-2, RAG-A double knock-out mouse that also lacks T-cells (Ma et al. 1995). This demonstrates the pivotal role played by the T-cell in chronic enteritis. Absence of IL-2 is thought to prevent activated T-cell apoptosis, which may explain the paradox that mice with deficiency in a major TH1 cytokine develop colitis.

30% of mice with a defective T-cell receptor (TCR) have a distal colitis that resembles UC. However, this does not occur in mice also lacking a B-cell response (TCR, RAG-A double knock-out) or in TCRα -/- mice following neonatal appendectomy (Mizoguchi et al. 1996). This suggests that lymphoid tissue within the appendix presents antigen to naïve T-cells soon after birth, with the antibody response normally regulated by tolerant (αβ) T-cells (Fedorak & Madsen 2000b).

Both colitis and arthritis occur in transgenic rats that overexpress the human major histocompatibility complex (MHC) molecule HLA-B27. However, in germ-free animals colitis only occurs following colonisation with *Bacteroides* species (Rath et al.

1996), especially *B. vulgatus* (Rath et al. 1999). This illustrates the importance of this MHC molecule in mediating both colitis and arthritis and demonstrates that certain bacteria have a greater pathogenic potential.

Mice deficient in IL-10, an anti-inflammatory cytokine, pivotal in the TH2 response, develop enterocolitis characterised by a TH1 response. This model emphasises the importance of homeostatic regulatory immune responses such as IL-10 (Kuhn et al. 1993a). Moreover, colitis can be prevented with anti-IL-12 but not anti-interferon-γ (Davidson et al. 1998), which demonstrates that IL-12 acts early in defining the TH1 phenotype.

Another murine colitis model has provided insight into immune tolerance in the intestine. TGFβ -/- mice develop a very severe inflammatory response including a diffuse gastroenteritis leading to a wasting syndrome and premature death (Kulkarni et al. 1993). T-cells in these mice are highly differentiated and activated, which may be due to up-regulation of both MHC class I and II receptors (Nakabayashi et al. 1997). Although this model does not parallel human IBD, it does underscore the importance of counter regulation of the immune response and demonstrates the important role of TGFβ in performing this function.

Reconstitution of the severe combined immune deficiency (SCID) mouse, deficient in both T- and B-cells, with naïve CD4$^+$ cells (CD45Rb^{high} cells) from normal mice, causes colitis in the recipients. However, synchronous reconstitution with memory CD4$^+$ cells (CD45Rb^{low} cells) prevents its development (Powrie et al. 1993). CD45Rb^{low} cells secrete TGFβ and IL-10 and comprise a regulatory T-cell subset (Powrie et al. 1996). This model illustrates that an unrestrained TH1 cell subset is normally regulated by a reciprocal TH2 subset.

Inbreeding selecting for disease modifying genes created the C3H/HeJ Bir mouse, 75% of whom develop a spontaneous patchy right sided colitis from 10-20 weeks of age (Sundberg et al. 1994). These mice do not respond to LPS and therefore may have reduced immune tolerance on subsequent challenge by the bowel flora (Sartor 2000).

Two germline models have been created that demonstrate the importance of an intact intestinal epithelium. Mice deficient in intestinal trefoil factor (Mashimo et al. 1996), a

protease resistant peptide that stabilises the mucus layer and promotes epithelial restitution (Playford 1997), develop a more severe colitis after DSS administration. Mice expressing dominant negative N-cadherin along the entire crypt-villus axis develop CD-like inflammation (Hermiston & Gordon 1995). Both these models provide evidence of the importance of epithelial integrity in preventing IBD.

Finally, colitis does not occur in germline models maintained in germ-free conditions (Elson et al. 1995) (Fedorak & Madsen 2000a), illustrating the role bacteria and the intestinal environment play in colitis.

Environmental models

Induced animal models rely on damaging the mucosal surface through the administration of chemicals, either by mouth or rectum. However, all resemble acute colonic injury rather than remitting / relapsing IBD. They include the administration of acetic acid (MacPherson & Pfeiffer 1978); TNBS / ethanol (Morris et al. 1989); immune-complex mediated colitis (Hodgson et al. 1978); indomethacin-induced colitis (Kent et al. 1969); DSS-induced colitis (Okayasu et al. 1990); carrageenan-induced colitis (Marcus & Watt 1969); and PG-PS enterocolitis (Sartor et al. 1985).

Although widely used, these models only provide clues to IBD aetiopathogenesis. For example, TNBS/ethanol bowel inflammation can be reproduced by subsequently applying TNBS/ethanol to the skin, indicating a role for gut-homing memory T-cells in this model.

The immune-complex model demonstrates that several factors may be required at the same time in order to precipitate colitis. In this model, dilute formalin damages the colonic epithelium, which allows injected human immune-complexes to enter the capillary bed and initiate chronic inflammation.

The non-selective cyclo-oxygenase inhibitor indomethacin produces small bowel ulceration in rats (but not mice) demonstrating the importance of prostanoids and leukotrienes in maintaining bowel integrity.

DSS causes a patchy colitis in mice that is equally severe in SCID mice (Dieleman et al. 1994) indicating that acute DSS-colitis is independent of T- and B-cell antigen

recognition. DSS-induced colitis does not persist and the mechanism of action is largely unknown (Ni et al. 1996).

Carrageenan causes colitis in guinea pigs by destroying intraepithelial tight junctions and allowing bacteria including *B. vulgatus* to translocate across the mucosa (Ling et al. 1988).

The PG-PS model produces a granulomatous colitis that requires a specified genetic background; demonstrates disease in the ileo-cecum and distal colon; causes extra-intestinal manifestations; and is caused by an immunologic response to components of the bowel flora. As such it is a reasonable model for CD but requires an experienced operator to perform the sub-serosal injection in the rat and is not a disease originating from within the bowel (Sartor et al. 1988).

The genetic background is important in determining disease phenotype after environmental colitis-induction. For example, colitis is more severe in Wistar compared to Fischer rats after acetic acid administration (Fabia et al. 1992); PG-PS causes a florid colitis in Sprague-Dawley rats, with extra-intestinal manifestations in Lewis rats but only minor effects in Buffalo & Fischer rats (McCall et al. 1994); and mouse strain modulates the degree of colitis after TNBS/ethanol (Elson et al. 1996) and DSS (Stevceva et al. 1999) (Mahler et al. 1998b). Disease susceptibility genes probably account for the absence of granulomas in murine models in contrast to their presence in the rat after TNBS/ethanol and PG-PS. Similarly, it is probable that non-primate species do not contain the gene complement necessary to cause a disease that mimics human IBD. Therefore animal models should be used to define possible disease mechanisms which can then be studied in man.

CHRONIC GRANULOMATOUS DISEASE

The link between CD and CGD

The bowel disease found in CGD is remarkably similar to CD (Ament & Ochs 1973a). In both diseases, a chronic colitis occurs, often separated by "skip" lesions of relatively normal mucosa. In addition, in both diseases ulceration extends to the submucosa and perianal disease is often present. It is possible therefore that a similar cellular abnormality occurs in both diseases.

Aetiology of CGD

In 1959, Sbarra & Karnovsky showed that the enhanced respiration of neutrophils was independent of mitochondria respiration (Sbarra & Karnovsky 1959). This implicated another metabolic pathway to account for the consumption of oxygen by these cells. About this time, the CGD phenotype was defined (Janeway et al. 1954). However, it was not until 1967 that a link was made between CGD and the enhanced oxygen consumption of neutrophils. In that year, the same group showed that neutrophils from patients with CGD were incapable of killing *S. aureus in vitro* (Quie et al. 1967) and demonstrated that neutrophil-specific oxygen consumption was absent in a patient with CGD (Holmes et al. 1967). This implicated the neutrophil respiratory burst as integral to host defence, which in 1973 Babior showed to be mediated by the reduction of oxygen to O_2^- (Babior et al. 1973). The molecular location for the enhanced phagocyte oxygen consumption was not defined until 1976, when Segal and Peters demonstrated that the oxidation defect in CGD localised to plasma-membrane-enriched fractions, providing physiological concentrations of NADH were used as the substrate (Segal & Peters 1976). This was confirmed two years later when Segal and Jones purified cytochrome b_{558} from human phagocyte vacuoles and suggested that it functioned to transport an electron from NADPH, generated by the hexose monophosphate shunt, to oxygen to generate superoxide (Segal & Jones 1978).

Components of the NADPH oxidase

The NADPH oxidase is now known to comprise six proteins, gp91^{phox}, p22^{phox}, p67^{phox}, p47^{phox}, p40^{phox} and Rac2; two haem groups; and flavine adenine dinucleotide (FAD). gp91^{phox} and p22^{phox} are membrane bound components, which together form the cytochrome b_{558} (also termed cytochrome b_{-245}).

gp91^{phox} is encoded by the gene *CYBB*, located on the X-chromosome at Xp21.1, and comprises a cytosolic hydrophilic C-terminal domain that binds FAD and NADPH; and a membrane bound hydrophobic glycosylated N-terminal domain that contains binding sites for two molecules of haem. p22^{phox}, encoded by the gene *CYBA* at 16q24, is very hydrophobic but contains a cytosolic proline rich tail that facilitates protein interactions with a src-homology 3 domain (SH3) domain of p47^{phox}. gp91^{phox} and p22^{phox} are stored in the specific granules and in the plasma membrane. Molecular defects in either protein result in the absence of both components, indicating instability of either protein alone (Segal 1987).

It was discovered that isolated membranes obtained from fractionated neutrophils regained oxidase activity on the addition of the cytosol component, providing amphiphiles such as sodium dodecyl sulfate (SDS) were present, termed the "cell-free system" (Bromberg & Pick 1984). This suggested that in addition to gp91^{phox} and p22^{phox} in the membrane, there existed protein components within the cytosol which were necessary for function of the NADPH oxidase. Subsequently, p47^{phox} was identified by analysing proteins that were not phosphorylated during the respiratory burst (Segal et al. 1985).

p47^{phox}, encoded by the gene *NCF1* at 7q11.23, comprises 390 amino acids (Segal et al. 1985), and contains an N-terminal PHOX domain, two SH3 domains that are important for protein folding by interacting with proline rich sequences; and a C-terminal serine rich domain involved in protein phosphorylation. It is expressed in phagocytes and in small amounts in lymphocytes (Rodaway et al. 1990).

p67^{phox} was discovered following the serendipitous production of p67^{phox} anti-sera during the search for neutrophil GTP-binding proteins (Volpp et al. 1988). It is encoded by *NCF2* at 1q25, and comprises 526 amino acids, contains two SH3 domains but in addition a binding site for the cytoplasmic protein Rac2. Within the cytosol it complexes with p47^{phox} and another cytosolic protein p40^{phox} (Wientjes et al. 1993).

Rac2, a member of the Ras superfamily of GTP-binding proteins, is an integral cytosolic component of the NADPH oxidase (Abo et al. 1991). Expressed predominantly in myeloid cells and active in the GTP-bound state, it interacts with p67^{phox} on stimulation of the oxidase (Diekmann et al. 1994). The activation of Rac2 is dependent on the disassociation of another protein, Rho-GDI (guanine nucleotide

disassociation inhibitor). Although Rac2 is required to reconstitute oxidase function in the cell-free system, interestingly there is only one report of human CGD resulting from Rac2 deficiency (D57N) (Williams et al. 2000). This mutation may exert some of its effects by interacting in a dominant negative fashion with Rac1, an ubiquitous GTP-binding protein (Gu et al. 2001). *Rac1* mutations are lethal and therefore it is possible that the other *Rac2* mutations have not been described because they interact more severely with Rac1.

Activation of the NADPH oxidase in the cell free system is minimally dependent on the presence of the cytochrome b_{558}, $p47^{phox}$, $p67^{phox}$ and Rac2. However, this system is artifactual and *in vivo* two additional proteins are involved, $p40^{phox}$ and Rap1A.

$p40^{phox}$ was identified following co-immunoprecipitation of $p67^{phox}$ with a 40 kDa protein (Wientjes et al. 1993). Since this protein co-precipitates with $p67^{phox}$, it is likely that these two proteins are complexed together in the cytoplasm, together with $p47^{phox}$. $p40^{phox}$ comprises 339 amino acids and contains an N-terminal PHOX domain, one SH3 domain and a C-terminal domain that binds to $p67^{phox}$. The importance of $p40^{phox}$ is uncertain however, since there are no reports of patients deficient in this protein. It binds to the cytoskeletal protein coronin and so may be involved in intracellular mobilisation during the respiratory burst (Grogan et al. 1997).

Rap1A, another small GTP-binding protein, is found in the membrane associated with the cytochrome. The function of this protein is unknown. NADPH oxidase activity increases when GTP-Rap1a is over expressed in the cell free system therefore this protein may function to modulate activity of the oxidase, possibly by controlling phosphorylation events, since Rap1A mutants that are unable to exchange GDP and GTP inhibit the oxidase (Maly et al. 1994).

$gp91^{phox}$ contains two haem groups and FAD (Segal et al. 1992). The haem groups bind to four histidines located within the cytochrome and receive electrons from FAD in a process that is regulated by both $p67^{phox}$ and Rac2 (Diebold & Bokoch 2001). The components of the activated NADPH oxidase are shown (Figure 4).
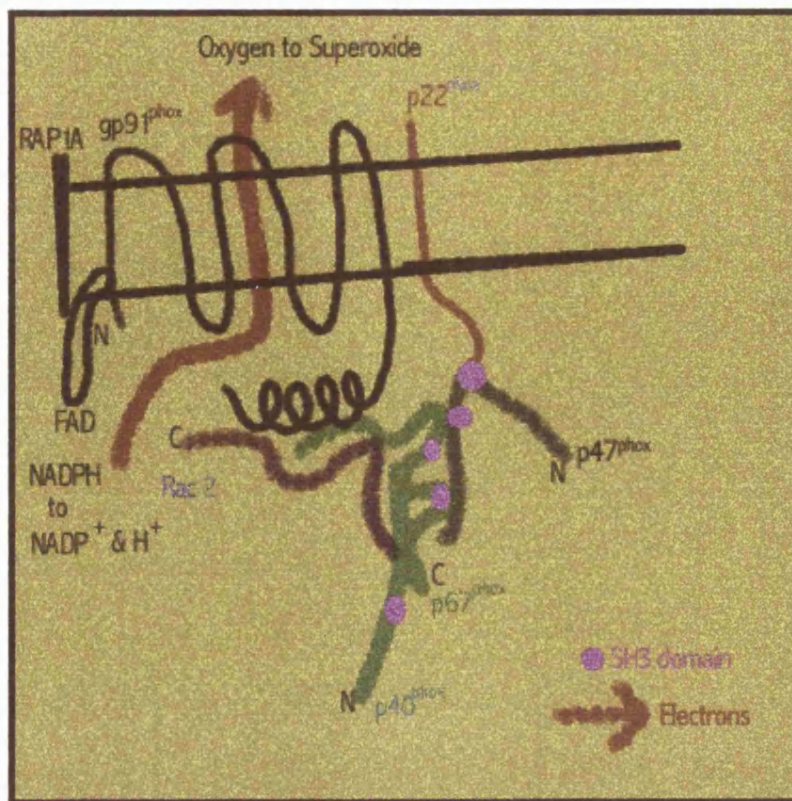
Figure 4: The activated NADPH oxidase proteins. During phagolysosomal formation, cytosolic p47phox, p67phox, p40phox and Rac2 translocate to the membrane and cause a conformational change in the cytosolic tail of gp91phox which allows the transfer of two electrons from NADPH to FAD, haem and then to oxygen to form O_2^-. Adapted from (Segal et al. 1999).

Activation of the NADPH oxidase

The intracellular signals that activate the oxidase are complicated and poorly understood. Several different pathways exist, depending on the receptor stimulated; for example the immunoglobulin receptors Fcγ-RII or Fcγ-RIII, the β2-integrin complement receptor, or the Toll-like receptors. On receptor stimulation, changes occur in the cytoskeleton to promote phagocytosis. This is associated with recruitment of the specific and azurophile granules to the phagosome, thereby incorporating cytochrome b_{558} into the phagolysosomal membrane. A cascade of intra-cellular signals results in phosphorylation of some of the nine serine residues at the C-terminal end of p47phox, the most important of which appears to be at position 379 (Faust et al. 1995). This causes the molecule to unbind the proline-rich C-terminal domain from its own N-terminal SH3 domain and translocate to the cytochrome, "dragging" into position p67phox and p40phox (Heyworth et al. 1991). Further phosphorylation events occur, facilitating binding of the N-terminal SH3 domain of p47phox to p22phox (Sumimoto et al. 1996) and separating a proline rich-C terminal sequence of p47phox (amino acids 323-

332) from p67^{phox} to allow it to bind to gp91^{phox} (De Leo et al. 1996). At the same time, Rho-GDI is released from cytosolic Rac2, which is phosphorylated to the GTP-bound state by a guanine exchange factor. Rac2 translocates to the membrane where it interacts with p67^{phox} (Prigmore et al. 1995). This causes a conformational change in the protein complex, thought to include deviation of the cytosolic tail of gp91^{phox} from FAD, allowing the transfer of two electrons from NADPH to FAD. The role of p40^{phox} and Rap1A in this process are not understood. Termination of the oxidase function is in part mediated by protein phosphatases, as shown by using the protein phosphatase inhibitor okadeic acid (Harbecke et al. 1997). An illustration of this process is shown (Figure 5).
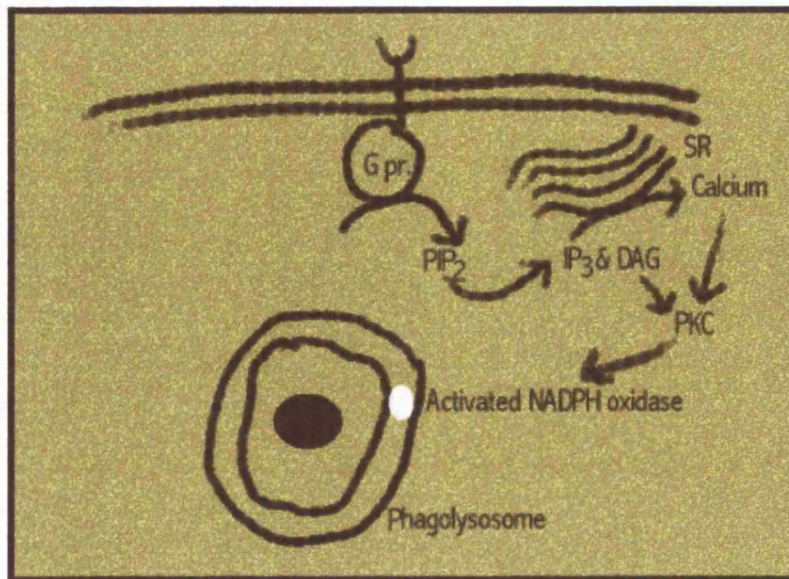
Figure 5: Activation of the respiratory burst. Opsonised or complement bound ligand binds to a G-protein coupled receptor and is internalised in a phagolysosome, causing release of inositol-triphosphate (IP₃) from phosphatidylinositol-diphosphate (PIP₂), which had been synthesized by phosphoinositide-3-kinase. IP₃ releases calcium from the sarcoplasmic reticulum. A cascade of protein interactions then occurs, principally involving activation of protein kinase-C by diacylglycerol (DAG) and calcium, resulting in activation of the NADPH oxidase. In addition, Ca++ independent pathways can be activated, e.g. phospholipase A₂ and phospholipase D, which release arachidonic acid, and phosphatidic acid and diacylglycerol, respectively, to then activate intracellular signalling cascades.

Function of the NADPH oxidase

The NADPH oxidase functions within the phagosome to transfer electrons from NADPH via FAD and haem to reduce oxygen to O_2^-. This then dismutates in the presence of protons to H_2O_2 ($2O_2^- + 4H^+ \rightarrow 2H_2O_2$). Meanwhile, "specific" and "azurophile" cell granules containing proteolytic enzymes will have fused with the phagolysosome. The mechanism for this process is not understood. Myeloperoxidase (MPO), an enzyme stored in the azurophil granules, can catalyse the formation of hypochlorus acid from chloride and H_2O_2 ($H_2O_2 + 2Cl^- \rightarrow 2HOCl$). HOCl will then react with bacterial nitrogen-containing groups to form chloramines, which results in microbial killing within the phagolysosome. At high concentrations, MPO can function as a catalase, converting H_2O_2 to water and oxygen. Therefore it may function in fact to reduce rather than promote phagosomal toxicity. In support of this, patients deficient in MPO do not have an increased susceptibility to infections (Stendahl et al. 1984) although under severe laboratory conditions MPO-deficient mice are more susceptible to *Candida* and *Pseudomonas* species (Aratani et al. 2000). Catalase or

glutathione peroxidase, present within the cell, can convert H_2O_2 to water; for this reason catalase producing microbes have been thought to be more virulent in CGD. Finally, H_2O_2 and O_2^- react in the presence of transition metal salts (Fe/Cu) to form $\cdot OH$, O_2 and OH^- in the Haber-Weiss reaction; and H_2O_2 and Fe^{2+} react to form Fe^{3+}, $\cdot OH$ and OH^- in Fenton's reaction (Edwards 1994) (Figure 6).
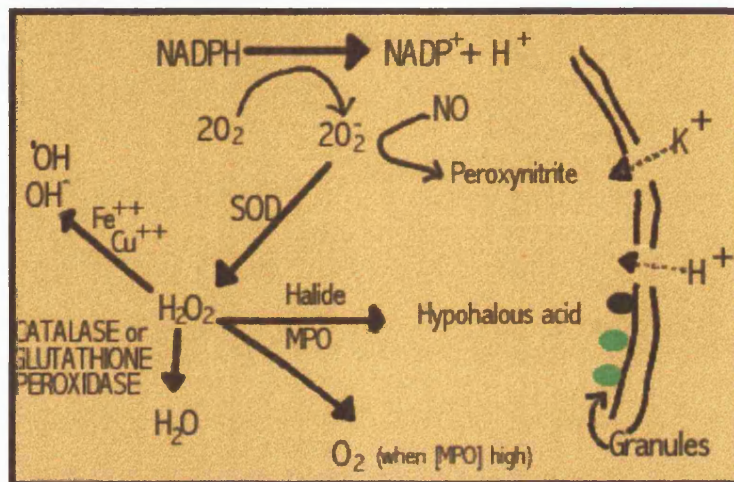
Figure 6: The fate of superoxide. An electron is transferred to oxygen, forming O_2^-, which is unstable and can be converted to peroxynitrite by the addition of nitrous oxide (NO); or which dismutates to H_2O_2 which itself is degraded to water by catalase, glutathione peroxidase or MPO, or reduced to the highly reactive hydroxyl radical ($\cdot OH$) in the presence of iron or copper. The electrostatic forces within the phagolysosome generated following electron transfer stimulate the influx of K^+, which displaces the ionic binding of proteases from the granule matrix. The increase in hydroxide ion concentration within the phagolysosome raises the pH to an optimal level for the granule proteases to function (Reeves et al. 2002).

However, things are not so simple. Radioactive iodide (which will mimic the reactions involving chloride) demonstrated iodination of neutrophil but not bacterial proteins following bacterial phagocytosis (Segal et al. 1983). This suggested that chloramines are not major microbicidal molecules.

So what is the role for H_2O_2 / O_2^- in the phagosome? Alone, they are not particularly toxic. However, $\cdot OH$ is very reactive, would damage bacteria in the phagosome and has been assumed to be the radical that is responsible for bacterial killing. Yet, there is no good evidence of the formation of significant quantities of $\cdot OH$ in the phagolysosome, despite the use of sensitive techniques such as electron paramagnetic resonance spectroscopy (Edwards 1994).

Recently, a novel role for phagosomal H_2O_2 / O_2^- has been reported by Segal and colleagues (Reeves et al. 2002) which explains the discrepancies surrounding the functioning of the oxidase. They showed that the large potential difference generated by electron influx into the phagosome is balanced initially by K^+ but not proton influx, thereby allowing the vacuole pH to rise due to the generation of OH^- from O_2^-. This accounts for the rise in lysosomal pH to 7.8 that occurs soon after phagocytosis (Segal et al. 1981), which will normalise the pH following fusion of the acidic granule contents producing an optimal pH for proteolytic enzyme activity. In addition, the ionic tension within the phagolysosome generated by the influx of K^+ was shown to release the granule proteases from the cationic granule scaffold to which they originally were bound by electrostatic forces. This suggests that the NADPH oxidase in fact functions to induce K+ influx into the phagosome, to raise phagosomal pH and to liberate granule proteases. Therefore, it may be the granule proteins and not OH^- that kills microorganisms.

Mutations causing CGD

CGD is caused by defects in the neutrophil NADPH oxidase complex of proteins, specifically in membrane bound p22^{phox} (5% of cases) and gp91^{phox} (65%); and cytosolic p67^{phox} (5%) and p47^{phox} (25%) (Thrasher et al. 1994). Mutations in *CYBB* (which encodes gp91^{phox}), *CYBA* (which encodes p22^{phox}) and *NCF2* (which encodes p67^{phox}) occur sporadically throughout each gene. Most result in complete absence of their protein, although mutations in 5% of X-linked patients and one mutation in p67^{phox} (Pro156Gln) result in the expression of non-functioning protein (Roos et al. 1993).

Mutations in *NCF1* (which encodes p47^{phox}) are both conserved and relatively common, in contrast to the other autosomal genes, *CYBA* and *NCF2*. In about 90% of cases of p47^{phox} deficiency, a GT deletion occurs at a dinucleotide repeat in exon 2, resulting in either an unstable or non-translated truncated protein of 51 amino acids. This mutation is believed to be common due to frequent aberrant homologous recombination events during meiosis between *NCF1* and a very similar "pseudogene" (Gorlach et al. 1997), which is also located at 7q11.23.

Clinical features of CGD

CGD is a rare genetic disease, with a prevalence of about 1/250,000 (Winkelstein et al. 2000). It presents with recurrent life threatening bacterial and fungal infections, and

with chronic inflammation, typically in the lung, liver, bone, cervical lymph nodes, skin and bowel. The natural history of the disease has been recently reported in 368 patients (Winkelstein et al. 2000), which suggests that X-linked patients have more severe disease. A small number of patients have partial oxidase activity (termed CGD variants) who generally have a less severe phenotype. The majority of patients are diagnosed in infancy and whereas twenty years ago death during childhood was common, the majority of patients now survive into adulthood, due to a better awareness of the disease and the prophylactic use of Septrin™ and Itraconazole™. Correspondingly, there is an increased prevalence of the chronic sequelae of the disease, particularly bowel inflammation (Figure 7). Chronic inflammation is probably stimulated by persistence of non-digested antigens within the cell; although may be in part due to reduced inactivation of pro-inflammatory mediators, which is normally mediated by reactive oxygen species (Clark & Klebanoff 1979).
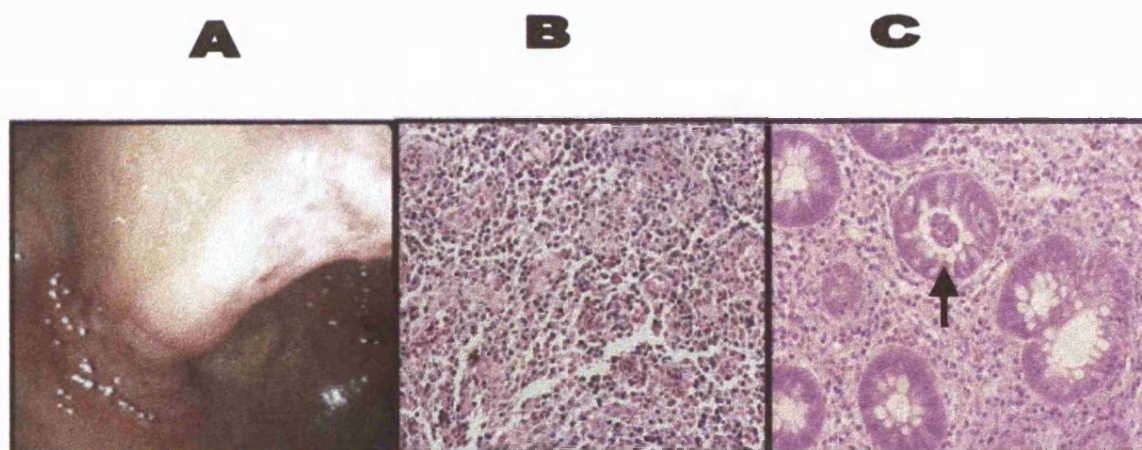
Figure 7: View of the sigmoid colon in a patient with X-linked CGD colitis. Discreet ulceration (A) is seen surrounding relatively normal mucosa. The ulcer contained granulation tissue (B) and an adjacent crypt abscess (arrow) associated with crypt distortion (C).

CGD enteritis

Gastrointestinal manifestations reported in the U.S. registry (Winkelstein et al. 2000) included perianal abscess formation in 13%, colitis in 17%, and granulomatous obstruction at the pylorus or cardia in 15%. The rates reported are likely to be under-estimates, since gastrointestinal complications will remain undiagnosed in some patients. Sub-clinical small intestinal malabsorbtion may occur in the majority of patients, based on a study in a small number of patients (Ament & Ochs 1973b). In addition, enteritis

will increase infection with gut-derived bacteria, for example *Salmonella, Klebsiella, Seratia,* and *Pseudomonas*.

The clinical aspects of CGD enteritis have been reported only in two small series, one of nine (Ament & Ochs 1973b) and the other seven (Schappi et al. 2001) patients. Macroscopically and histologically the appearance is most often compared to CD (Ament & Ochs 1973b; Yogman et al. 1974; Mitomi et al. 1999) although in one case report the features were comparable to UC (Sloan et al. 1996). Typically, the colon contains lightly pigmented lipid laden histiocytes; and in children, a paucity of neutrophils together with multiple degranulating eosinophils (Schappi et al. 2001). Interestingly, colon granulomas are rare in children, which may reflect a time-dependence to their formation.

The disease exhibits genetic and phenotypic heterogeneity, since only 20% of CGD patients develop enteritis and within those patients the spectrum of disease can be very variable. This suggests that between individuals there are disease modifying genes and disease modifying environmental factors, which may be important in disease initiation or disease phenotype. It is interesting therefore that in a study of 129 CGD patients, polymorphisms in two disease modifying genes (out of seven candidate host defence genes tested) were associated with an enteropathy. Polymorphisms in MPO were associated with perianal disease; and polymorphisms in Fcγ-receptor IIIb were associated with obstructive enteropathy (Foster et al. 1998). None were individually associated with colitis. However, combining the susceptible polymorphisms produced a strong association with all gastrointestinal manifestations (colitis; perianal disease; and obstructive enteropathy). It would be interesting to evaluate whether these polymorphisms are also susceptibility genes for CD.

Treatment of CGD enteritis

Reports of effective prophylactic and therapeutic modalities for CGD enteritis are limited due to the rarity of the disease. In a double blind, placebo controlled study involving 128 patients, recombinant human interferon-γ was an effective prophylactic agent, although the study was under-powered to determine enteritis prophylaxis (The International Chronic Granulomatous Disease Co-operative Study Group 1991). Recombinant human G-CSF administration has been associated with colitis remission in two brothers with CGD (Myrup et al. 1998) but in our experience ineffective in an

adult with p67^{phox}-deficient CGD, and in a child with X-linked CGD (J. Walker-Smith – personal communication). Non-myeloablative, matched, related bone marrow transplantation can successfully reduce gastrointestinal granulomas (Horwitz et al. 2001) but still carries a mortality of 30% (possibly higher in adults). Exogenous neutrophil transfusion has been used in CGD to treat life threatening infections. However, it is limited by cost, difficulty in provision and administration, potential transmission of blood born pathogens, and the risk of anti-HLA antibody development, both reducing efficacy and prejudicing future bone marrow engraftment. In our experience, neutrophil transfusion was an ineffective treatment for severe colitis in the p67^{phox}-deficient patient referred to earlier, despite targeted neutrophil administration via the superior and inferior mesenteric arteries. Proctocolectomy for CGD colitis can be successful (Werlin et al. 1982) but carries a high risk of major complications such as breakdown of surgical suture margins, especially surrounding an ileostomy (A.W. Segal FRS – personal communication).

Patients with gastrointestinal obstructive symptoms are usually treated with oral steroids and antibiotics, and with mesalazine derivatives for colitis (Segal et al. 2000). However, effectiveness of these treatments is unknown, since descriptions include only case-reports (Chin et al. 1987; Renner et al. 1991; Hiller et al. 1995; Ruiz-Contreras et al. 1998) and one small series (Schappi et al. 2001). In the latter, five of seven children responded to a mesalazine derivative. Immunosuppression with cyclosporine (Rosh et al. 1995) or azathioprine (Schappi et al. 2001) has been successful but imposes large risks on patients already immunocompromised. Antibodies to tumour necrosis factor alpha (TNFα) (Infliximab™), of proven value in chronic active CD (van-Dullemen et al. 1995; Targan et al. 1997; Present et al. 1999; D'Haens et al. 1999) will also pose a considerable risk to the CGD patient but should be evaluated in very severe enteritis in the absence of therapeutic alternatives.

Rationale for the production of a murine model of CGD enteritis

A knock-out mouse model was available in which the p47^{phox} sub-unit is defective. Phagocytes from this mouse do not produce O_2^- (Tkalcevic et al. 2000), therefore it represented a murine model for CGD. Although spontaneous colitis did not appear to occur in this mouse, it was hypothesised that environmentally-induced acute bowel inflammation would persist as a chronic colitis. If so, this would produce a model that could be used to investigate the colitis of both CGD and CD. In addition, the

generation of the model would enhance the understanding of the environmental factors that predispose to colitis; avoidance of these factors could prevent enteritis and liver abscess formation in some patients.

SUMMARY

The presence of a CD-like colitis in CGD suggests abnormalities in the innate immune response may also cause CD. The recent discovery of *NOD2* polymorphisms in CD (Hampe et al. 2001) (Hugot et al. 2001) (Ogura et al. 2001a) reinforces this since NOD2 is expressed in monocytes.

The discovery of gene mutations in CD will allow characterisation of patients by genotype. Not only will this provided enormous impetus to researchers interested in the causes of CD, also it will increase the sensitivity of future genetic and biological studies. As more of the jigsaw puzzle is completed, further associated genetic factors will be discovered.

However, determining the effect of the CD genotypes on protein structure and function still provides a huge challenge, in particular understanding the complex interactions between the bacterial flora and the mucosal immune system. Until such time as complete genetic profiles in CD patients are available, research must focus on likely aetiopathogenic factors in CD. It is most likely that these will include abnormalities in innate immunity, specifically in the macrophage and perhaps the neutrophil.

Currently, gene therapy for CGD is still in development and bone marrow transplantation is only possible in those patients that have an HLA-matched sibling. Consequently, there is a need for elucidation of the environmental factors that precipitate enteritis in about 20% of patients. Indeed, this is also true for CD. Modulation of these factors could reduce the incidence of CGD enteritis and CD.

MURINE CHRONIC GRANULOMATOUS DISEASE ENTERITIS

AIM

To develop a model of chronic enteritis in p47^{phox}-/- mice.

INTRODUCTION

The commonest autosomal recessive cause of CGD, affecting 25% of patients (Winkelstein et al. 2000), is caused by defects in the p47^{phox} subunit of the NADPH oxidase. A mouse model of CGD was created at University College London by disrupting this subunit, in order to increase the understanding of the biochemistry and cell biology of this disease. These mice provide the opportunity to investigate environmental factors that initiate enteritis in CGD.

The presence of spontaneous colitis was not addressed in the description of the original p47^{phox} -/- mouse (Jackson et al. 1995) and there was no evidence of spontaneous colitis in our p47^{phox}-/- mouse. In an attempt to create a model of CGD enteritis, it was speculated that chronic colitis, defined as significant histological differences present at least 4 weeks following gastrointestinal challenge, would persist in p47^{phox} -/- mice after mucosal ablation with DSS, due to an inability to digest invading bacteria completely. It was hypothesised that concomitant administration of a human faecal flora preparation would cause a more severe colitis.

In addition, it was decided to assess the effect of chronic NSAID administration in the p47^{phox}-/- mouse, since NSAID's are frequently taken by CGD patients, who may be more susceptible to their enteropathic effects. The deleterious effect of NSAID's on the human terminal ileum and colon is recognised (Aabakken & Osnes 1989), including the formation of ileal diaphragms (Lang et al. 1988). Oral administration of NSAID's to mice causes ileitis (Ettarh & Carr 1996) but NSAID-induced murine colitis has not been described.

METHODS

Compounds were purchased from Sigma Chemical Co. (Sigma Chemical Co., Poole, Dorset, UK) unless stated otherwise. Experiments on animals were carried out according to the regulations under the Home Office (UK) Animals (Scientific Procedures) Act 1986.

Generation of the CGD mouse

A p47^{phox} -/- mouse created by Dr. Jürgen Roes and Dr. David Power has been described (Harbord et al. 2002). In brief, the gene encoding p47^{phox} was isolated from a P1 mouse ES cell library (Strain 129Sv; Genome Systems, St Louis, MO) and partially characterized. A neomycin resistance gene was inserted into the third exon of the p47^{phox} gene as an Xho1/Sal1 fragment after conversion of a BspM1 site in exon 3 into a Sal1 site by linker ligation. A Pst1 site 5' of exon 3 was converted into Xho1 by linker ligation, to provide a unique restriction site for linearisation of the targeting vector before transfection. The *hsv-tk* gene was attached to the 3' end of the region of homology to permit negative selection of clones with random integration of the targeting vector. Gene targeting in mouse embryonic stem cells was performed as described (Roes & Rajewsky 1993). p47^{phox} -/- mice were established on the 129 background by mating transmitting chimeras with 129Sv females. Some of the mice were born following in-utero embryo transfer into specific pathogen free (SPF) surrogate females. Colonies of CGD and wild type mice were established, maintained in standard or SPF conditions in the animal facility of University College London. Mice were maintained in conventional housing facilities. All mice received water and standard mouse chow (SDS-3 expanded, Lillico, UK) ad libitum.

Induction of acute colitis with DSS

A dose ranging study administering for one week 1 %, 2 %, 3 % or 5 % DSS (40 kDa) (ICN Pharmaceuticals, Hampshire, U.K.), to mice maintained under standard conditions, established the sub-lethal dose that caused acute colitis effectively (LD$_{50}$ 3% DSS for one week). Thereafter, colitis was induced in cohorts of 6 to 10 p47^{phox}-/- mice and matched controls, by two weekly cycles of weekly oral administration of 1 %, 1.5 % or 2 % DSS. The solutions were changed every two days.

There is evidence that luminal helminths can down-regulate intestinal inflammatory responses by inducing TH2 immune responses. Therefore, to assess the effect of pin-

worm infection, 1 % or 2 % DSS was administered to SPF (pin worm free) and matched standard condition (pin worm infected) p47^{phox}-/- mice for one week (experiments SPF1 & SPF2)

Augmentation of DSS-induced inflammation by human enteric flora administration

Human enteric flora from the ascending colon was obtained at colonoscopy from 4 volunteers with normal mucosa on histological examination. The flora were combined, electrically homogenised on ice to reduce particulate matter, washed twice with water (4 °C, 2000xg, 20 min), resuspended in 50 % glycerol at an optical density (OD) at 600 nm of 18.25 and stored within 1 h of collection in aliquots at –20 °C. For the administration of fresh faecal flora, this was collected from a single donor each day, maintained on ice, homogenised, washed twice and re-suspended in water to an OD at 600 nm of 18.25. Subsequent histological examination of colonic biopsies from all donors demonstrated no abnormality.

p47^{phox} -/- and wild type mice were starved overnight 4, 7 and 10 days after the start of a cycle of DSS administration. After light halothane (Merial, Dublin, Ireland) sedation, 100 µl of freshly thawed or fresh human enteric flora were introduced into the stomach by gastric gavage then into the proximal colon using a 3.5 Fr. catheter (Vycon, France) lubricated with aqueous jelly, carefully inserted 4 cm into the colon. The mouse was held vertical by the tail for 1 min to allow the injected solution to bathe the proximal colon (experiments F1, F2, F4-6).

In one cohort of mice, freshly thawed human enteric flora were added to fresh drinking water every 2 days, at an OD at 600 nm of 0.30, four days after the start of a single cycle of 1.5% DSS administration, continued until the end of the study (35 days). The quantity of flora consumed is equivalent to a single daily oral gavage of 100 µl of flora with an OD of 18.25, assuming a mouse consumes 6 ml of water daily (experiment F3).

Control experiments comprised DSS administration with oral & colonic water gavage; two cycles of oral & colonic faecal flora gavage alone; or no challenge (experiments C1-C3). Mice were culled 5, 7 or 12 weeks following the start of the experiment.

Induction of enteritis with diclofenac sodium

Enteritis was induced by 7 weeks continuous oral administration of 64 µg/day or 128

µg/day soluble diclofenac sodium (Voltarol™, Novartis, UK) to p47^{phox} -/- and wild

type mice, equivalent to a standard daily human dose of 75 mg or 150 mg respectively.

The mice were culled at 7, 11 or 15 weeks (experiments V1-V5). A summary of all the

experiments is shown (Table I).

Table I: Experimental conditions to induce/augment inflammation in p47^{phox}-/- mice

Expt.	Experimental condition	Augmentation	Challenge to cull (Weeks)	Mean age (weeks +/- S.D.) at cull [n]	
				WT	p47^{phox}-/-
F1	1 cycle of 1.5% DSS	Stored feces	4	18.8+/-2.2 [6]	22.3+/-5.2 [6]
F2	1 cycle of 1.5% DSS	Fresh feces	4	24.0+/-0.0 [6]	27.0+/-3.3 [6]
F3	2 cycles of 1.5% DSS	Continuous feces	2	19.8+/-1.8 [8]	25.9+/-0.4 [7]
F4	2 cycles of 1.5% DSS	Stored feces	2	18.7+/-0.5 [6]	19+/-0.0 [4]
F5	2 cycles of 1.5% DSS	Stored feces	4	21.4+/-2.9 [9]	21.9+/-4.9 [7]
F6	2 cycles of 1.5% DSS	Stored feces	9	28.3+/-0.5 [10]	29.0+/-6.0 [7]
C1	-	-	-	30.0+/-0.0 [8]	30.0+/-0.0 [7]
C2	-	Stored feces	4	19.0+/-0.0 [5]	16.0+/-0.0 [4]
C3	2 cycles of 1.5% DSS	-	4	29.0+/-0.0 [4]	25.0+/-0.0 [6]
V1	Diclofenac 0.064 mg/d	-	0	41.9+/-0.6 9 [8]	42.5+/-1.6 [6]
V2	Diclofenac 0.064 mg/d	-	4	31.0+/-0.0 [8]	25.0+/-0.0 [7]
V3	Diclofenac 0.128 mg/d	-	0	20.0+/-0.0 [8]	19.0+/-0.0 [7]
V4	Diclofenac 0.128 mg/d	-	4	27.4+/-1.3 [10]	24.3+/-0.5 [6]
V5	Diclofenac 0.128 mg/d	-	8	34.5+/-3.4 [12]	29.0+/-0.0 [7]
				Standard	SPF
SPF1*	1 cycle of 1% DSS	-	4	33.4+/-17.0 [5]	27.3+/-9.8 [6]
SPF2*	1 cycle of 2% DSS	-	4	31.0+/-17.3 [4]	31.7+/-12.2 [6]

* p47^{phox} -/- mice in SPF & standard conditions compared, wild type mice not included
S.D., standard deviation

Monitoring of the mice

An observer unaware of mouse identity assessed the mice three times a week.

Weight change from baseline was recorded. Mouse appearance, rectal bleeding and

faecal consistency were scored based on a previous report (Cooper et al. 1993) as shown

(Table II). In order to control for the subjective scoring of appearance and stool

consistency, a significant difference was defined as 3 consecutive significantly different

readings.

Table II: Disease Activity Index

SCORE	Faecal consistency	Rectal bleeding	Appearance
0	Normal	None	Normal
1			Lack of grooming
2	Loose (adherent to cage)		Hunched, pilo-erection
3			Moribund
4	Diarrhea (adherent to anus)	Macroscopic blood	

Culling and examination of the mice

The mice were culled by CO_2 asphyxiation. Venous blood obtained following cardiac puncture was analysed for haemoglobin and leukocyte concentration on a Coulter Gen-S (Beckman Coulter, CA, USA). The colon was dissected free from its mesentery, opened longitudinally and faecal contents gently washed away with normal saline. After gentle blotting on filter paper, length and weight were recorded to determine thickness (Neurath et al. 1995), indicative of colonic inflammation. Colon, ileum, the major mesenteric lymph nodes, lung, heart, spleen, liver and kidney were fixed in 10% phosphate buffered formalin. 5 μm sections were stained with haematoxalin/eosin and viewed by light microscopy by a histopathologist unaware of the specimen identity. The scoring system used was based on a previous report (Dieleman et al. 1994) as shown (Table III). 8 SPF mice and 9 mice maintained under standard conditions were assessed for pinworm infestation by examining the faeces by zinc sulphate precipitation (Theinpont et al. 1979) and by histological review.

Table III: Histology score

SCORE	EXTENT	INFLAMN.	INFLAMN. LOCATION	NECROSIS	REGENERATION
0	None	None	None	None	None (normal)
1	Single focus	Mild	Mucosal	Mild	Re-epithelized
2	One segment	Moderate	Sub-mucosal	Moderate	Partial healing
3	More than one segment	Severe	Muscle or trans-mural	Severe	Persistant ulcer

Inflamn., inflammation;

Statistics

All values are expressed as mean ± standard deviation. Significant differences ($p < 0.05$) were determined using the unpaired, 1-tailed t test.

RESULTS

Generation of p47[phox] deficient mice

The CGD mouse to which I had access was created by gene targeting by Dr. Jürgen Roes. In brief, the p47[phox] gene was disrupted in mouse embryonic stem cells by insertion of a neomycin resistance gene into the third exon of the p47[phox] gene. Two targeted embryonic stem cell clones (9.3, K1) were obtained and used to generate chimeric mice. Chimeras transmitting the mutation were used to establish mutant mouse lines on the 129 genetic background (Figure 1). This mutation neither affected neutrophil development nor recruitment, but leads to complete inactivation of the neutrophil respiratory burst in response to stimulation with phorbol myristate acetate (PMA) or C. *albicans* as shown previously (Tkalcevic et al. 2000).

Figure 1: Targeted mutagenesis of the p47[phox] locus in mouse embryonic stem cells. A, the structure of the genomic locus (top), the targeting construct (middle), and the targeted locus after homologous recombination (bottom). Solid boxes represent exons, the open boxes indicate either the neomycin resistance gene, the HSV-Tk gene as indicated, or remaining plasmid vector sequences. The triangles represent loxP sites flanking this version of the neomycin gene. B = BamH1, C = Cla1, E = EcoR1, P = Pst1. B, southern blot analysis of genomic DNA samples digested with BamH1 and probed with a genomic fragment (probe A) derived from the p47[phox] locus. Targeted insertion of the neomycin resistance gene leads to an increase of the BamH1 restriction fragment of wild type (wt) clones by 1.5 kb, evident in clones 9.3 and K1.

Chronic colitis does not develop in p47^{phox} -/- mice after DSS

Spontaneous colonic inflammation was not found in p47^{phox} -/- or control mice (histological scores 0 ± 0 (n = 20; age 8.1 ± 3.2 months) and 0 ± 0 (n = 10; age 7.0 ± 1.7) respectively.

Rectal bleeding, diarrhoea, abnormal appearance and weight loss occurred during and in the week following DSS administration, equally in p47^{phox} -/- and control mice. Only 2 weeks following 8 cycles of alternate weekly DSS were histological appearances markedly more severe in the p47^{phox} -/- cohort (Table IV). There was a mild excess mortality in p47^{phox} -/- mice, which coincided with DSS administration and therefore can not be attributed to the development of a chronic colitis.

Table IV: Effect of oral DSS on mortality and colon histology.

Challenge	Genotype (n)	Challenge to cull (weeks)	Mortality	Histology score (0-15 +/- S.D.)	p
1 cycle of 1% DSS	p47 (2)	6	0	0+/-0	-
	WT (1)	6	0	3	
4 cycles of 1% DSS	p47 (3)	6	0	2.7+/-5.7	0.63
	WT (2)	6	0	6.0+/-1.4	
4 cycles of 1% DSS	p47 (8)	14	1	3.7+/-2.6	0.65
	WT (7)	14	0	3+/-3.1	
8 cycles of 1% DSS	p47 (6)	2	1	9.8+/-3.1	0.06
	WT (6)	2	1	4.4+/-4.4	
2 cycles of 1.5% DSS	p47 (6)	4	0	6.7+/-2.0	0.26
	WT (6)	4	2	5.8+/-2.2	
1 cycle of 2% DSS	p47 (4)	4	2	7.0+/-1.4	0.85
	WT (3)	4	0	7.3+/-2.1	
1 cycle of 2% DSS	p47 (4)	8	0	6.25+/-1.5	0.1
	WT (4)	8	0	3.5+/-2.4	
1 cycle of 2% DSS	p47 (6)	11	2	4.2+/-3.4	0.51
	WT (5)	11	0	2.6+/-3.6	
1 cycle of 2% DSS	p47 (5)	22	1	2.5+/-3.0	0.57
	WT (5)	22	0	4.0+/-3.6	

Luminal pin worm do not abrogate DSS inflammation in p47^{phox} -/- mice (experiments SPF1 and SPF2)

Gastrointestinal helminths (*Syphacia* species) were present in 7 of 9 p47^{phox} -/- mice maintained in standard conditions but 0 of 8 p47^{phox} -/- mice maintained in SPF conditions. Gastrointestinal helminths promote a TH2, immune tolerant, mucosal cytokine profile (Finkelman et al. 1997). Therefore it was hypothesised that DSS-induced inflammation would be more severe in SPF, pinworm free p47^{phox} -/- mice compared to pinworm infected mice maintained in standard conditions.

After DSS there was no difference between SPF and standard condition mouse phenotype (appearance, diarrhoea, rectal bleeding, weight change) or post-mortem parameters (anaemia, leukocytosis, histological appearance) except for reduced colon thickness in SPF mice (experiment SPF1 (SPF 29.4 ± 7.3 mg/cm, standard condition 39.3 ± 4.9 (p = 0.02); experiment SPF2 (SPF 38.9 ± 7.9 mg/cm, standard condition 51.7 ± 12.8 (p = 0.04)).

Intra-colonic human faecal flora increase DSS-induced inflammation in p47^{phox} -/- mice (experiments F1 to F6)

Faecal consistency and bleeding scores were more severe in p47^{phox} -/- cohorts in 5 of the 6 experiments performed. Appearance scores were more severe in the p47^{phox} -/- mice in all 6 experiments, significant in experiment 2 (p = 0.02), 3 (p = 0.02) and 6 (p = 0.04).

Although weight reduction occurred coincident with DSS administration, relative weight loss persisted in p47^{phox} -/- mice in the majority of experiments (Figure 2). Most excess deaths in p47^{phox} -/- mice occurred during or within one week of DSS administration, as a consequence of acute colitis.

Anaemia and leukocytosis occurred in experiments F3 and F4 (Figure 3). Colon thickness was increased in p47^{phox} -/- mice, but only in experiment F1 (p47^{phox} -/- 38.9 ± 7.0 mg/cm; wild type 29.6 ± 4.6 (p = 0.01)).

Figure 2: Percent weight change from baseline for p47phox -/- and wild type mice surviving for the study duration in experiments F1-F6 (*p < 0.05). ■ wild type; □ p47phox -/-; † death of wild type mouse; † death of p47phox -/- mouse.
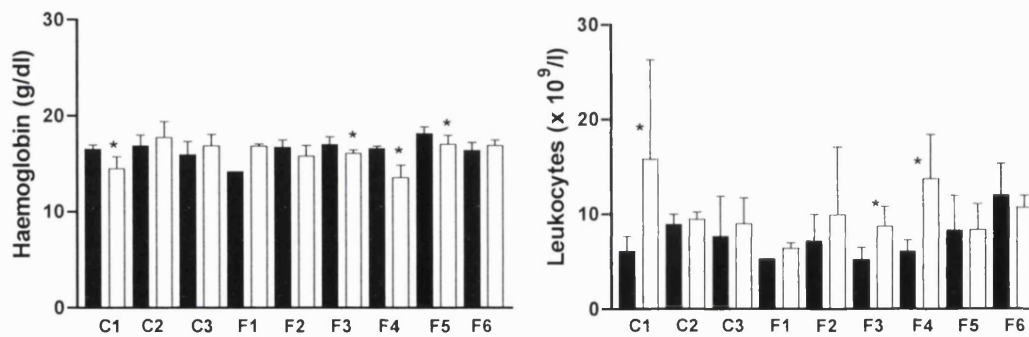
Figure 3: Haemoglobin and leukocyte count for p47phox -/- and wild type mice surviving for the study duration in experiments C1-C3 and F1-F6 (*p<0.05). ■ wild type; □ p47phox -/-.

Histological scores were more severe in $p47^{phox}$ -/- mice in all experiments, significant in experiment F2 (p = 0.04) and F4 (p = 0.03) (Figure 4). In all studies, the distal segment was affected more severely. There was no correlation between histological findings and other disease severity parameters.

Figure 4: Histology scores for p47phox -/- and wild type mice surviving for the study duration in experiments C1-C3 and F1-F6 (*p<0.05). Score was 0 in all mice in C1 and C2. ■ wild type; □ p47phox -/-.

The small bowel was normal in all the mice. Three $p47^{phox}$ -/- mice (experiments F4 & F5) had multiple liver abscesses with cholangitis; two wild type mice (experiments F6 & C3) had cholangitis, suggestive of portal pyaemia.

Faecal flora alone does not cause colitis (experiments C1 to C3)

Unchallenged $p47^{phox}$ -/- mice lose weight after 26 weeks compared to wild type mice (experiment C1), which may account for some of the later weight difference in

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experiment F6 and V5. At post-mortem, these mice had a relative anaemia and leukocytosis, which probably reflects their age (Figure 3). These mice did not develop colitis.

Challenge with faecal flora alone (experiment C2) did not cause colitis. The relative weight gain in p47^{phox} -/- mice is unexplained but in part may be due to their relative immaturity (Figure 5).

Challenge with DSS alone (experiment C3) produced similar changes in appearance, rectal bleeding and stool consistency to challenge with DSS & faecal flora but caused less severe changes in weight and histology (Figures 4 & 5).

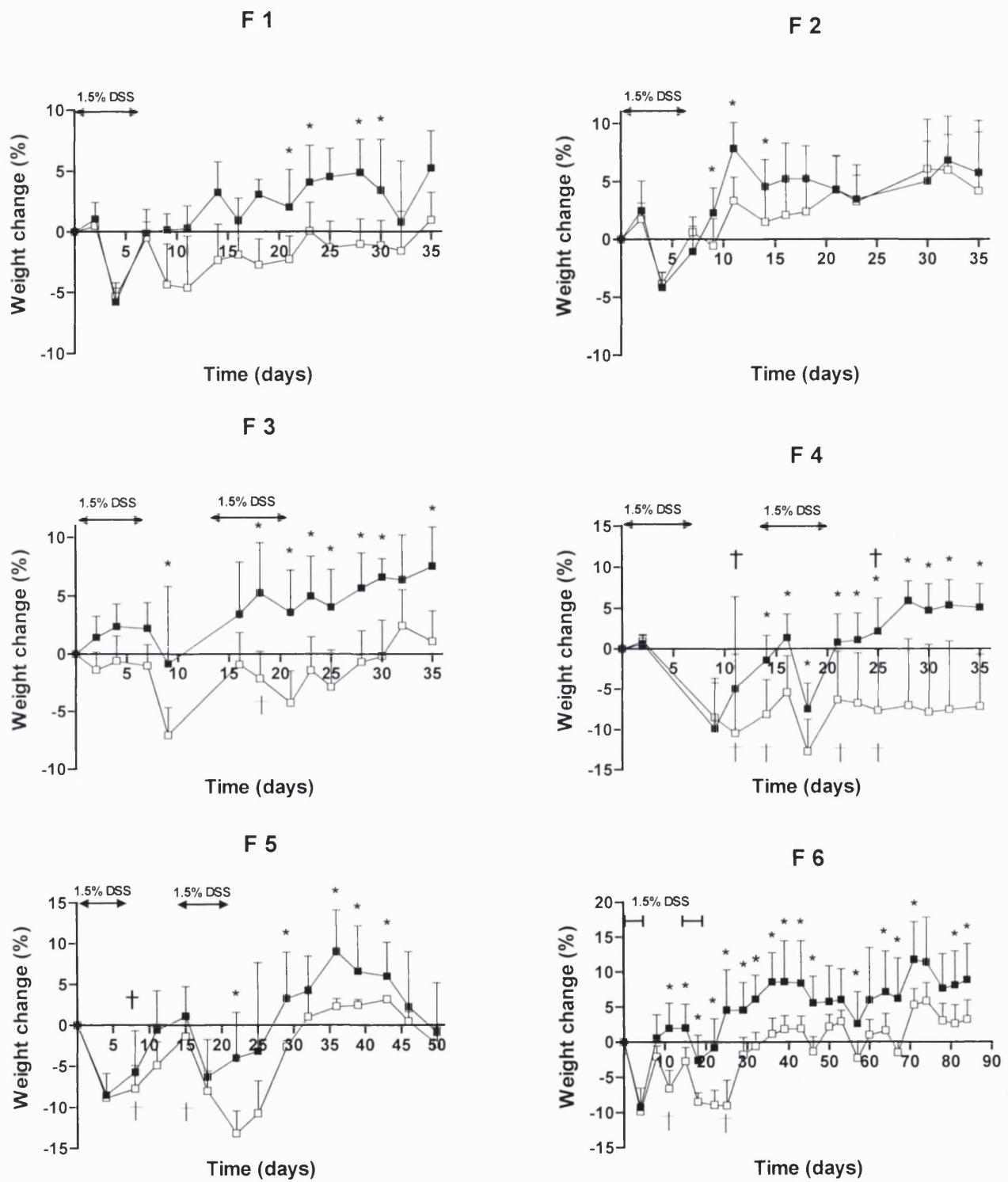Figure 5: Percent weight change from baseline for p47^{phox} -/- and wild type mice surviving for the study duration in experiments C1-C3 (*p < 0.05). ■ wild type; □ p47^{phox} -/-; † death of wild type mouse; † death of p47^{phox} -/- mouse.

Diclofenac induces chronic colitis in 129Sv mice

After 7 weeks administration of 64 µg/d diclofenac (experiment V1), moderate acute colitis was seen in the CGD mice (Figure 6), with persistence of moderate chronic colitis 4 weeks after administration had ceased (experiment V2). Significantly looser stools (days 31 - 38), leukocytosis and increased colon thickness (p47^{phox} -/- 47 ± 6.1 mg/cm; wild type 37.2 ± 5.5 (p = 0.001)) were present in p47^{phox} -/- mice in experiment V2.
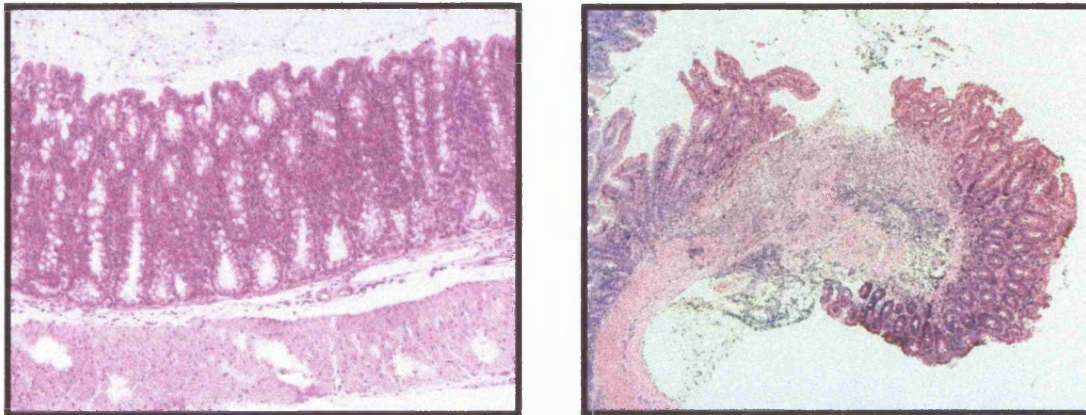
Figure 6: Left, normal colon mucosa from a wild type mouse and severely ulcerated mucosa with transmural chronic inflammation from a p47^{phox} mouse following 7 weeks diclofenac (64 µg/d).

7 weeks oral administration of 128 µg/d diclofenac (experiment V3) caused a severe colitis in both p47^{phox} -/- and wild type mice, with greater colon thickness in p47^{phox} -/- mice (50.8 ± 6.8 mg/cm; wild type 42.8 ± 7.7 (p = 0.03)).

There was excess mortality in p47^{phox} -/- mice administered 128 µg/d diclofenac for seven weeks and culled a further 4 or 8 weeks later (experiments V4 and V5), with 4 deaths out of 10 and 7 deaths out of 14 p47^{phox} -/- mice respectively, with no deaths in wild type mice. This probably occurred as a result of a severe diclofenac-induced colitis rather than intercurrent infection to which these mice are susceptible, since coincident mortality was absent in other p47^{phox} -/- mice maintained by us in the same animal facility. A significant leukocytosis (Figure 7) but only mild colitis (Figure 8) was seen in the surviving p47^{phox} -/- mice in these cohorts. In contrast to experiments F1-F6, relative weight loss in p47^{phox} -/- mice was not so apparent (Figure 9), and the inflammation was greater in the proximal colon.

Surprisingly, there was no ileitis. Gastritis in 2 wild type mice and transmural gastric inflammation in a p47^{phox} -/- mouse occurred in V2. Severe gastric ulceration was seen in 3 WT and 4 p47^{phox} -/- mice in V3, associated with a sub-phrenic abscess and a hepatic abscess respectively. Duodenal ulceration occurred in 3 p47^{phox} -/- mice in V3. In a minority of both p47^{phox} -/- and wild type mice in V4 and V5 there was evidence of healing of gastric inflammation but no active ulceration.
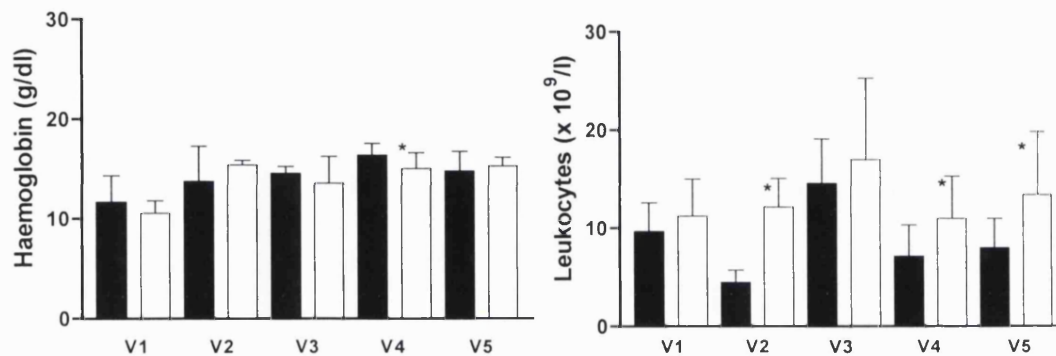
Figure 7: Haemoglobin and leukocyte count for p47^{phox} -/- and wild type mice surviving for the study duration in experiments V1-V5 (*p < 0.05). ■ wild type; ☐ p47^{phox} -/-.
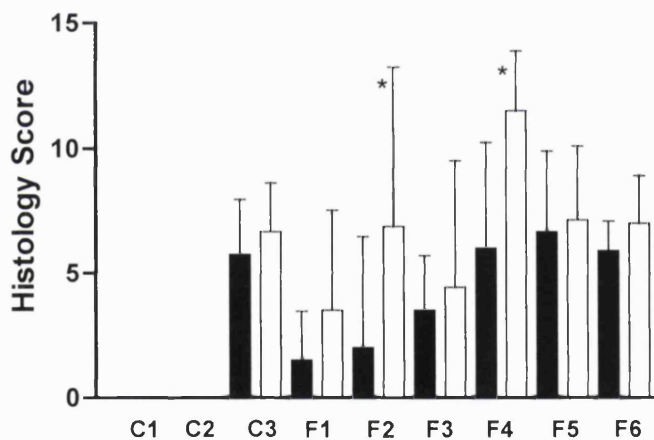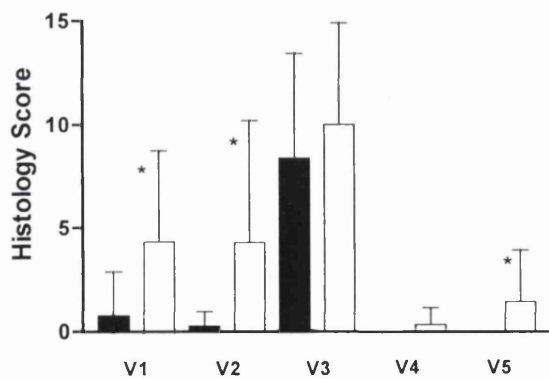
Figure 8: Histology scores for p47^{phox} -/- and wild type mice surviving for the study duration in experiments V1-V5 (*p<0.05). Score was 0 in wild type mice in V4 and V5. ■ wild type; ☐ p47^{phox} -/-.
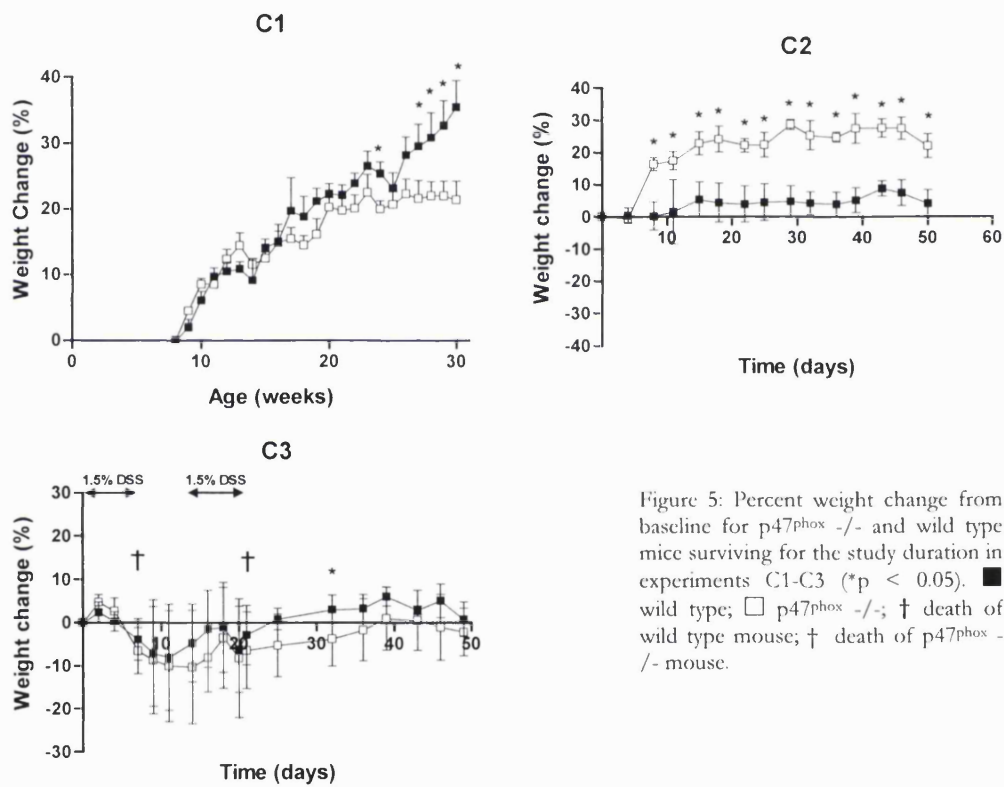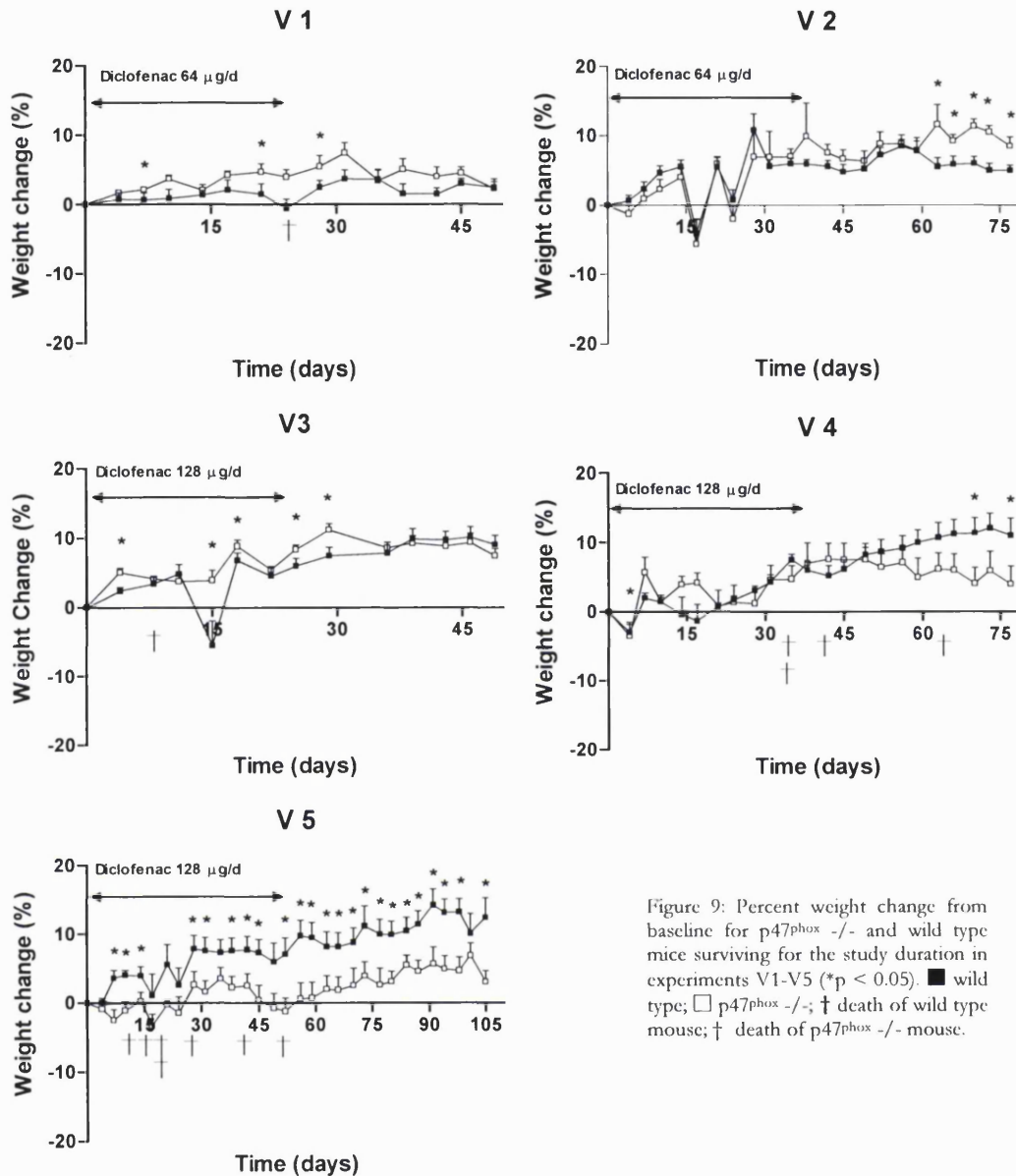
Figure 9: Percent weight change from baseline for p47phox -/- and wild type mice surviving for the study duration in experiments V1-V5 (*p < 0.05). ■ wild type; □ p47phox -/-; † death of wild type mouse; † death of p47phox -/- mouse.

Diclofenac was associated with extra-intestinal histological abnormalities, particular in p47^{phox} -/- mice (Table V). However, the prominent pulmonary abscesses seen in V2, V4 and V5 were probably due to infection in the p47^{phox} -/- colony, since identical appearances were seen in unchallenged p47^{phox} -/- mice (n=5; aged 27.6 ± 3.6 weeks), alive at the same time. In addition, copious pulmonary Ym1 crystals were seen in all p47^{phox} -/- mice, which are associated with inflammation in the p47^{phox} -/- mouse (Harbord et al. 2002). Splenomegaly was noted in unchallenged p47^{phox} -/- mice (experiment C1: p47^{phox} -/- 300 ± 200 mg; wild type 80 ± 10 (p = 0.03)) but this was not exaggerated after diclofenac administration.

Table V: Extra-intestinal abnormal histological appearances (frequency) after oral diclofenac

	WT	P47^{phox}-/-
V1	Mild pneumonitis (1)	Peri-nephritis (2) Ascending cholangitis (1)
V2	Mild pneumonitis (1) Pneumonia (1)	Pneumonitis and pulmonary abscesses (6) Pericarditis (1)
V3	Hepatic abscesses (3)	Hepatic abscesses (3) Pulmonary fungal abscesses (1)
V4	Pulmonary carcinoid (1)	Pulmonary fungal abscesses (6) Peri-splenitis (1)
V5	Granulomatous hepatitis (1) Pneumonitis (2) with hepatic abscess (1) Pericarditis (1)	Pulmonary fungal abscesses (7)

DISCUSSION

The development of transgenic and gene knockout animals has provided investigators with very important tools to study disease pathogenesis and evaluate treatment strategies. They are less artificial than the *in vitro* study of cells in culture, which often employ pharmacological inhibitors whose specificity of action is unknown. However, there are three major limitations to knockout and transgenic models. Firstly, alternative cellular pathways often compensate for loss or gain of function and so mask an effect. Secondly, knocking-out a gene is an extreme action that may create entirely artificial cellular responses. Thirdly, the effects observed apply to the species investigated and often do not translate to human pathophysiology.

Many rat and mouse genetic models of colitis have been created (Fedorak & Madsen 2000); however, the inflammation produced does not accurately resemble a human phenotype. Therefore, at best, these models provide only clues as to the causes of IBD. For this reason, it was hoped that the murine model of CGD would develop spontaneous colitis, which occurs in approximately 20% of CGD patients. This would be the first model genetically homologous to a human colitis and would be very useful in screening for potential therapies in CGD colitis and perhaps also in CD.

There are considerable difficulties in determining the severity of murine colonic inflammation. Macroscopic bleeding, visible in severe colitis, may not be seen in milder disease. An assay for occult bleeding (Hem-occult™, YSI (UK) Ltd., Hampshire, UK) was found to be too insensitive. Faecal consistency and mouse appearance, although widely used, are subjective and insensitive measures; and appearance, weight change, mortality and blood parameters will be affected by extra-intestinal infection and inflammation, which often occur in CGD mice. Therefore, histological assessment is probably the most sensitive measure, but subject to sampling error and difficulties in grading severity. In addition, variability in inflammation with DSS (Cooper et al. 1993) seen in both $p47^{phox}$ -/- and wild type cohorts means that large numbers of mice are required to achieve statistical significance.

The murine model of CGD did not develop chronic colitis after DSS-induced acute colitis. It was speculated that this was due to intra-luminal pinworm reducing the inflammatory response by inducing TH2 cytokine responses, as demonstrated previously in TNBS-induced colitis with intra-peritoneal *Schistosoma mansonii* and with

intra-luminal *Trichuris muris* oocytes (Elliott DE et al. 1999). However, pinworm-free SPF mice did not develop a more severe colitis. Although this suggests luminal pinworm do not modulate inflammation in the CGD colon, this is complicated by the difficulty of assessing subtle changes histologically; by the presence of alternative pathogens in the mice maintained under standard conditions; and may reflect the age of mice challenged with DSS, since TH2 responses predominate after oral challenge with self-peptide in neonatal but not adult *Syphacia* sp. infected mice (Agersborg et al. 2001).

Experimental mice are maintained in a stable environment, with little variation in food, pathogens, and stress, markedly different to the human condition. In order to try and establish chronic colitis, the diversity of antigens and bacteria present within the CGD murine colon were increased by instilling human faeces into the stomach and proximal colon. A similar approach has been used to produce colitis in non-tolerant, germ-free mice (Fukushima et al. 1999). Histology scores were more severe in all p47^{phox} -/- cohorts and in general correlated with excessive mortality and relative weight loss. However, the more severe p47^{phox} -/- colitis present following faecal and DSS challenge did not persist beyond four weeks. Thus instillation of foreign bacteria at the time of DSS challenge causes a self-limiting but not a chronic colitis.

Animal models of colitis have demonstrated that enteric bacteria are intrinsic to the inflammatory process. The faecal flora were not maintained under anaerobic conditions during sample preparation, therefore, the more severe colitis after administration of fresh faeces (F2) compared to stored faeces (F1) may be due to increased instillation of viable, toxic anaerobes. It is possible that anaerobic faecal challenge would have resulted in a more severe colitis in p47^{phox} -/- mice, analogous to *Bacteroides* species in the HLA-B27 transgenic rat (Rath et al. 1996; Rath et al. 1999).

Prolonged oral administration of diclofenac (64 μg/d) caused a more severe, persistent colitis in p47^{phox} -/- mice. The effect of challenge with double this dose was masked by excess mortality in the p47^{phox} -/- mice, possibly resulting in only those with mild histological abnormalities surviving. In all the experiments there was a wide variability in colon histology scores following gastrointestinal challenge, sometimes with very severe changes and virtually no changes present in littermates. Therefore, if the death of a mouse is equated with severe colitis, the histological scores in experiments V4 and V5 would be significantly higher in p47^{phox} -/- mice. Despite this, eight weeks

following diclofenac administration, the colitis was still statistically significantly worse in the p47^{phox} -/- mice. The relative protection from a relatively more severe colitis in p47^{phox} -/- seen in V3 may in part be due to the young age of the cohort studied.

The finding of enhanced susceptibility to colitis following diclofenac administration in CGD mice is important for three reasons. Firstly, NSAID-induced murine colitis has not been described previously. Secondly, CGD patients may be especially susceptible to NSAID's precipitating acute and perhaps even chronic colitis. Thirdly, NSAID's increase gut permeability and therefore may increase the penetration of organisms into the mucosa and therefore the frequency of enteric-derived infections in CGD patients.

The p47^{phox} -/- mice did not appear to be protected from gastric lesions following NSAID challenge. This contrasts with the report of reduced stomach and small bowel damage in gp91^{phox} -/- (CGD) mice, compared to controls, three hours after intra-peritoneal indomethacin administration (Beck et al. 2000). However, the studies differ in design, in particular in the use of prophylactic antibiotics, mouse strain, NSAID type and route of administration, and most importantly in the timing of analysis. The differences can be explained by reduced neutrophil protease function in CGD mice (Reeves et al. 2002), which will lessen the very early inflammatory response but will ensure chronicity through a reduced ability to digest residual antigenic matter. Of note, there was no comment on the effect of indomethacin on the colon in gp91^{phox} -/- mice (Beck et al. 2000).

Why then do CGD mice not develop relapsing colitis analogous to the human condition? Genetic polymorphisms in MPO and Fcγ receptor IIIb increase colitis susceptibility in CGD patients (Foster et al. 1998). It is possible that the CGD mouse lacks the requisite genes or genetic polymorphisms necessary for the propagation of inflammation. The degree of inflammation after DSS depends on the strain of mouse (Mahler et al. 1998) and it maybe that different strains of p47^{phox} -/- mice, e.g. C3H/HeJ, would contain a more appropriate genotype to be susceptible to chronic colitis.

In summary, it has been demonstrated that murine CGD colitis may be dependent on the composition of the bowel flora. More importantly, the severity of colitis induced

by diclofenac in the p47^{phox} -/- mouse, at a dose equivalent to human use, means that the safety of NSAID's in human CGD patients should be re-assessed.

YM1 PROTEIN

AIM

To identify and characterise crystals found in p47^{phox} -/- murine lungs.

INTRODUCTION

When the tissues of the p47^{phox} -/- mouse were examined microscopically, crystals were noted in the lungs, most noticeable in the older animals. Such crystals had not been described in the report of the original p47^{phox} -/- mouse in 1995, created by disrupting exon 7 of the p47^{phox} gene (Jackson et al. 1995). It was decided to investigate this phenomenon and to identify the crystals.

Unidentified pulmonary crystalline deposits have been described in other transgenic, immune deficient mice (Zhu et al. 1999) and in certain strains of immune competent mice, particularly on a 129Sv or C57BL/6 background (Murray & Luz 1990), especially during experimental chronic pulmonary infection (Huffnagle et al. 1998). These were previously thought to be Charcot-Leyden-like crystals but it now seems likely that they are composed of Ym1 protein.

During the course of this work, Ym1, a member of a family of mammalian proteins that share sequences with lower organism chitinases, was described as forming crystals in the lungs of three immunodeficient mice: the moth-eaten mouse, the CD40L-deficient mouse and a transgenic mouse with lung specific human tumour necrosis factor receptor expression (Guo et al. 2000).

Ym1 mRNA is expressed in the spleen and lung and mRNA from a highly homologous gene, *Ym2* in the thymus, stomach and kidney (Jin et al. 1998). The distribution of Ym1 protein within the cells of these and other tissues has not been determined although it has been assumed to be a macrophage protein since it was first discovered in a peritoneal macrophage cDNA library (Chang et al. 2001). Recently, enhanced macrophage Ym1 gene expression, mediated by a homologue of macrophage migration inhibitory factor, has been found associated with TH2 cytokine inducing helminth infection (Falcone et al. 2001).

METHODS

Purification of lung crystals

Lungs from 12-month-old p47^{phox} -/- mice were placed in iced water, divided into small pieces with a sharp blade, gently Dounce homogenised, incubated at 37 °C for 90 min with collagenase, (1 mg/ml in water; 5 mg/lung), DNAse (30 μg/ml in water) and protease inhibitors (1 μg/ml leupeptin; 1 μg/ml pepstatin A; 1 μg/ml tosyl-lysine chloro-methyl ketone; 0.5 mM phenylmethylsulfonyl fluoride). The digest was layered on top of Lymphoprep (Nycomed Pharma, Oslo, Norway) and centrifuged (1000xg, 4 °C, 20 min). The pellet containing the crystals was washed in water and centrifuged (100,000xg, 4 °C, 30 min) 3 times.

Although the crystalline structures were stable in water or 50% ethanol and fragmented by sonication or cycles of freezing, they were very soluble under alkaline conditions. This allowed a novel method to be developed which enabled purification to homogeneity. Enriched crystals were dissolved in 10 mM CHES pH 9.5 (250 μl/lung) for 1 h and then 100 mM MES (pH 6) added to the supernatant for 18 h (1:3 v/v; 4 °C, resultant pH 6.06) to re-crystallise the protein. This was washed 3 times in water and further purified by re-crystallisation. Protein size and purity was assessed by staining with Coomassie Brilliant Blue (BDH Laboratory Supplies, Poole, England) after 10% SDS polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) and yield determined by the Bio-Rad protein assay (Bio-Rad Labs, Munchen) (Bradford 1976).

To determine the effect of pH on Ym1 solubilisation, 50 μg of purified, crystalline Ym1 (25 μg/μl in water) was added to 98 μl of buffer at pH 1 to pH 10. Buffer solutions contained 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM NaCl. For pH 1.25 & 2, they comprised 0.2 M KCl / 0.2 M HCl; for pH 2.5, 3, 4, 5 & 6, 50 mM citric acid / 100 mM Na$_2$HPO$_4$; for pH 7, 7.5, 8, 9 & 10, Tris 50 mM / HCl. After 15 min incubation at 4 °C, the remaining crystalline protein was removed by centrifugation (20,000xg, 4 °C, 10 min). Ym1 in solution was quantified by analysis of the supernatant by 10% SDS-PAGE using image analysis software (Scion Corporation) and compared to the complete solubilisation of an equivalent amount of purified Ym1 by standard sample buffer (62.5 mM TRIS-HCl pH 6.8, 2% w/v 20% SDS, 2.5% w/v 50% glycerol, 2.5 mM DTT, bromophenol blue). In a separate experiment, an equivalent amount of Ym1 was incubated at 37 °C for 2 h in 50 mM TRIS-HCl buffer (pH 7.0-8.5, in 0.1

increments) and supernatant protein concentration determined by the Bio-Rad protein assay.

Mass spectrometric identification of the purified protein as Ym1

After purification of the crystalline protein, 4 protein bands were identified by SDS-PAGE. A small part of the centre of each band was cut from the gel, destained, dried and digested with modified trypsin (Promega Corp., WI, USA) (Hellman et al. 1995). The digest was analysed by matrix assisted laser desorbtion ionisation (MALDI) mass spectroscopy using a Bruker Biflex III mass spectrometer (Bruker UK LTD). The identity of the protein was determined using the programmes Mascot <www.matrixscience.com>, PeptIdent <www.expasy.ch> and MS-FIT http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm to search the protein databases.

Direct sequencing of the purified protein

Purified protein was separated by 10% SDS-PAGE containing 2 mM mercaptoacetic acid, soaked in transfer buffer (25 mM TRIS; 190 mM glycine; 10% methanol; 0.00375% SDS) and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon PSQ, Millipore Corp., MA (Matsudaira 1987). The membrane was stained with Coomassie Brilliant Blue (BDH) and the dominant protein band at 43 kDa excised, destained, washed and dried. The bands were commercially sequenced by automated Edmann degradation using an ABI Procise protein sequencer (Applied Biosystems, Foster City, CA, USA) (Edman & Begg 1967).

Production of affinity purified polyclonal antibody

Two New Zealand white rabbits were immunised with 1 mg purified crystalline Ym1 in 1 ml phosphate buffered saline (PBS) (8.1 g NaCl; 0.74 g KCl; 1.56 g NaH_2PO_4; in 1 l water (pH 7.4)) emulsified in Complete Freund's Adjuvant and Incomplete Freund's Adjuvant (Freund 1956) at 4 and 6 weeks. Serum was harvested at 8 weeks. 4 mg of purified Ym1 was conjugated to a HiTrap affinity column (Amersham Pharmacia Biotech, UK). 85% of the Ym1 protein that had passed through the column remained bound. 5 ml of rabbit sera was passed through the column. Affinity purified antibodies were eluted with 100 mM glycine (pH 2.5) and with fresh 100 mM triethylamine (pH 11.5) (Harlow & Lane 1988). Protein concentration was assessed as before.

Light microscopy

Formalin fixed tissues for light microscopy were paraffin imbedded and 5 μm sections stained with haematoxalin/eosin or Wright/Giemsa.

Immunohistochemistry

Miss Phillipa Musden kindly performed this study. 3 μm sections from paraffin-embedded tissue were dried overnight at 60 °C, dewaxed for 10 min in alcohol with 0.5% H_2O_2 in methanol and rinsed thoroughly in water. They were digested for 10 min at 37 °C by a 0.1% solution of chymotrypsin containing 0.1% calcium chloride (pH 7.8), rinsed in water, then in 0.05% Tween 20 in TRIS-buffered saline (TBS) (11.69 g/l NaCl; 6.06 g/l Tris-HCl (pH 7.5)). Endogenous biotin activity was blocked (Avidin/Biotin Blocking Kit, Vector, California, USA), the sections incubated for 10 min in 1/10 normal swine serum (Dako LTD, Ely, UK), then incubated for 60 min with the primary antibody (1/3000 anti-Ym1 or rabbit polyclonal anti-murine thyroglobulin as a non-specific control). They were developed using a biotinylated 1/200 swine anti-rabbit secondary antibody (Dako) and a streptavidin-biotin-peroxidase complex (Dako) and visualised with DAB (Kem-en-Tec, Copenhagen, Denmark).

Electron microscopy

Dr. Giorgio Landon kindly performed this study. Briefly, lung samples for electron microscopy were retrieved from formalin-fixed paraffin embedded $p47^{phox}$ -/- tissue using a combined toluene/osmium retrieval method. Silver to gold ultra-thin sections were cut on a Reichart-Jung Ultracut II (Leica Microsystems UK Ltd.) and double-stained with Reynold's lead citrate and uranyl acetate. Sections were examined and photographed on a Jeol 1200 EX transmission electron microscope (Jeol UK Ltd.).

Immunoblotting

To determine the time course of the accumulation of Ym1 and its tissue distribution, organs from two $p47^{phox}$ -/- and two wild type mice aged 3 weeks, 6 weeks, 5 months and 10 months were homogenised in a tenfold volume (v/w) of ice-cold buffer (50 mM TRIS pH 6.8, containing protease inhibitors). Bone marrow was obtained by flushing the central cavity of each femur with 400 μl buffer. Gastrointestinal tract contents, removed without any mucosal disturbance, were centrifuged (15,000xg, 4 °C, 10 min) and the supernatant collected. The protein was quantified by 10% SDS-PAGE. 2 μg of protein was resolved by 10% SDS-PAGE and the Ym1 content quantified by

western blotting (Towbin et al. 1979), by comparing to pure Ym1 standards (50 ng; 10 ng; 1 ng). Both the dominant band (43 kDa) and degradation bands were quantified. In detail, nitro-cellulose membrane (BDH) and filter paper (Whatman, Maidstone, UK) were soaked in Towbin transfer buffer (14.4 g/l glycine; 0.374 g/l SDS; 25 ml/l 1 M TRIS-HCl (pH 8.0); 200ml/l methanol). The gel and underlying membrane were "sandwiched" free of air bubbles between three sheets of filter paper and the proteins electrically transferred (0.8 mA/cm^2 for 1 h) (Bio-Rad, USA). The membrane was stained with 0.2% Ponceau S for 10 min and rinsed several times over 2 min with PBS to visualise and annotate transferred proteins. Then it was incubated for 90 min at 25 °C with TBS containing Tween 0.05%, 3% milk protein (Marvel, Premier Brands UK, Lincs., UK) and 0.02% sodium azide, to block non-specific antibody binding, before incubating for 18 h at 4 °C or 1 h at 25 °C with affinity purified anti-Ym1 in an air-free sealed bag (1 µg/ml in TBS / Tween 0.05% containing 3% marvel and 0.02% sodium azide; 10 ml/15 cm^2 membrane). It was then washed three times for 10 min with at least 400 ml TBS / Tween 0.05% and incubated for 1 h at 25 °C with the secondary antibody in an air-free sealed bag (anti-rabbit IgG conjugated to horse radish peroxidase (Amersham Life Science, UK) for qualitative evaluation or ^{125}I-labelled anti-rabbit Ig (Amersham Pharmacia Biotech, England) for quantitative evaluation (1/1000 in TBS / Tween & 1% marvel; 10 ml/15 cm^2 membrane). It was washed as before then visualised using ECL western blotting detection reagents (Amersham Pharmacia Biotech, UK) as per the manufacturers' instructions and exposed to photographic film (Ilford) for qualitative evaluation or detected using the FLA3000 PhosphorImager (FujiFilm, Japan) for quantitative evaluation.

Pulmonary crystal modulation

It was hypothesised that amantadine, a weak base used to treat Parkinsons disease and influenza, would raise the pH of the neutrophil phagolysosome which is excessively acidic in CGD cells (Segal et al. 1981) and therefore reduce crystallisation of Ym1. p47^{phox} -/- mice received amantadine (0.07 mg/g mouse weight, which is 10% of the LD$_{50}$ in humans) in the drinking water from birth and were culled aged 3 and 6 weeks (two in each group). A further two p47^{phox} -/- mice aged 10 months were provided oral amantadine for 50 days. Animal tissues were extracted and prepared as described and Ym1 concentrations compared with untreated litter mates.

It was hypothesised that particulate matter within p47^{phox} -/- lungs might predispose to Ym1 crystal formation. Consequently, pulmonary Ym1 concentration in two mice aged 4 months that had always been maintained in filtered air cages, which filter the air to 0.3 μm, was compared with that in two mice maintained in standard cages and with two mice maintained in standard cages that had received the oral antibiotic Batril™ since weaning.

Cellular location of Ym1

0.8 ml of 3% thioglycolate was injected into the peritoneal cavity of 3-month-old wild type mice. Macrophages were retrieved 5 days later by peritoneal lavage with ice cold PBS containing heparin (5 u/ml) (Leo Laboratories, Bucks, UK), 0.5% bovine serum albumin (BSA) and 0.01% sodium azide. Neutrophils were similarly obtained 18 h after thioglycolate administration. To purify neutrophils from bone marrow, cells were collected by flushing the central cavity of femurs of 5 mice administered thioglycolate five days previously, with 400 μl of buffer as above. The cells were filtered through 60 μM gauze (Millipore Corp., Bedford, MA), and stained in the dark for 15 min at 4 °C with GR1- fluorescein isothyocyanate (FITC) (Pharmingen) specific for neutrophils (Lagasse & Weissman 1996) or F4/80-FITC (Serotec) specific for macrophages (Austyn & Gordon 1981) (1 x 10^6 cells/20 μl). They were then washed, recounted and resuspended in FACS buffer (0.1 g sodium azide, 0.19 g EDTA, 10 ml fetal calf serum, to a final volume of 500 ml with PBS) (1 x 10^7 cells/ml). At least 1 x 10^6 cells/ml were unstained as a control. Neutrophils and macrophages were sorted at 4 °C using an EpicsElite cell sorter, orifice size 75 μm (Coulter, UK; Expo 300 software, Applied Cyto Systems, UK). T cells were obtained by gently grinding the thymus between the roughened glass of 2 microscope slides and the cells washed free with buffer. They were confirmed as greater than 98% pure T-cells by morphology. Purified 129sv murine B cells in sample buffer were kindly provided by Dr. Balthazar Cazac. 20 μg cellular protein (equivalent to 25,000 neutrophils) was analysed by immunoblotting for Ym1 and neutrophil elastase (Tkalcevic et al. 2000) and for Ym1 by immunocytochemistry of cytospin smears (Cytospin 2, Shadon, UK).

Subcellular fractionation of wild type murine neutrophils

2.5 x 10^7 thioglycolate induced peritoneal neutrophils were obtained from 8 wild type mice. 2 μl of di-isopropyl fluorophosphate (1 M in ethanol) was added to the cell

pellet, followed by 2.5 ml of break buffer (10 mM PIPES pH 7.1; 100 mM KCl; 3 mM NaCl; 3.5 mM MgCl$_2$, protease inhibitors). The cells were sonicated (Soniprep 150, Sanyo, Japan) on ice (3 cycles of 5 s), centrifuged (1000xg, 4 °C, 10 min), the post-nuclear supernatant layered onto a 10 ml continuous sucrose gradient (15% w/w to 55% w/w in break buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) pH 7.4; 5 u/ml heparin; protease inhibitors) and centrifuged in a Beckman TLS55 head (Beckman, CA, USA) (150,000xg; 4 °C, 1 h). The gradient was collected in 40 fractions by aspiration from the upper surface. Sucrose concentration was determined by refractometry. Fractions were analysed for murine Ym1, lactoferrin (kindly donated by Dr S.H. Li of The Institute of Biological Chemistry, Taipei, Taiwan), B2 microglobulin (Santa Cruz Biotech. Inc.), and cathepsin G (Santa Cruz) by immunoblotting. Fractions were also analysed for MPO enzymatic activity (Methods Enzymol. 1986). Briefly, 10 µl aliquots were added to 525 µl substrate (0.05% Triton X 100; 0.003% H$_2$O$_2$; 0.32 mM 0-dianisidine; in 0.1 M citrate buffer pH 5.5). After 5 min, the reaction was stopped by the addition of 35% perchlorate / 20% DMSO in water. Absorbance was read at 560 nm in a Uvikon 860 spectrophotometer. The results were compared to known quantities of horse-radish peroxidase. The fractions were also analysed for lactate dehydrogenase (LDH) (Methods Enzymol. 1986). Briefly, 50 µl aliquots were diluted with 450 µl of water and added to fresh substrate solution (0.2 ml PBS (pH 7.4); 0.1 ml 5 mM sodium pyruvate; 0.1 ml 1 mM NADH; 0.1 ml 1% Triton X-100) in a quartz cuvette and the decrease in absorbance at 340 nm was measured. Values were obtained over two minutes and the maximal rate of reduction calculated.

Confocal microscopic examination of wild type murine neutrophils

Dr. Guillermo Lopez kindly helped with this study. Thioglycolate induced peritoneal neutrophils were suspended in PBS (7 x 10^5/ml). 500 µl aliquots were placed onto cover slips & allowed to adhere over several minutes. The cover slips were washed three times in PBS, fixed with fresh 3% paraformaldehyde / 0.1% gluteraldehyde for 60 min, washed and permeabilised with 0.5% Triton X-100 in PBS over 15 min. Four washes in PBS, two 15 min incubations with fresh sodium borohydride (1 mg/ml) and a further four washes in PBS were performed. The cells were incubated for 18 h at 4 °C with 5% goat serum, then for 48 h at 4 °C with polyclonal anti-elastase or polyclonal anti-Ym1 (both 1:50 in 5% goat serum); or for double labelling experiments with 1:100 polyclonal anti-Ym1 antibody (rabbit anti-mouse) and 1:25 anti-cathepsin G (goat anti-

mouse) (Santa Cruz); or anti-Yml and 1:25 anti-B-2-microglobulin (goat anti-mouse) (Santa Cruz), in 0.5% BSA in PBS, followed by a 1 h incubation at room temperature. After 6 washes, the cells were incubated with fluorescein labelled goat anti-rabbit Fc-IgG antibody (Pierce, Chester, UK) (1:200 in 5% goat serum in PBS); and for double-labelling experiments, also with rhodamine-labelled donkey anti-goat Fc-IgG antibody (Pierce). A further 6 washes in PBS and two washes in water were performed. The dried slides were sealed in Mowiol mount (Calbiochem, Nottingham, UK) and visualised in a DMRBE confocal microscope (Leica, Wetzlar, Germany), using Leica TCS NT software, version 1.6.587.

Immunogold electron microscopy of peritoneal neutrophils

Prof. Giorgio Gabella kindly performed this study. Wild type thioglycolate induced peritoneal neutrophils, fixed in fresh 2% paraformaldehyde containing 0.5% glutaraldehyde in PBS, were dehydrated through a graded series of alcohol, followed by 2 changes of propylene oxide and embedded with araldite CY212 resin. Silver to gold ultra-thin sections were cut with a diamond knife, collected onto 200-mesh carbon-coated nickel grids, incubated with 1% periodic acid for 10 min, washed with distilled water, incubated with 50 mM glycine for 5 min, washed in PBS and incubated for 5 min in PBS containing 0.5% BSA, 0.1% Triton X100, 0.1% lysine and 0.1% sodium azide. After overnight incubation in 1:2000 anti-Yml, the cells were washed with PBS and incubated for 1 h with 1:100 nanogold goat anti rabbit Fab' conjugate (Nanoprobes NY, USA; particle size 1.5 nm) in PBS containing 2% normal goat serum. After washing for 30 min in PBS the sections were post-fixed with 1% glutaraldehyde for 10 min and washed for a further 15 min. The detection of gold particles was enhanced with HQsilver (Nanoprobes). Equal parts of initiator and moderator were mixed before adding activator and mixing thoroughly. The mixture was put on ice under safe lights and the sections were floated for between 2 and 10 min, followed by washing for 15 min. The sections were stained with uranyl acetate and lead citrate before viewing in a transmission electron microscope operating at 80 kV.

Yml functional studies

It was hypothesised that Yml might function as a non-specific opsonin. Thioglycolate induced, peritoneal neutrophils from 2 wild type mice aged 6 weeks were harvested in RPMI (Gibco) containing BSA (0.1 mg/ml). They were shown to be greater than 99% viable by trypan blue exclusion. 1×10^7 *C. albicans*, grown overnight

in culture, were incubated with 20 mM mouse sera protein, 20 mM crystalline Yml or with RPMI for 90 min at 37 °C, then washed three times, resuspended in RPMI at 4°C containing BSA and recounted. Subsequently, 1 x 10^6 *C. albicans* were incubated with 5 x 10^6 neutrophils. Killing was assessed with aliquots taken at 0, 30, 90 min and 4 h and 24 h for duplicate colony counts on LB-agar following 10 min incubation in water at 4 °C to lyse the neutrophils. Phagocytosis was assessed with aliquots taken at 0, 10, 30, 90 min and 4 hours for Gram stain. Briefly, the cells are smeared on a microscope slide that is allowed to air dry, then fixed by passing quickly through a flame. The slide is then covered with 1% crystal violet for 2 min, washed with water, covered with Lugol's iodine for two min, and washed with water during which time concentrated acetone is allowed briefly to wash over the slide. The slide is then counter-stained with safranin for two min; washed with water and carefully dried on tissue paper. Counts were made of the number of *C. albicans* (coloured blue) internalised per 250 sequentially viewed neutrophils (coloured red). In separate duplicate experiments, 4 x 10^3 *C. albicans* were grown in the presence or absence of 1 mg/ml crystalline Yml at pH 3.0 or pH 7.6 and aliquots taken at 0, 8 and 24 h for colony counts; and 10^5 *C albicans* were incubated with 2 x 10^6 murine neutrophils at pH 7.4 in the presence or absence of 18 μg anti-Yml in a final volume of 1 ml and aliquots taken at 0, 60 and 120 min for colony count estimation.

Since the amino-acid sequence of Yml is homologous to microbial chitinase, it was hypothesised that Yml would bind chitin and therefore perhaps act as a lectin. 30 μg pure, soluble Yml protein or 30 μg soluble *S. marcesens* chitinase was added to 10 mg ground, washed, crab shell chitin; chitin beads; or 2 x 10^7 *C. albicans* in RPMI, containing 0.1 mg/ml BSA in a final volume of 100 μl, adjusted to pH 7.5 or 8.0 or to pH 1, 2 or 3 with buffers as detailed before. This was incubated at 100 rpm for 30 min at 37 °C. After centrifugation (13000xg, 10 min, 4 °C), 10% of the supernatant and washed pellet was resolved separately by 12.5% SDS-PAGE. Enzyme samples without substrate were included as controls. If no binding occurred, the proportion of Yml in the supernatant in control and experimental samples would be similar.

Enzyme Kinetics of Yml

Purified Yml crystals (18 mg/ml in water) were solubilised by adding 11 volumes of 10 mM CHES pH 9.5. 3 volumes of acidic buffer (pH 2.5 - 5.5) were added to the

supernatant.   Under these conditions Ym1 remained in solution, providing the concentration of Ym1 was < 400 μg/ml.   50 μg aliquots of soluble Ym1 or buffer alone were incubated at 37 °C in a white, flat based 96 well plate (Costar) with 110 μM of 4-methyl-umbelliferyl-N acetyl BD glucoseaminide (MU-[GlcNAc]$_1$), 4-methyl-umbelliferyl diacetylchitobioside (MU-[GlcNAc]$_2$) or 4-methyl-umbelliferyl triacetylchitotrioside (MU-[GlcNAc]$_3$) (dissolved respectively in DMSO; DMF; and DMSO) at a final pH 2.5 to 5.5, in 0.5 pH unit increments.   These substrates are widely used to assess n-acetyl glucoseaminidase, chitobiase and chitinase activity respectively. Net fluorescence (emission 445 nm; excitation 365 nm) was measured over 80 min (Cytofluor multi-well plate reader, Perceptive Biosystems, MA, USA).   4 methyl-umbelliferone (MU) released was determined against standard curves at each pH of MU in DMF.   In the original method (McCreath & Gooday 1992) an alkaline stop buffer was added to enhance the measured fluorescence from MU.   However, equivalent results were obtained for enzymatic activity with or without the addition of alkaline stop buffer since fluorescence from MU is stable at acidic pH (McCreath & Gooday 1992). This allowed readings to be taken over time.   Kinetic properties for the cleavage of MU-[GlcNAc]$_1$ were determined by creating a Lineweaver-Burke plot of rate measurements of the reaction between 50 μg Ym1 and 0, 11, 22, 44, 66, 88 and 110 μM of substrate.   All assays were performed in triplicate using 2 preparations of Ym1. Concurrent experiments were performed using Chitinase from *S. marcescens* as a positive control.   Pepsin (final pH 2.5 - 5.5 in increments of 0.5 units) and trypsin (final pH 4 & 8) were combined with Ym1 for one experiment and fluorescence was calculated to assess a synergistic effect.

Subcutaneous injection of crystalline and soluble Ym1

Pure Ym1 protein was solubilised in 10 mM CHES, sterilised by passage through a 0.22 μM filter (Millipore, Bedford, MA), returned to pH 7.4 in crystalline form by the addition of RPMI or returned to pH 7.4 in solution by keeping the Ym1 concentration < 400 μg/ml.   2 μg or 20 μg soluble Ym1 was injected subcutaneously into two p47[phox] -/- and two wild type mice.   20 μg albumin (pH 7.4) was injected as a control.   The injection sites were excised after 1 and 5 h and assessed histologically.   20 μg crystalline Ym1 (in 73 μl PBS) was injected subcutaneously into four p47[phox] -/- and four wild type mice.   20 μg uric acid (pH 7.4) was injected as a control.   The injection sites were excised after 10 days and assessed histologically.   In 2 mouse pairs, Ym1 was also

injected 1 day and 5 h before excision.   Sterility of the injected material was confirmed before and after the procedure by aerobic culture and purity confirmed by SDS-PAGE.

RESULTS

Crystal location

Spontaneous pulmonary crystal formation was noted in 25 of 33 p47[phox] -/- mice at a mean age of 5.5 ± 2.6 months (mean ± standard deviation) but not in the lungs of 33 wild type mice, mean age 5.6 ± 1.9 months. In the CGD mice, the crystals were first seen at 2 months of age and increased in density until by 6 months of age up to 60% of the lung contained crystals. The correlation coefficient (r^2) for estimated crystal density against age in the 25 mice was 0.39. The distribution was patchy, heaviest in basal and peripheral regions.

The crystals were mainly extracellular, multifaceted, approximately 10 μm to 100 μm in length. They were often associated with giant cells and macrophages (Figure 2, overleaf). In addition, they were frequently noted in chronically inflamed bile ducts of p47[phox] -/- mice following oral DSS, and in spontaneous skin abscesses. The crystals were found in 2 wild type mice; in each case they were in severely inflamed bile ducts following treatment with DSS where colitis had been produced.

Crystal purification

Following digestion of lung tissue with collagenase and DNase, the crystals were further purified by centrifugation. It was discovered that the crystals disappeared from the enriched crystal preparation at extremes of pH but were insoluble near their iso-electric point (the pI of Ym1 is 5.7) (Figure 1).
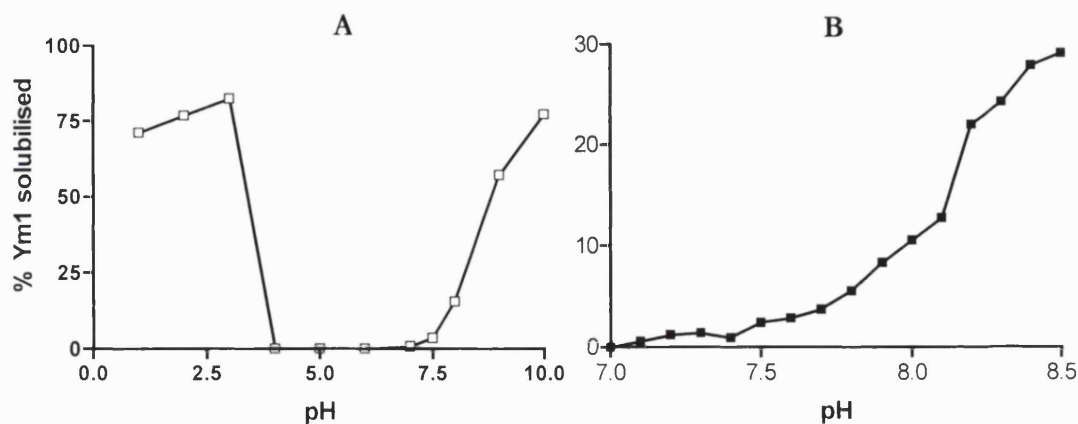
Figure 1: Ym1 is insoluble near neutral pH. Purified crystalline Ym1 solubilisation after 15 min incubation at pH 1 – 10 (A) or 2 h incubation at pH 7.0-8.5 (B).

A   C   E

B   D   F
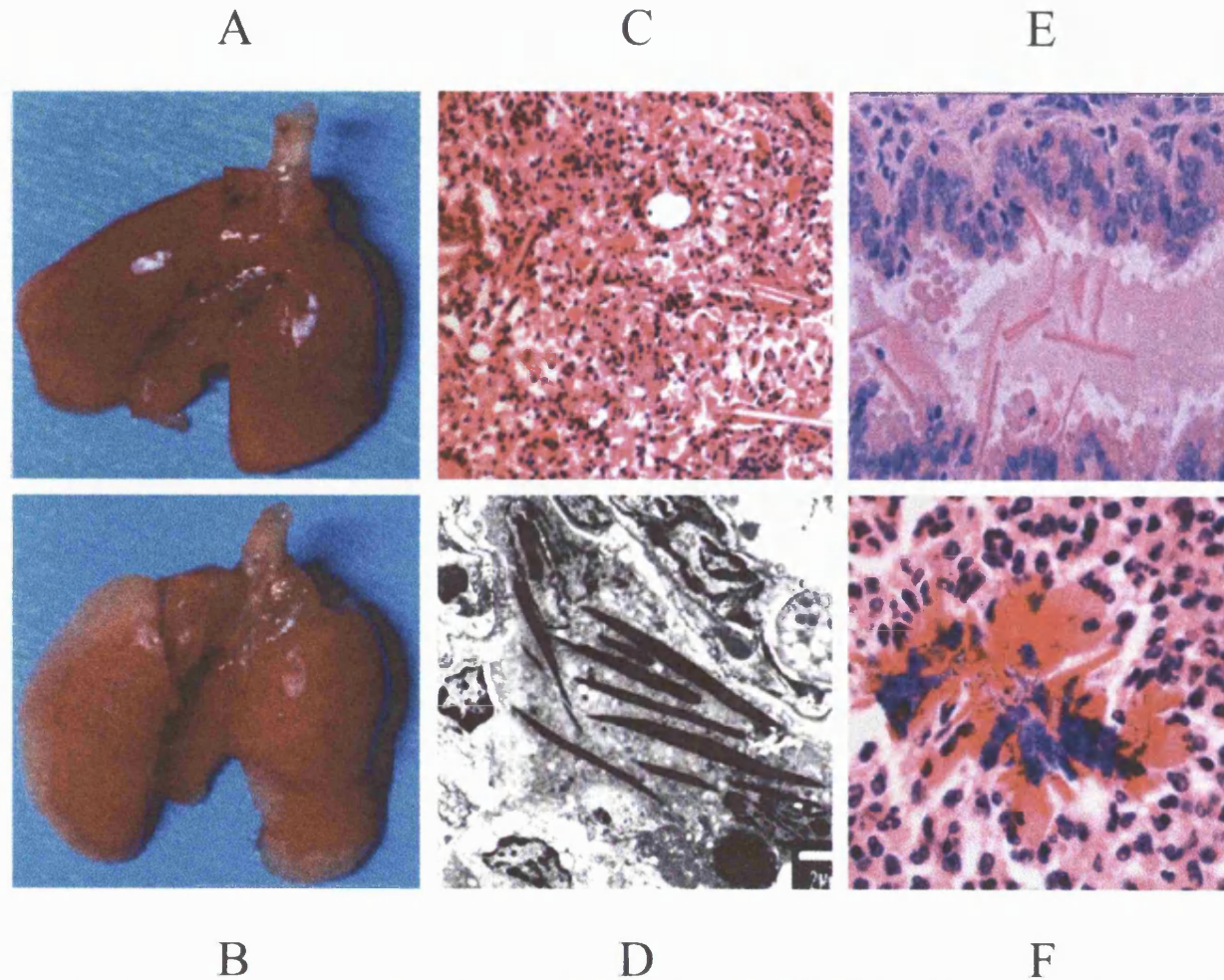
Figure 2.   Ym1 crystal location in the CGD mouse.   Dorsal view of lungs from 10-month-old (A) wild-type and (B) p47^{phox} -/- mice.   The pale areas at the periphery of (B) are sites of consolidation with a chronic inflammatory cell infiltrate composed predominantly of macrophages surrounding crystals (C).   Electron micrograph of the p47^{phox} -/- mouse lung shows intra-cytoplasmic, needle shaped crystals within alveolar macrophages (D).   A 6-month-old p47^{phox} -/- mouse with chronic liver and skin abscesses had crystals within the bile ducts (E) and adjacent to bacteria in the skin (F).

This allowed the purification of approximately 2.5 mg of protein from each aged p47^{phox} -/- mouse by repeated solubilisation and re-crystallisation (Figure 3).
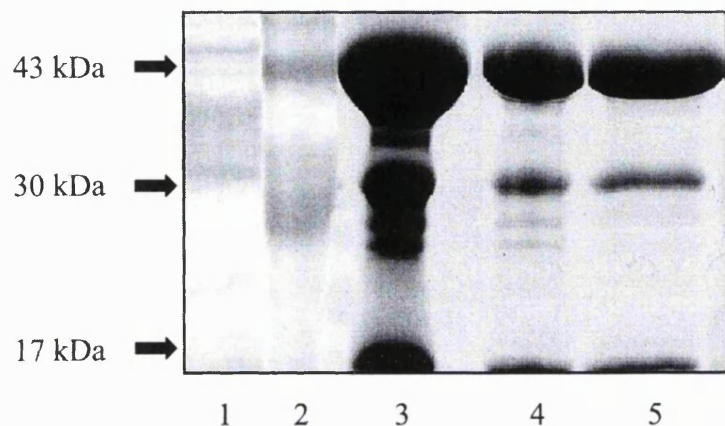
Figure 3: 10% SDS-PAGE separation of Ym1 protein at different stages of purification. Wild-type lung homogenate (lane 1); p47^{phox} -/- lung homogenate (lane 2), after alkaline solubilisation (lane 3), re-crystallisation (lane 4); and re-alkalisation (lane 5). The 4 bands (at 43; 31; 29 and 14 kDa) in lane 5 were Ym1 or Ym1 breakdown products.

Mass spectroscopic and direct sequence identification of the purified protein as Ym1

All four protein bands obtained by the purification process were analysed by tryptic digestion and mass fingerprinting. This demonstrated convincingly that the major band was mouse secretory protein Ym1; and that the other three bands were Ym1 degradation products. For the major band at 43 kDa, the Mascot probability based Mowse score was 105 (p <<< 0.05) with 10/13 peptide matches (Figure 4, overleaf). The identity of Ym1 was confirmed using PeptIdent and MS-Fit, programmes that use different algorithms to search the protein databases. Direct analysis of the tryptic digest of the purified crystals also produced a significant Mowse score of 72 with 8/13 peptide matches.

In order to confirm this finding, gel purified protein was sequenced by Edmann degradation and the first six amino acids were found to agree with the N-terminal of Ym1 after secretary leader sequence cleavage (YQLM?[1]Y). These findings were independent and predated the report by Guo et al (Guo et al. 2000), which has identified pulmonary crystals in other mice strains as Ym1.

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[1] The fifth residue could not be identified. This commonly occurs when the residue is cysteine, as found in Ym1.
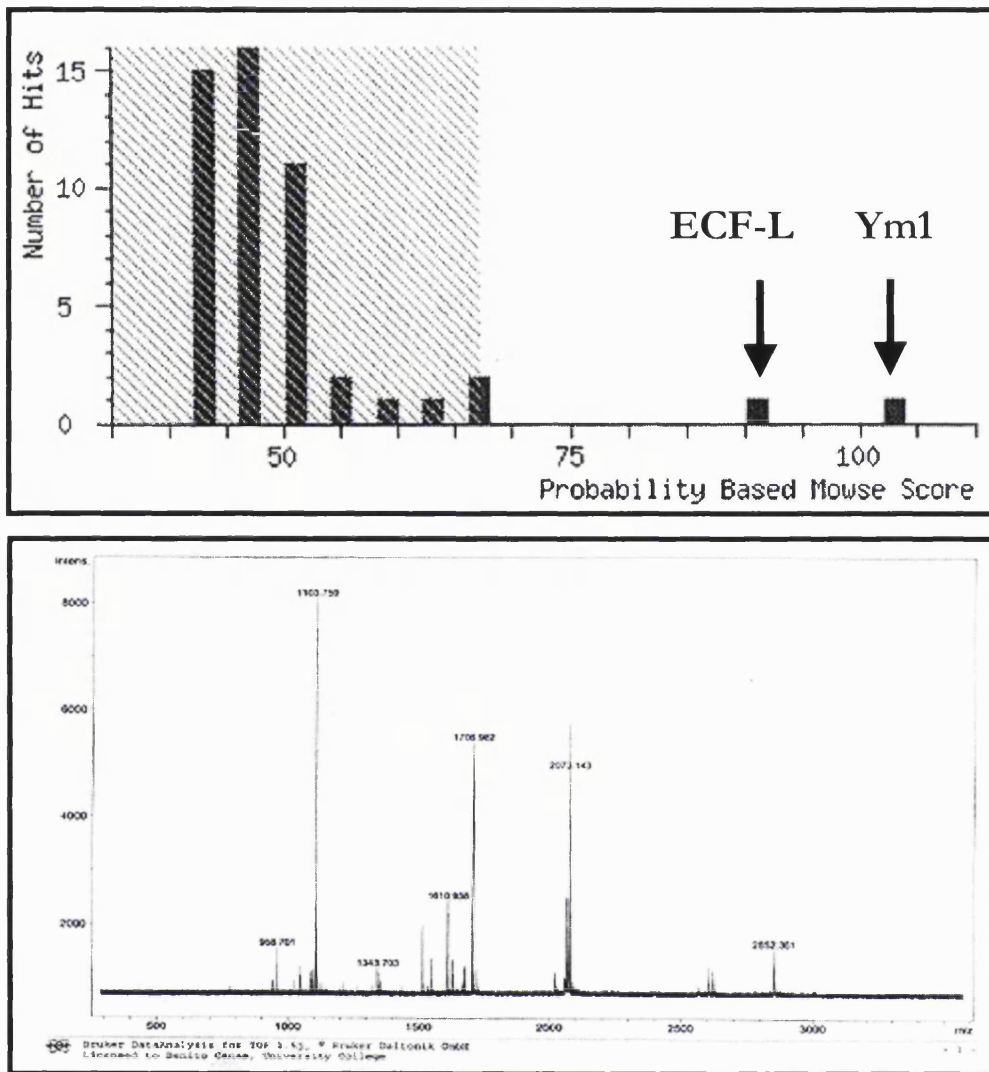
Figure 4: Mass spectra and Mascot Mowse score identified the dominant band as Ym1. The less significant score represented ECF-L, a very similar protein to Ym1.

Anti-Ym1 affinity purified polyclonal antibody

Maximally, acid and alkali eluted 0.91 mg/ml and 0.57 mg/ml affinity purified antibody respectively. Purity of the elute was confirmed by SDS-PAGE. The affinity purified antibody was capable of detecting 200 pg of Ym1 by western blotting. Linear quantification of Ym1 standards up to 50 ng was possible using ^{125}I–labelled anti-rabbit IgG as the secondary antibody (Figure 5). It was largely adsorbed out by pre-incubation with the pure protein (Figure 6).
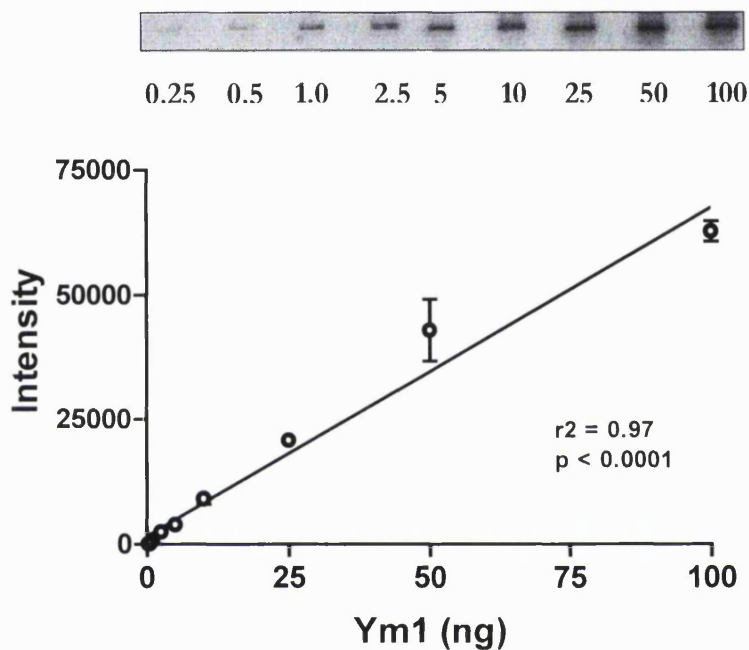
Figure 5: Anti-Ym1 could be used to assay Ym1 in solution. There was a linear interaction between anti-Ym1 and purified Ym1 standards with a lower sensitivity of 200 pg.
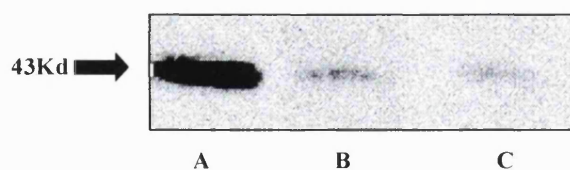
Figure 6: Pre-adsorption of anti-Ym1 by pure Ym1 confirms ligand binding. 2 ng of purified Ym1 was probed with A: 0.1 μg/ml anti-Ym1; B: 0.01 μg/ml anti-Ym1; and C: 0.1 μg/ml anti-Ym1 pre-incubated with 1 μg/ml of crystalline Ym1 for 30 min at room temperature which was removed by centrifugation at 13000xg for 10 min.

Ym1 tissue distribution

Protein expression was greatest in the lung, spleen, bone marrow and gastric antrum of both normal and $p47^{phox}$ -/- mice. The results were confirmed by immunohistochemistry (Figure 7).

A

43 kDa ➡

a b c d e f g h i j k l m n o p

B

Spleen Bone Marrow Gastric Antrum

Lung Bile Duct Skin Abscess
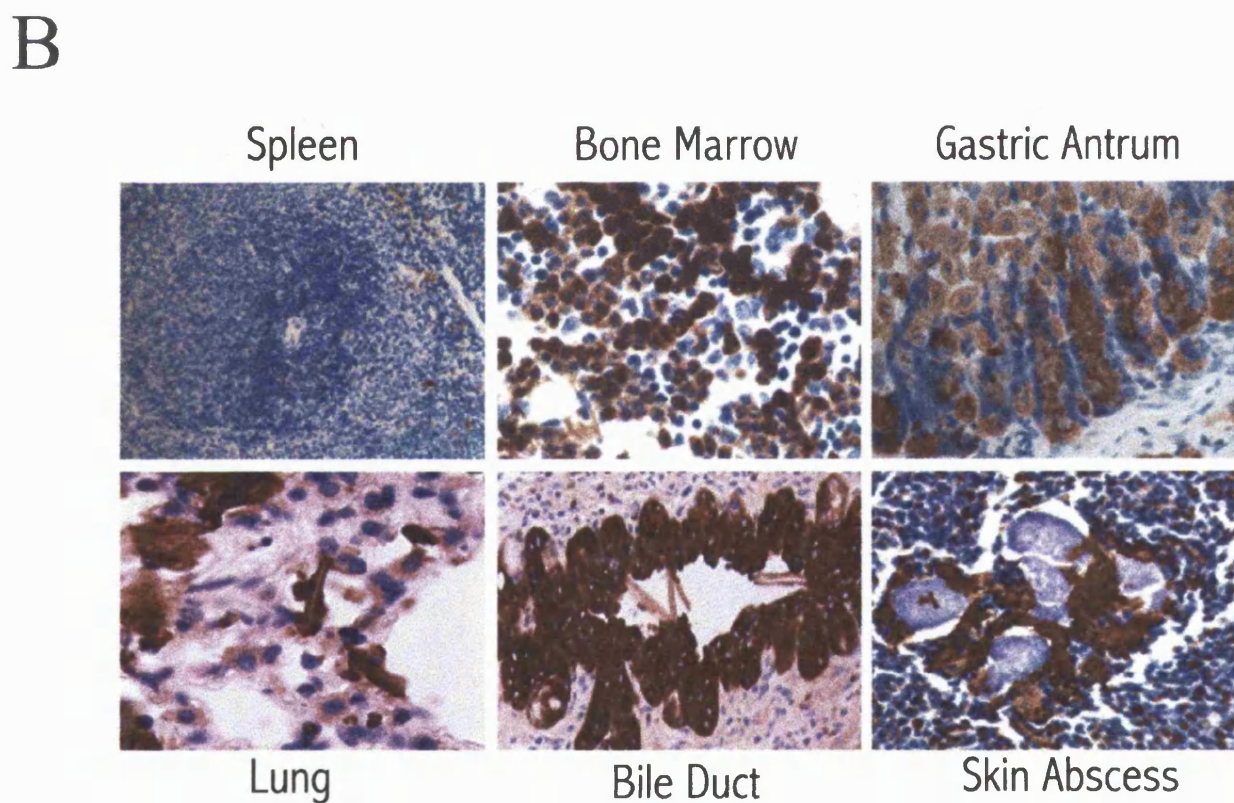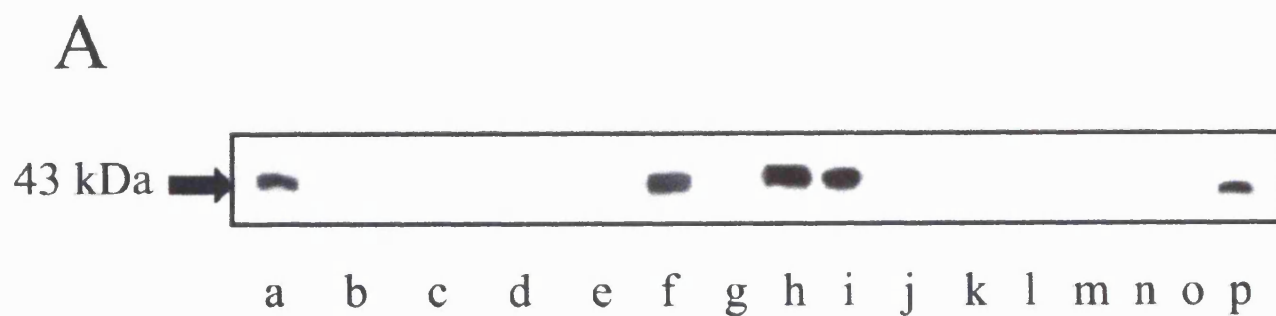
Figure 7: Ym1 expression in the wild type and CGD mouse.   A, anti-Ym1 (1 μg/ml) immunoblot of wild-type mouse organs demonstrating the major sites of expression; a: Ym1 (1 ng); b: venous blood; c: colon; d: ileum; e: duodenum; f: gastric antrum; g: proximal stomach; h: bone marrow; i: lung; j: brain; k: heart; l: thymus; m: kidney; n: testis; o: liver; p: spleen.   50 μg of protein was loaded in each lane.   B, immunohistochemistry for Ym1 confirmed the major tissues of protein expression in the p47phox -/- mouse as spleen showing scattered positive cells in the red pulp, bone marrow showing staining of the granulocytic cell lineage, non-specialized type gastric mucosa in the gastric antrum showing both parietal and chief cell staining and lung showing staining of crystals and macrophages.   Immunoreactivity within an inflamed bile duct and skin abscess is also shown.

Smaller amounts of Ym1 were seen in the liver, kidney, brain, heart, proximal duodenum, and submandibular gland and in one mutant mouse in the thymus. There was similar Ym1 expression (with a gradual increase with mouse age) in p47[phox] -/- and wild-type mice, except in the aged p47[phox] -/- lung, where very high levels were observed consistent with the development of crystals (at 42 weeks p47[phox] -/- 20.6 $\pm$ 1.0 ng Ym1/μg lung protein; wild type 0.9 $\pm$ 0.2 ng/μg) (Figure 8).
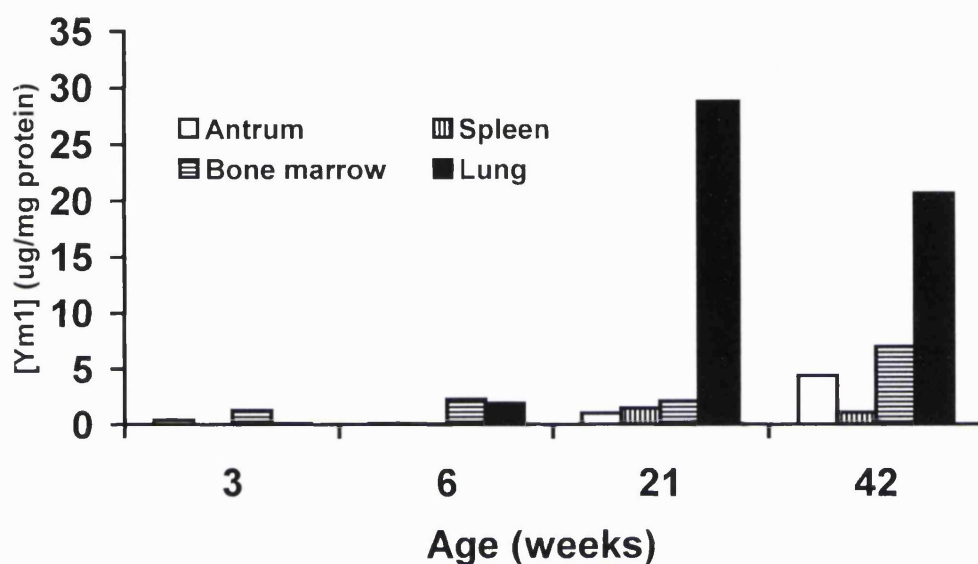
Figure 8: Accumulation of Ym1 protein in the p47[phox] -/- mouse lung increases with time. Tissue Ym1 was quantified by immunoblotting. Results are the mean concentration Ym1 in the organs from two p47[phox] -/- mice and two control mice, at each time point.

Attempts to modify crystal deposition

Murine pulmonary crystallisation was less in mice that had received amantadine (control mice aged 3 weeks 0.08 $\pm$ 0.01 μg/mg cell protein cf. 0.03 $\pm$ 0.04 amantadine treated mice; control mice aged 6 weeks 1.89 $\pm$ 2.67 c.f. 0.32 $\pm$ 0.45). However, there was an increase in lung Ym1 of 10 month old mice treated with amantadine (20.61 $\pm$ 1.36 c.f. 42.50 $\pm$ 24.25). The numbers of mice analysed were small which may explain a lack of statistical significance. There was no reduction in Ym1 lung concentration in mice that were maintained in filtered air cages (26 and 18 μg Ym1/mg lung protein) or that had received prophylactic antibiotics (28 and 8 μg Ym1/mg lung protein) compared to control mice (12 and 7 μg Ym1/mg lung protein).

Gastrointestinal Ym2

The immunoreactive proteins from gastric antrum had higher and lower molecular weights than that of Ym1 extracted from other tissues (45 kDa and 42 kDa) (Figure 9B).   This probably represents antibody cross-reactivity with Ym2, which has been recently shown to cause gastric hyalinosis in CYP1A2-null B6, 129 mice (Ward et al. 2001).

The finding of Ym2 immunoreactivity within the gastric mucosa raised the question whether Ym2 might be secreted into the stomach lumen.   This was suggested by reactivity being found throughout the gastrointestinal tract (stomach 0.8 $\pm$ 0.9 ng Ym2/μg protein; ileum 3.8 $\pm$ 2.1; colon 5.9 $\pm$ 4.1) (Figure 9A).   There was considerable variability in the level of gastrointestinal Ym2 within the lumen, which may reflect variability in feeding between the animals at the time of sacrifice.   Bile and urine from two $p47^{phox}$-/- and two control mice contained no Ym1 (data not shown).
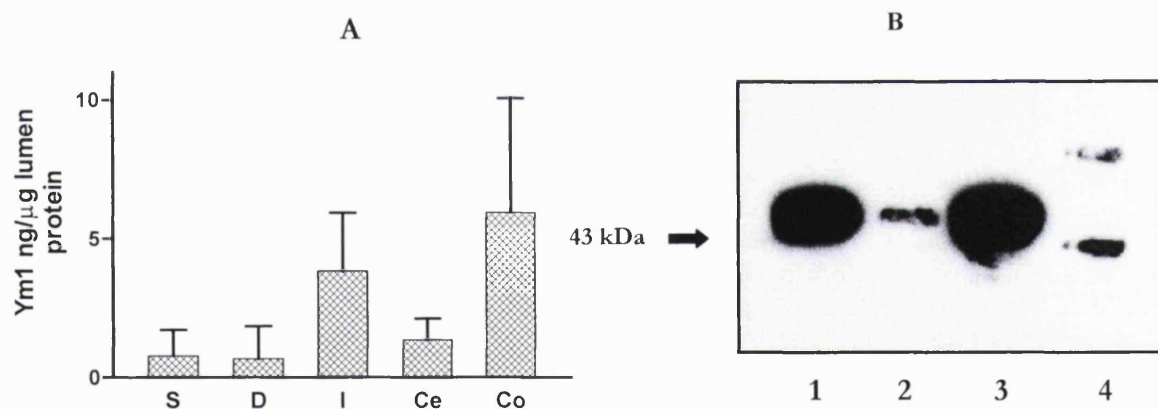
Figure 9: The protein recognised by anti-Ym1 found within the gastrointestinal tract contents has a different molecular weight to pulmonary Ym1.   A, protein distribution from the stomach, S; duodenum, D; Ileum, I; cecum, Ce; and colon, Co.   2 μg of total soluble protein obtained from three wild type mice aged 12 months and analysed by immunoblotting against affinity purified anti-Ym1 (1 μg/ml).   B, comparative immunoblot demonstrating protein mass from 1, lung; 2, spleen; 3, bone marrow; 4, gastric antrum.

Ym1 is a neutrophil protein

Before this study, the main cell of origin of Ym1 was presumed to be the macrophage and the subcellular location was unknown.   However, the primary problem in CGD affects the neutrophil.   It was discovered that Ym1 was neutrophil derived (2.5 ng/μg neutrophil protein), present equally in both peritoneal and bone marrow derived

neutrophils (Figure 10). Ym1 was present within purified peritoneal macrophages at a much lower concentration (0.2 ng/µg). Ym1 was not detected in T- or B-cells.
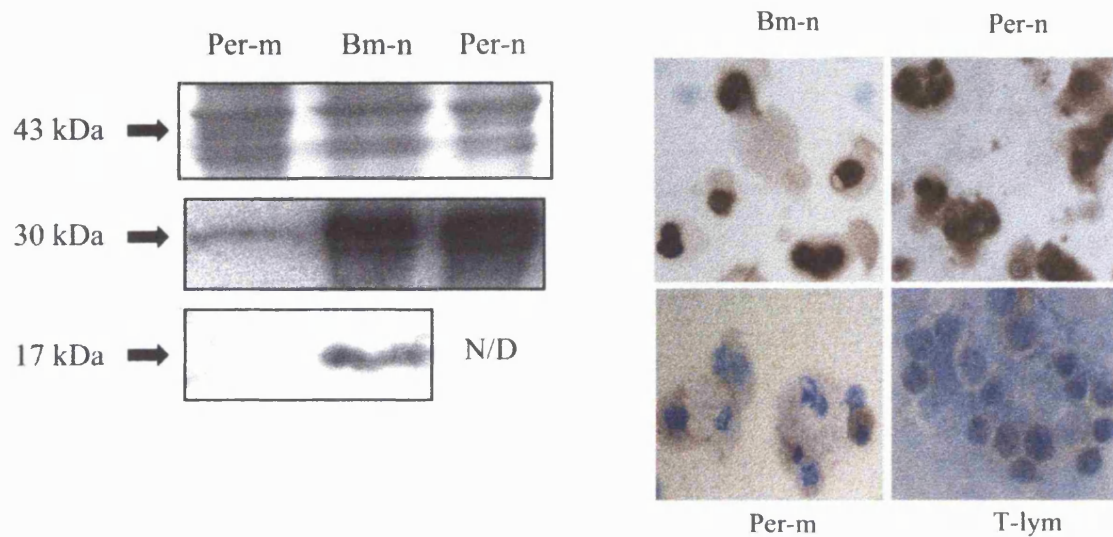
Figure 10: Ym1 is preferentially expressed in bone marrow (Bm-n) and peritoneal (Per-n) neutrophils compared to peritoneal macrophages (Per-m). A, coomassie brilliant blue stained gel (top) demonstrates equal protein loading of gels with anti-Ym1 (middle); anti-elastase (bottom) confirms correct sample assignation; N/D: not done. B, cytospin preparations of Bm-n, Per-n, Per-m and T-lymphocytes (T-lym) stained with anti-Ym1. Occasional Ym1 staining in the thymus extract was within macrophages. Anti-thyroglobulin produced no immnuoreactivity in any of the specimens.

The variable staining of neutrophils within tissue specimens may have been due to degranulation of some of the cells causing a reduction in staining. In support of this, neutrophils within blood vessels stained more than neutrophils within inflammatory lesions. This suggested that Ym1 may be a granule protein.

Ym1 is found within neutrophil granules

Subcellular fractionation by sucrose density centrifugation showed that Ym1 co-localises with other neutrophil granule proteins. The peaks of Ym1, lactoferrin and MPO occurred at sucrose densities of 37 to 39% but Ym1 and MPO were also present in denser fractions (43 to 45%) (Figure 11). This suggested Ym1 may be present in the azurophil granule as it coincided with the azurophilic granule protein MPO rather than with the specific granule protein, lactoferrin (Gullberg et al. 1997). As is commonly found, there was contamination of the less dense layers by granule contents (Gullberg et al. 1997). LDH was only seen in fractions 1-12, in keeping with its known cytosolic localisation (not shown). Commercial antibodies to the specific granule proteins β2-microglobulin and to the azurophilic protein cathepsin G were too insensitive to enable confirmation of Ym1 granule sub-type by western blotting, despite their use at a 1 in 25 dilution.
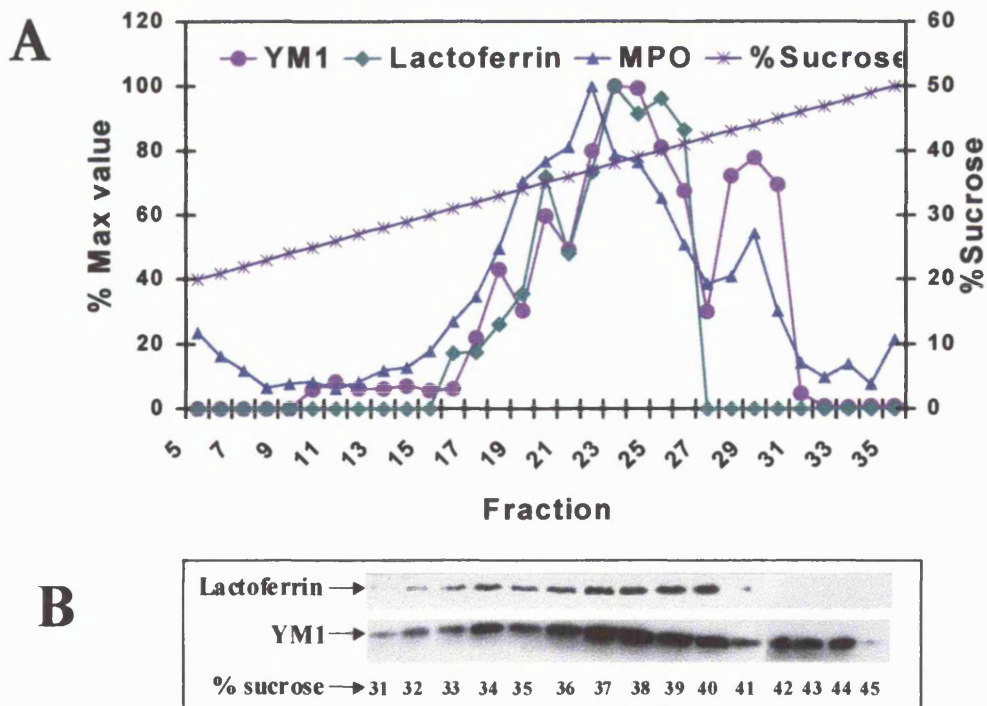
Figure 11: Ym1 is a neutrophil granule protein. A, aliquots of fractions from a sucrose density gradient (✱) separation of murine neutrophils probed with anti-Ym1, anti-lactoferrin and assayed for MPO. Ym1 and MPO are in similar proportions in the same subcellular fractions of the neutrophil. Lactoferrin is not seen in the denser fractions. B, anti-Ym1 and anti-lactoferrin immunoblot.

The granular distribution of Ym1 was clearly confirmed by immuno-gold electron microscopy of murine neutrophils (Figure 12).
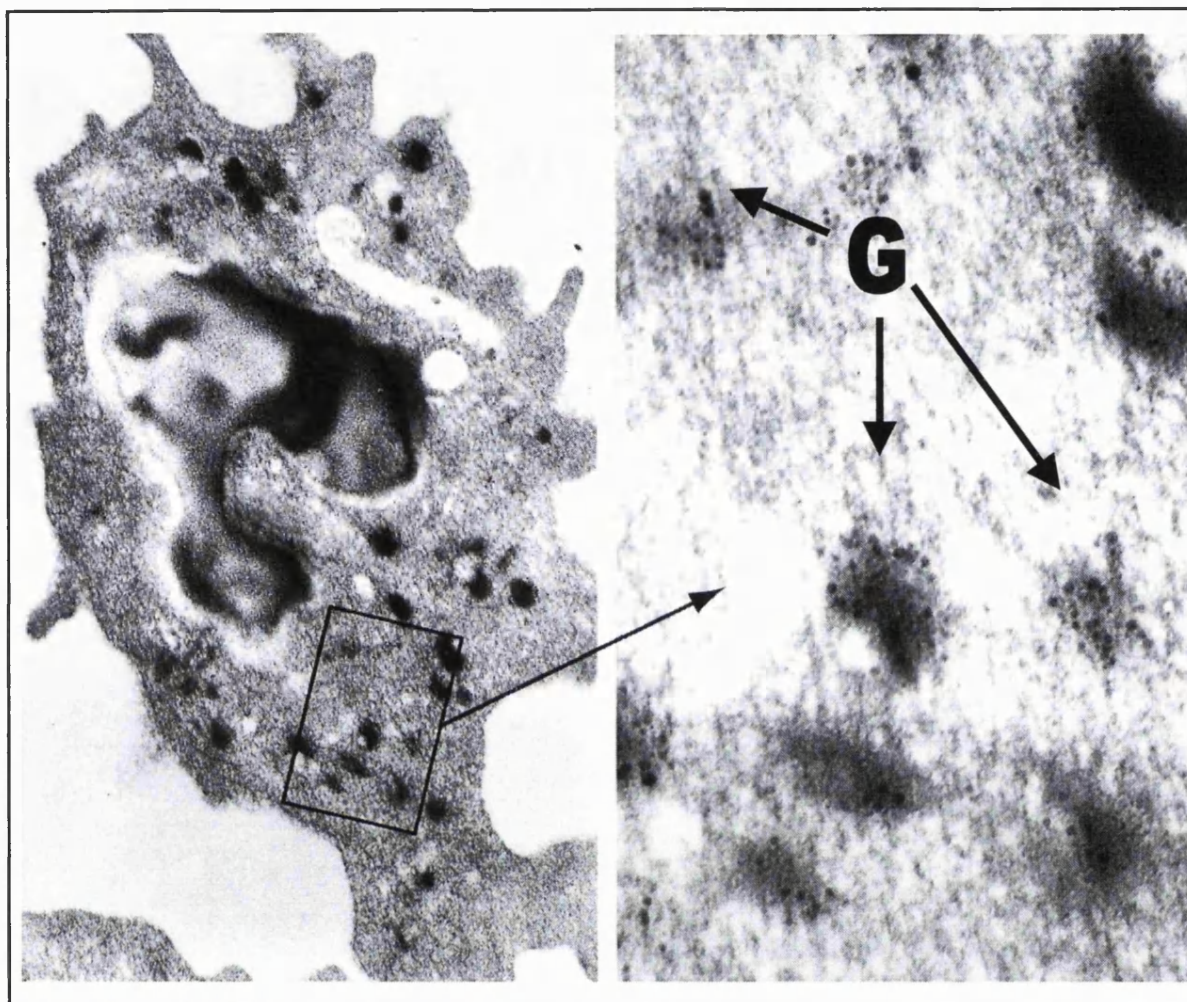
Figure 12: Electron micrograph of a neutrophil section (16,000x) and a further magnified section through the cytoplasm (40,000x). The cytoplasmic granules (G) are heavily labelled with silver-enhanced gold particles coupled to anti-Ym1 antibody.

Confocal examination of neutrophils with antibodies to Ym1 and elastase (present in azurophilic granules) suggested a similar granular distribution in the cell periphery. Antibody co-localisation did not occur after double labelling with antibodies to Ym1 and β2-microglobulin, suggesting Ym1 is found in azurophilic rather than specific granules. This was supported by possible anti-Ym1 co-localisation with anti-cathepsin G, which is found in azurophil granules (Figure 13).

83

84
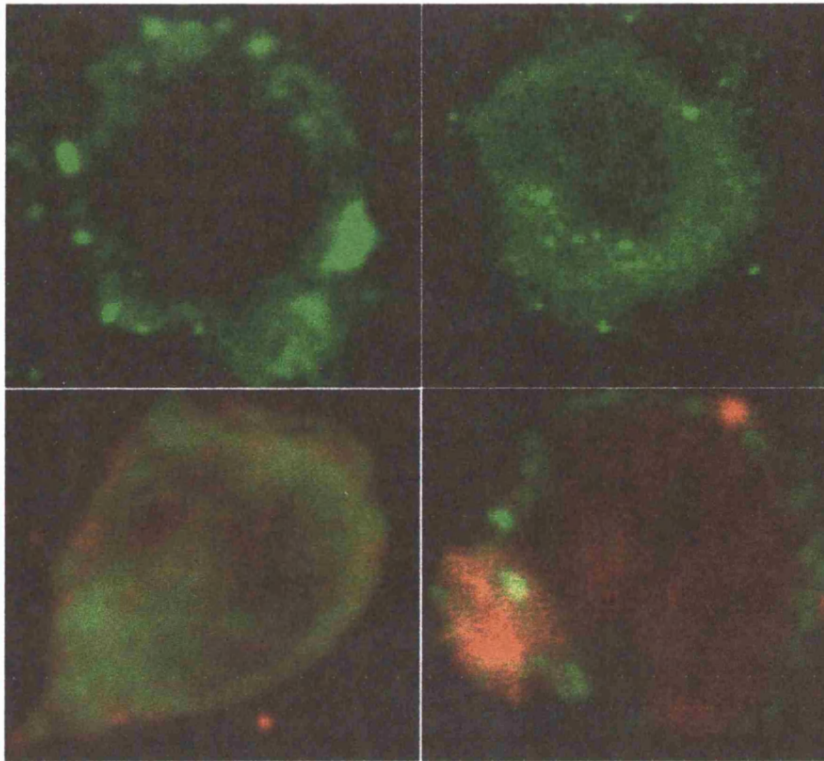
Figure 13: Confocal microscopy demonstrates a granular distribution of Ym1 (green), with possible co-localisation with the azurophilic granule protein cathepsin G (red, bottom right) and not the specific granule protein β2-microglobulin (red, bottom left). Top, left, anti-Ym1 and right, anti-elastase binding to permeabilised wild type neutrophils. Bottom, double labelling of permeabilised, wild-type neutrophils with anti-Ym1 and left, anti-β2-microglobulin and right, anti-cathepsin G.

Ym1 does not function as a fungistatic agent nor fungal opsonin

Ym1 is related to lower organism chitinases therefore pilot experiments were undertaken to see whether Ym1 has a fungicidal or fungistatic effect on *C. albicans* or whether Ym1 facilitates neutrophil killing of *C. albicans* by acting as an opsonin. Ym1 did not inhibit *C. albicans* growth in culture at pH 3 or pH 7.6. There was no clear increase in neutrophil phagocytosis or killing of *C. albicans* in the presence of Ym1 (Figure 14). In addition, there was no significant reduction in neutrophil killing of *C. albicans* in the presence of anti-Ym1 (% of control at 60 min, 114%; at 120 min, 84%).
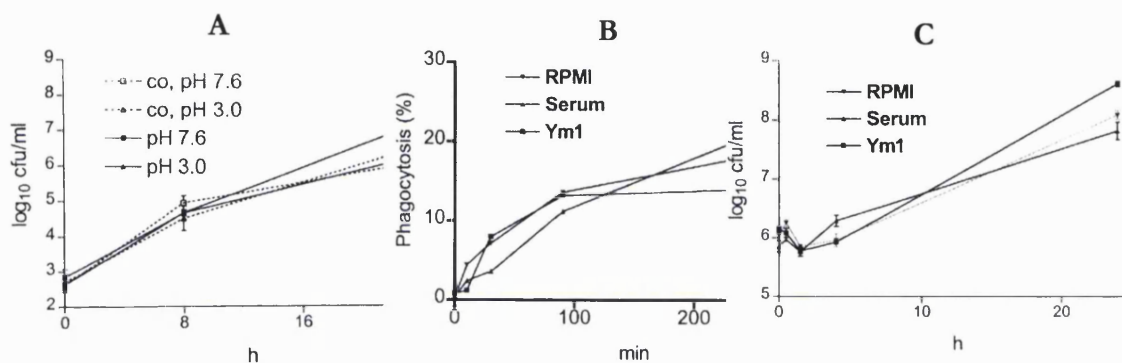
Figure 14: Ym1 is not fungistatic nor functions as an opsonin. A, 1 mg/ml Ym1 at pH 3 & 7.6 did not affect the growth of *C. albicans* in LB broth; pre-incubation of *C. albicans* with 20 mM Ym1 does not affect rate of murine neutrophil phagosytosis (B) nor killing (C).

Ym1 does not bind chitin or C. albicans. Ym1 may function as a chemoattractant in vivo

In addition, Ym1 did not appear to bind chitin since there was no appreciable difference in supernatant or pellet Ym1 concentrations (Figure 15). However, binding was not assessed between pH 4 and pH 7.
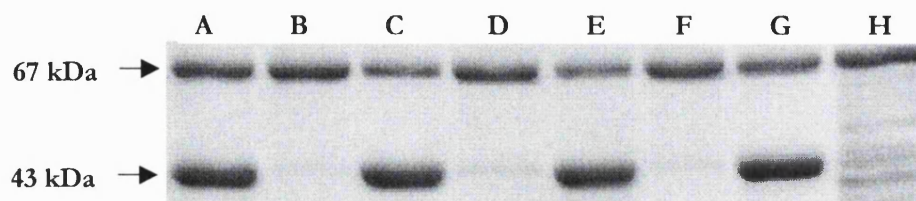
Figure 15: Ym1 does not bind chitin or *C. albicans* at pH 1-3 or 7.5-8 (a representative gel at pH 3 is shown). Ym1 is present in solution (A) but not in the insoluble fraction (B). Ym1 remains in solution on addition of crab shell chitin (C), chitin beads (E) or *C. albicans* (G), and still is not present in the insoluble fractions (D), (F) and (H) respectively. BSA accounts for the bands seen at 67 kDa and confirms equal protein concentrations within each lane.

Subcutaneous injection of soluble Ym1 caused a dose dependent inflammatory reaction with abscess formation in both wild type and mutant mice. There was no abnormality at the sites of albumin injection.

CGD mice can not degrade Ym1 crystals in vivo

In order to understand the mechanism of crystal production, purified crystals were injected into the subcutaneous tissues of CGD and wild type mice. It was found that whereas all the crystals had been solubilised in the normal mice 10 days after injection, they persisted in 3 of 4 of the CGD mice. They were associated with acute inflammation with abscess formation, a giant cell response, fat replacement in the dermis, and pseudo epitheliomatous hyperplasia. Neither Ym1 crystals nor an inflammatory response was seen at sites where the Ym1 crystals were replaced with equal quantities of uric acid crystals, suggesting that the findings were not due to crystal formation in response to the introduction of skin bacteria at the time of injection. Ym1 and urate crystals were seen in all mice killed 5 h after injection.

Ym1 is a β-N-acetylhexosaminidase

The production of pure Ym1 in solution enabled its chitinase activity to be measured accurately for the first time. It was discovered that purified Ym1 could be maintained in solution at normal pH providing the concentration was always less than 400 μg/ml. This allowed the analysis of pure, soluble Ym1 through an acidic pH range (Figure 16).
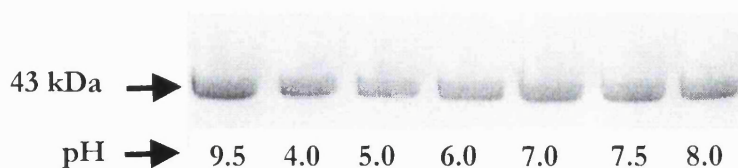
Figure 16: At low concentrations, Ym1 remains in solution under mildly acidic conditions. 7.5 μl aliquots of Ym1 (1 mg/ml, in 10 mM CHES (pH 9.5)) were added to 22.5 μl of buffer (50 mM citric acid / 100 mM Na_2HPO_4 pH 4-6; 50 mM TRIS / HCl pH 7-8) or to 22.5 μl 10 mM CHES (pH 9.5) and incubated at room temperature for 30 min, then centrifuged at 13000xg for 5 min. 20 μl of the supernatant was added to 10 μl of 5x sample buffer and 15 μl was resolved by 12.5% SDS-PAGE (equivalent to 5 μg of protein in each lane).

The enzymatic activity was assessed using sensitive assays based on the release of free MU from molecules of N-acetyl-glucoseamine [GlcNAc]. There was maximal cleavage of the chitobiose [GlcNAc]$_2$ analogue, MU-[GlcNAc]$_1$, at pH 4 & 4.5 but no significant cleavage of MU-[GlcNAc]$_2$ or MU-[GlcNAc]$_3$. Ym1 has a small amount of autofluoresence, equivalent to a maximum of 0.07 n.mol/μg of protein, which represents part of the small amount of net fluorescence emitted (Figure 17).
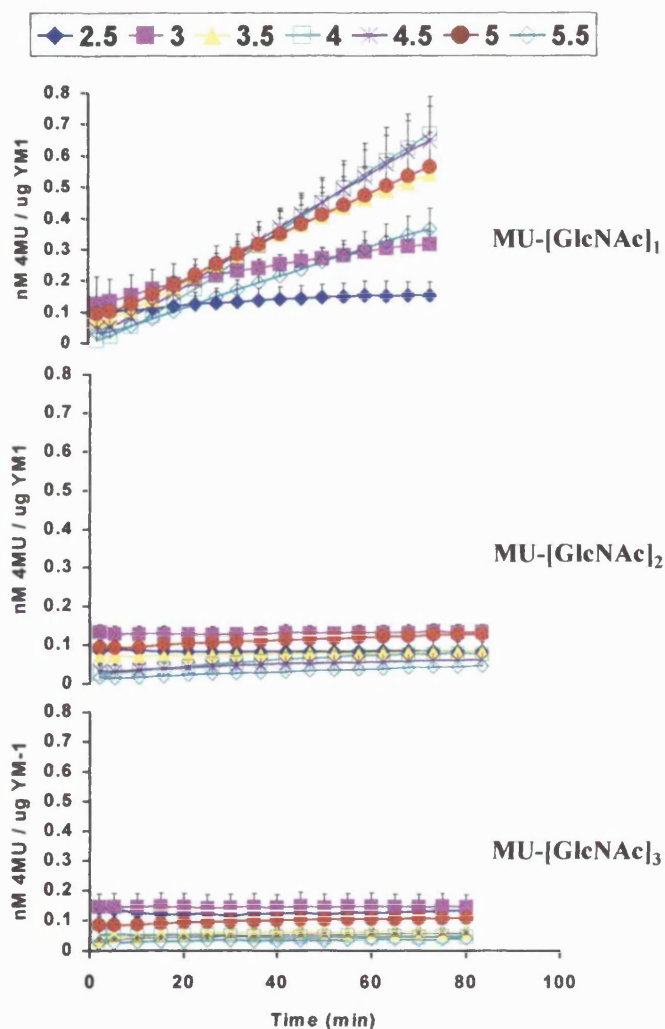
Figure 17: Ym1 is a weak β-N-acetylhexosaminidase. Cleavage of the substrate MU-[GlcNAc]$_{1,2,3}$ at pH 2.5 (◆), 3.0 (■), 3.5 (Δ), 4.0 (□), 4.5 (✳), 5.0 (●) and 5.5 (◇) by pure Ym1 in solution to release MU demonstrates only β-hex activity, maximal at pH 4 and 4.5.

This defined Ym1 as having β-hexoseaminidase (β–hex) and not chitinase activity (Overdijk et al. 1996). The finding that cleavage was specific to the β-D-linkage of [GlcNAc] (Guo et al. 2000) was confirmed since Ym1 did not cleave MU-N-acetyl-α-

D-glucosamine nor chitin azure, a γ-chitin derivative (data not shown). The Vmax and Km were 0.023 μmol/min/mg Ym1 and 120.8 μM respectively (Koshland-DE & Neet 1968). The Km is similar to known values for chitinases found in *Serratia marcescens* (Brurberg et al. 1996) but the Vmax is several orders of magnitude less (Figure 18).
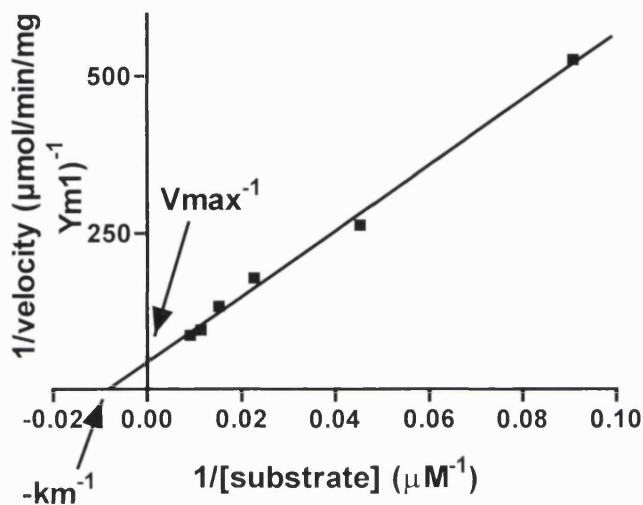
Figure 18: A Lineweaver-Burke plot for MU-[GlcNAc]₁ provides values for the Vmax & Km at pH 4.

Chitinase from *S. marcescens* cleaved MU-[GlcNAc]₁, MU-[GlcNAc]₂ and MU-[GlcNAc]₃ equally, with an activity approximately 1000 fold more than Ym1 (data not shown). Since Ym1 is found in the gastrointestinal lumen, synergy with two major gastric peptides was assessed. There was no effect of pepsin or trypsin on β−hex activity due to Ym1.

DISCUSSION

Lungs from aged p47^{phox} -/- mice showed patchy consolidation with a heavy intra-alveolar chronic inflammatory cell infiltrate composed predominantly of macrophages together with numerous crystals, which were identified as the chitinase-like protein Ym1. Similar crystals were also demonstrated within the inflamed biliary tree, immunoreactive to anti-Ym1.

The protein was confirmed as Ym1 and not the very similar protein ECF-L (Owhashi et al. 2000) since the mass of the tryptic peptide 102-117 was 1722.82 Da, corresponding to proline at position 106 and not serine as in ECF-L. Nor was it derived from a highly homologous gene *Ym2* (Jin et al. 1998) since the mass of the tryptic peptide 220-231 was 1343.70 Da, which corresponds to aspartate at position 220 and not asparagine as in Ym2.

Increased expression of Ym2 has been found in a murine model of asthma, believed to be macrophage derived and dependent on CD4^{+} T cells (Webb et al. 2001). Gastric hyalinosis in CYP1A2-null B6, 129 mice has recently been identified as Ym2 (Ward et al. 2001) and in the same study, anti-Ym1 immunoreactivity demonstrated to pulmonary macrophage-associated crystals. In contrast to the CYP1A2-null B6, 129 mouse, no protein crystals were seen within the stomach. This may be due a more acidic environment or a lower Ym2 concentration in the p47^{phox} -/- mouse.

Chitinases, members of the 18-glycosyl-hydrolase family, cleave consecutive n-acetyl-glucosamine [GlcNAc] moieties of chitin. They are found in most organisms, including plants, fungi, bacteria, insects, marine invertebrates, fish and protozoans (Flach et al. 1992). Some of these organisms contain chitin and require chitinases to grow. Others, including some bacteria and higher plants contain no chitin in which case chitinases facilitate pathogen destruction or pathogen entry into a chitin containing host (Vinetz et al. 2000).

Mammals do not contain chitin although ten mammalian proteins are similar to chitinases of lower organisms, and as such termed "chitinase-like proteins" (Hakala et al. 1993; Morrison & Leder 1994; Sendai et al. 1995; Renkema et al. 1995; Hu et al. 1996; Jin et al. 1998; Saito et al. 1999; Owhashi et al. 2000; Boot et al. 2001) (Table I). Only chitotriosidase and TSA-1902L have been shown clearly to have endochitinase

activity (Hollak et al. 1994; Saito et al. 1999), defined as the ability to cleave the insoluble chitin polymer (Flach et al. 1992).

TABLE I

Mammalian chitinase-like proteins' molecular weight and cellular origin (where expression known)

Name	Mass (kDa)	Cellular origin
Human YKL-39	43039	Articular chondrocyte (Hu et al. 1996)
Human HCgp-39 (YKL-40)	42613	Macrophage (Krause et al. 1996); neutrophil (Volck et al. 1998); articular chondrocyte (Hakala et al. 1993)
Human oviductal glycoprotein	75421	N/K
Human chitotriosidase	51681	Monocyte (Hollak et al. 1994)
Human TSA1902-L	40082	N/K
Mouse AMCase	50000	N/K
Mouse ECF-L	44448	N/K
Mouse Ym1	44528	Neutrophil (Harbord et al. 2002); gastric epithelia (Ward et al. 2001)
Mouse BRP39	43001	N/K
Mouse oviductin	78807	N/K

N/K, not known.

Ym1 has been assumed to be macrophage derived (Webb et al. 2001) since it was sequenced from an activated peritoneal macrophage cDNA library (Chang et al. 2001)

and microscopically Ym1 crystals are associated with macrophages (Guo et al. 2000). However, Ym1 was shown to be mainly a neutrophil granule protein (Figure 19). What is the explanation for this disparity?
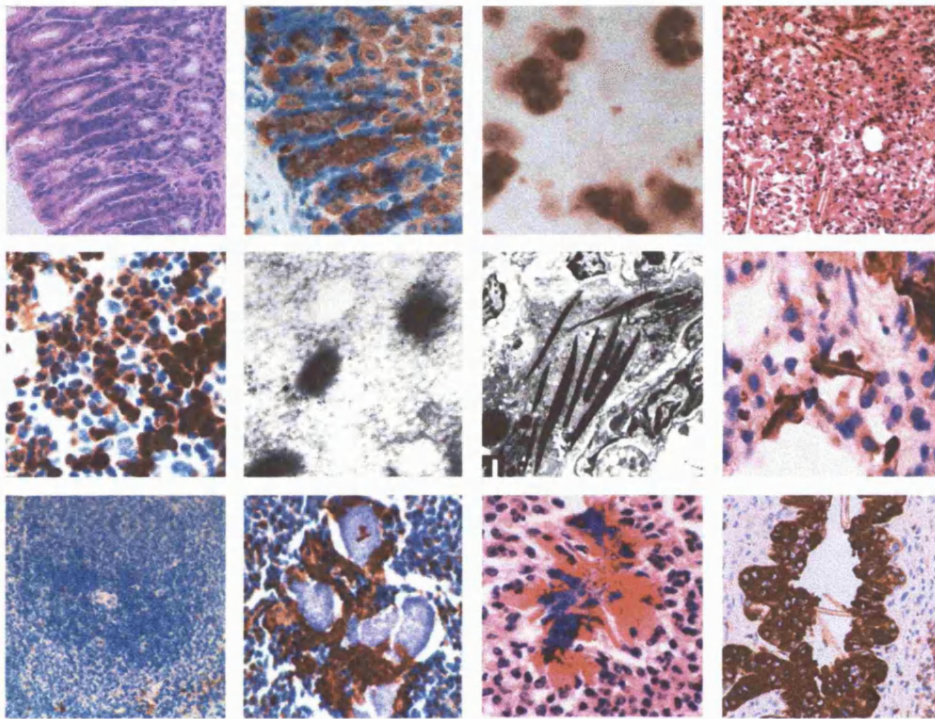
Figure 19: Ym1, a chitinase-like protein, spontaneously crystallises in p47^{phox} knockout (chronic granulomatous disease) mice. Coloured images show clockwise from top right Ym1 crystals within the lung, an inflamed bile duct and a skin abscess; and anti-Ym1 immuno-reactivity in the spleen, bone marrow, distal stomach and neutrophil. Immunogold electron microscopy co-localised Ym1 with the neutrophil granule.

Neutrophils have a circulation half-life of about 6 to 8 h and are the predominant cell type present during the first 24 h of an inflammatory stimulus (Lord et al. 1991). They apoptose, are phagocytosed and digested by macrophages, which have a life span of weeks to months (Green et al. 1977) and can very rapidly degrade apoptotic neutrophils (up to 1 neutrophil/macrophage/30 min) (Savill et al. 1989). Therefore, extra-cellular non-degradable crystalline Ym1, or Ym1 derived from apoptotic neutrophils, will appear to be mainly associated with macrophages. This is compatible with the low concentration of Ym1 found in thioglycolate induced peritoneal macrophages, where the majority of cells will have been recently synthesised from bone marrow promonocytes and blood monocytes (Van-Furth et al. 1973) and therefore will not have had the opportunity to phagocytose many neutrophils.

It is noteworthy that another member of this family of proteins, YKL-40, has been found within the specific granules of human neutrophils (Volck et al. 1998) and that chitinase activity in human leukocytes has been shown to be mainly from granulocytes (Escott & Adams 1995). It is possible that Ym1 is expressed also by eosinophils since both alveolar macrophage crystals in C57/BL6 mice (Huffnagle et al. 1998) and Ym2 expression during experimental allergy (Webb et al. 2001) increased with peripheral eosinophilia.

What can be deduced about protein crystallisation from these findings? Firstly, crystals may form due to a high local concentration of Ym1 secondary to neutrophil degranulation occurring during repeated episodes of inflammation. This is analogous to the deposition of α1-antitrypsin variants within the liver, where polymerisation of the protein is enhanced during episodes of inflammation (Parmar & Lomas 2000); and to Charcot-Leyden crystal formation, where lysophospholipase, a granule protein comprising 10% of an eosinophil's protein (Ackerman et al. 1993), crystallises during asthmatic inflammatory reactions.

In support of this, Ym1 crystals increased with age; were found within the lung, a major portal of antigen entry, in peripheral and basal sites suggesting a response to inhaled matter; and increased anti-Ym1 immuno-reactivity and Ym1 crystals occurred at extra-pulmonary sites of inflammation.

Secondly a mildly acidic pH milieu outside the cell or within the CGD phagolysosome may contribute to crystallisation. This suggestion is supported by the reduction in pulmonary Ym1 crystals after treatment with amantadine, which increases phagolysosomal pH (Maurin et al. 1992). Inflammation causes a mildly acidic extracellular environment (Hunt et al. 2000) and the phagolysosome of the human CGD neutrophil has been shown to be abnormally acidic (6 min post phagocytosis, pH 6.1 cf. pH 7.4 in control subjects (Segal et al. 1981)). Assuming similar pH changes occur in the murine CGD phagolysosome, the concentration of Ym1 protein may exceed the crystallisation threshold due to excessive degranulation. This indeed may be the case since there is 1 ng Ym1 in 10000 murine neutrophils; the volume of granules in one human neutrophil is 52.5 fl (Edwards 1994); assuming human and murine neutrophils have a similar granule volume, the granule concentration of Ym1 is 1.9

µg/µl. At neutral pH, Ym1 crystallises *in vitro* when the concentration exceeds 0.4 µg/µl.

Thirdly crystalline Ym1 may be resistant to degradation within the CGD macrophage due to absence of functional NADPH oxidase. This is also likely since skin injection experiments showed that only wild-type mice were able to clear Ym1 crystals from their subcutis.

After invasion by chitin containing micro-organisms, chitin and other foreign antigens must be digested in order to prevent a granulomatous tissue response. Ym1 may facilitate this since chitin can be degraded with a binary enzyme system using a combination of a chitinase and an N-acetyl-β-glucosaminidase (Brurberg et al. 1996). Ym1 can be classified as an N-acetyl-β-glucosaminidase since it cleaved β-1, 4-linked N-acetylglucosamine (GlcNAc) from the chitobiose $(GlcNAc)_2$ analogue, MU-$(GlcNAc)_1$. It did not function as a chitobiase or chitinase since it did not cleave MU-$(GlcNAc)_2$ or MU-$(GlcNAc)_3$ (Flach et al. 1992). This is compatible with the mutated chitinase active site of Ym1 (Boot et al. 1995). Incidentally, the minimal activity with MU-$[GlcNAc]_2$ and MU-$[GlcNAc]_3$ shows that Ym1 can not cleave N-acetylglucoseamine monomers sequentially from the same molecule of MU-$[GlcNAc]_2$ or MU-$[GlcNAc]_3$, as has been described for chitinase A & B of *S. marcescens* (Brurberg et al. 1996) (Figure 20).
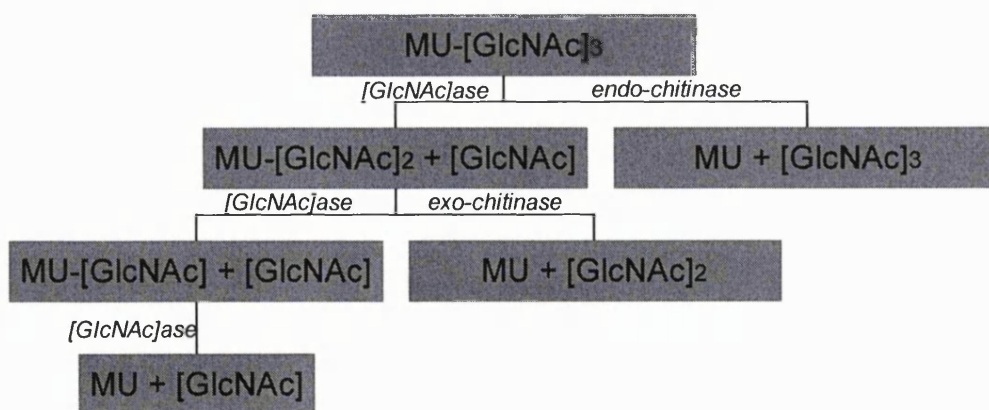
Figure 20: The degradation pathways for MU-[GlcNAc]₃. Enzymes are shown in italics ([GlcNAc]ase = n-acetyl-β-glucosaminidase). The release of the flurogenic substance MU can be measured. Endo-chitinase cleaves within the chitin polymer, exo-chitinase (sometimes called chitobiase) releases chitobiose and [GlcNAc]ase releases β-1, 4-linked N-acetylglucosamines [GlcNAc] monomers from chitin.

Contrary to our findings, Guo *et al* (Guo et al. 2000) suggested that Ym1 also could cleave MU-(GlcNAc)$_{2,3}$. However, their preparation contained cellular debris, which may have included chitinases of a similar molecular weight as Ym1, or enzymes capable of sequential β-n-acetylglucosaminidase or exo-chitinase activity, allowing Ym1 to release 4MU from MU-(GlcNAc)$_1$. Such problems in distinguishing between exo- and endo-chitinase activity have been described (Ren et al. 2000).

It is noteworthy that the enzyme activity had the same pH dependency as shown recently for Ym1 binding to N-unsubstituted glucosamine moieties (Chang et al. 2001), although binding sites and cleavage sites within the protein may be distinct. Finally, although the enzyme activity demonstrated here is weak, this may under-estimate *in vivo* kinetics since functionality could require synergy with other proteins, shown previously for chitinase (Davies & Pope 1978).

It is interesting that a protein that may play a role in the digestion of bacteria and fungi is found as crystal deposits in the CGD mouse, which is susceptible to repeated bacterial and fungal infections. Could a similar phenomenon occur in humans? There are no reports of similar crystals in human patients with CGD. There was no immuno-reactivity by anti-Ym1 against human blood nor human inflamed colon (data not shown) although this could be due to a lack of inter-species antibody cross reactivity. After comprehensive searches against protein, nucleotide and Expressed Sequence Tag databases using the Basic Local Alignment Search Tool (BLAST), no known human homologue for Ym1 was found. Although there is 61% protein sequence homology to a human lung gene, TSA1902 (Saito et al. 1999), the human gene has a different murine homologue called acidic mammalian chitinase (AMCase), recently reported by Boot et al (Boot et al. 2001). Interestingly, AMCase is similar to Ym1 and Ym2 since both proteins are expressed predominantly by gastric epithelium with maximum activity in an acidic milieu.

The function of these mammalian, chitinase-like proteins is unknown but it has been suggested that they remodel the extra-cellular matrix, that they are fungicidal or fungistatic. Generally, they are associated with sites of longstanding inflammation, for example their concentration has been found to be increased in a rat model of pulmonary silicosis (Guoping et al. 1997); in a guinea pig model of systemic *Aspergillus fumigatus* infection (Overdijk et al. 1999); and in humans with periodontal inflammation

(Van-Steijn et al. 1999), with colorectal cancer (Cintin et al. 1999), with breast cancer (Johansen et al. 1995), in macrophages within atherosclerotic human arteries (Boot et al. 1999) and in osteoarthritic synovial fluid (Johansen et al. 1996).

A specific function has been ascribed to two proteins. Firstly, Gp38K (Shackelton et al. 1995), the porcine homologue of HCgp-39, has been shown to promote HUVEC migration and chemotaxis (Malinda et al. 1999). Secondly, ECF-L has been shown to be chemotactic for murine eosinophils (Owhashi et al. 1998; Owhashi et al. 2000). ECF-L differs from Ym1 at positions 196-204, including seven non-homologous amino-acids.

The function of Ym1 *in vivo* is unknown although recently the same researchers have both deduced the protein structure, which contains a saccharide binding site (Sun et al. 2001) and shown that Ym1 can function as a lectin, binding multivalent hexosamines and heparin (Chang et al. 2001). Soluble Ym1 caused an intense inflammatory reaction *in vivo* that suggests it may also function as a chemoattractant. Analogously, undefined human neutrophil granules have been shown to contain chemoattractants for T cells and monocytes (Taub et al. 1996).

The function of Ym1 is unlikely to be fungicidal, since neither Ym1 nor anti-Ym1 had anti-*C. albicans* activity *in vitro*, nor did Ym1 augment *C. albicans* phagocytosis or killing by granulocytes *in vitro*, at either an acidic or normal pH. However, there are several criticisms of the pilot experiments undertaken. Firstly, neutrophil preparations may have contained opsonins, explaining why the rate of phagocytosis was similar with or without *C. albicans* opsonisation. Secondly, *C. albicans* were present as both hyphae and spores, which were hard to quantify when assessing phagocytosis. Spore forms would have predominated had they been cultured at 35 °C. Thirdly, at both pH 7.4 or pH 3.5, Ym1 has reduced solubility and therefore only a proportion would have been functionally available. Ym1 maximally cleaves $(GlcNAc)_2$ at pH 4.0-4.5, therefore it is possible that soluble Ym1 could inhibit fungi at this pH.

As a neutrophil protein probably found within azurophil granules, it is likely that YM1 is a digestive enzyme (Gullberg et al. 1997). These proteins may be present in mice to digest chitin containing micro-organisms in the lung or stomach, since these types of pathogens are in high concentration in the habitats in which mice evolved.

Alternative functions of Ym1 remain speculative. For example, neutrophil O_2^- production & elastase release are inhibited by N-acetyl-galactosamine and [GlcNAc] (Kamel & Alnahdi 1992), which could provide a mechanism for Ym1 to act as an anti-inflammatory agent. Also, rodent macrophages have been shown to recognise apoptotic thymocytes due to exposed [GlcNAc] (Duvall et al. 1985); it is conceivable that Ym1 could function to enhance [GlcNAc] exposure and therefore increase phagocytosis of apoptotic neutrophils.

In summary, it has been demonstrated that Ym1 is a neutrophil granule protein with weak β-hex activity at pH 4. However, the lack of a human homologue limits the relevance of this protein to CD and CGD.

CANTHARIDIN BLISTERS TO INVESTIGATE ACUTE INFLAMMATION IN INFLAMMATORY BOWEL DISEASE

AIM

To develop a technique to investigate acute inflammation in humans using cantharidin induced skin blisters. To apply this technique to investigate chemokine expression and neutrophil migration in IBD.

INTRODUCTION

The recruitment of cells to acute inflammatory sites is dependent on the release of vasoactive and chemotactic factors that increase regional blood flow, increase microvascular permeability and promote the exudation of leukocytes from the circulation into the tissues (Suffredini et al. 1999). Techniques that create aseptic inflammatory reactions have provided information regarding the regulation of acute inflammation.

Skin-window chamber methods create a lesion or "window" in the stratum corneum with either a surgical scalpel (Rebuck & Crowley 1955), high-speed drill (Senn et al. 1969) or by tape stripping (Mass et al. 1975), which cause leukocytes within dermal capillaries to migrate through the skin. Suction blisters, created by applying negative pressure to intact skin, have also been used to examine the cellular composition during acute inflammation (Kiistala & Mustakallio 1964). However, it is not easy to standardise the size and depth of lesions with these methods; and by destroying the microanatomy they provide a good model for trauma but a poor model for localised acute inflammation. Therefore, an alternative method to induce an acute inflammatory lesion was required.

Cantharidin, a protein phosphatase 1 and 2α inhibitor (Honkanen 1993) can be purified from the hemolymph of blister beetles (e.g. the common Southern European species *Lytta vesicatoria*) (Figure 1) which secrete it from the limb joints when attacked by other insects. On ingestion, it causes severe bleeding of the mucous membranes of the gastrointestinal and renal tracts. Such is its potency that ingesting 1 blister beetle can kill a small child. Very dilute cantharidin was present in "Spanish Fly", a topical

aphrodisiac used in the 18th and 19th centuries, reputedly by Louis XV (Presto, III & Muecke 1970), which probably caused its effect by irritating the skin. It does not function as an aphrodisiac when administered to rats and modern preparations of "Spanish Fly" (luckily) contain no cantharidin.

Figure 1: Post-prandial mating of blister beetles on Texas mountain laurel

When applied to the skin it induces vesico-bullous blistering by causing acantholysis (Pierard & Pierard 1988), defined as the loss of cohesion between keratinocytes with consequent intra-epidermal blister formation. This occurs due to activation of neutral serine proteases, causing tonofilament detachment from the desmosomal plaque (Bertaux et al. 1988).

In conjunction with Dr. R. Day of Imperial College, a novel skin window technique has been developed using cantharidin to create blisters on the forearm of subjects (Day et al. 2001) in which cells and acute inflammatory mediators accumulate. This has been used to investigate IBD.

Previous studies have demonstrated a reduction in the number of neutrophils migrating into skin windows from five hours in patients with CD (Segal & Loewi 1976; Morain et al. 1981; Wandall & Binder 1982a) and UC (Wandall & Binder 1982b). Migration was independent of disease site and activity and therefore may be causal rather than consequential. Indeed, delay in the accumulation of neutrophils during the

early stages of inflammation in CD could explain granuloma formation (Schmitz et al. 1984), poor post-operative wound healing (Scammell & Keighley 1986) and the clinical course of exacerbations and remissions, through the persistence of non-digested material within the bowel wall provoking enhanced macrophage responses.

The effect of chemokines and cytokines on neutrophil migration during the first 24 hours of acute inflammation in IBD is unknown. It was hypothesised that neutrophil dysfunction is an aetiopathogenic factor in CD, possibly mediated by reduced chemokine function. This was examined using cantharidin blisters.

METHODS

The Ethics Committee of participating hospitals had approved each study involving human subjects and all participants provided informed consent.

Patient recruitment

Patients with quiescent IBD and non-inflammatory controls were recruited from the out-patients' clinics of University College London Hospitals and St. Mark's Hospital. Healthy medical students were also recruited. The diagnosis of IBD was confirmed by histology (CD 18; UC 20) or, where unavailable, radiological examination (CD 5). Disease activity was recorded based on a previously published modification of the Crohn's Disease Activity Index that has been used in both CD and UC, ranging from 0 (no clinical disease) to 15 (severe disease) (Kozarek et al. 1989). Exclusion criteria (Table I) and subject characteristics (Table II) are shown. All subjects were Caucasian except for Asian, 1 CD; 2 UC; 2 control subjects; Chinese, 1 control subject; and Afro-Caribbean, 1 control subject.

Table I: Exclusion criteria for the investigation of IBD using cantharidin blisters

Age < 18 years or > 75 years
Intercurrent active infection
Non-IBD active inflammatory process
Pregnancy, lactation
Chronic liver or renal disease
Infection with HIV or hepatitis viruses
Intercurrent neoplastic lesion
Bleeding tendency
Immunosuppression (excl. azathioprine and steroids)
Surgery within the previous 6 week
Uncertain diagnosis of IBD
History of severe atopy or contact dermatitis

Table II: Subject characteristics for the investigation of IBD using cantharidin blisters (mean ± standard deviation)

	CD	UC	Control
Number	23	20	21
Age (years)	42.5 +/- 12.3	44.8 +/- 10.5	40.6 +/- 15.7
Male	13	14	12
Smoking ^"	9	0	6
Body mass index	24.4 +/- 3.7	26.5 +/- 3.4	25.4 +/- 3.7
Objective activity index	1.9 +/- 1.3	1.7 +/- 1.9	-
Oral steroid use	3	5	0
Mesalazine use	4	6	0
ESR (mm/hr) *^	18.2 +/- 12.0	12.5 +/- 6.3	5.9 +/- 3.0
CRP (mg/l)	7.8 +/- 6.1	1.8 +/- 1.3	4.1 +/- 3.0
Leukocyte count (x10^9/l)	8.5 +/- 2.1	6.9 +/- 1.6	7.0 +/- 1.6
Neutrophil count (x10^9/l)*^	5.8 +/- 1.9	4.2 +/- 1.4	4.2 +/- 1.0
Monocyte count (x10^9/l)	0.6 +/- 0.2	0.5 +/- 0.2	0.6 +/- 0.2
Haemoglobin (g/dl)	13.8 +/- 1.1	14.5 +/- 0.7	14.4 +/- 1.0

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein
Significance ($p < 0.05$) assessed by the unpaired two-tailed t test
*CD : control; ^CD : UC; "UC : control

Generation and sampling of cantharidin blisters in human subjects

The skin on the volar aspect of the subject's forearm was sterilised with an alcohol wipe (Seton Healthcare, Oldham, UK) and allowed to air dry. Two 0.8 cm^2 diameter discs of Whatman qualitative No.1 filter paper (Whatman Ltd., Maidstone, England), prepared using a wad punch (RS Components Ltd, Northants, UK), were placed on the forearm. 25 µl of 0.1% cantharidin (Cantharone; Dormer Laboratories Inc., Rexdale, Canada) in acetone (Analar; BDH Chemicals, Dorset, UK) was applied to each disc, whilst maintaining the disc in direct contact with the skin. After air drying, the discs were covered individually with a dressing prepared by placing 2 cm^2 moisture proof film (Nescofilm, Nippon Shoji Kaisha Ltd, Osaka, Japan) in the centre of 3 cm^2 Mefix

adhesive dressing (Mölnlycke Healthcare, Sweden). Subjects were then advised to wear loose clothing at the site of blistering since hyperaesthesia was present in the skin underlying the disc, and to keep the blister dressing dry.

After 24 h, the blister dressing was carefully removed to avoid breaking the surface of blister and the skin sterilised with alcohol and allowed to air dry. One side of the blister was lanced with a 25 gauge sterile needle (Terumo Europe, Leuven, Belgium), avoiding any dermal contact that would cause pain and bleeding into the blister fluid. A pipette tip was rolled across the surface of the blister, applying very light pressure only. The blister fluid was collected as it was released using a pipettor fitted with a siliconised tip and placed into a siliconised microfuge tube (Novara Group Ltd., Leicestershire, U.K.). The blistered skin was covered with a Comfeel™ ulcer dressing (Coloplast, Denmark), to be removed after 4 days. The fluid used for phenotype and respiratory burst analysis was maintained on ice and at room temperature respectively. Venous blood samples were taken for standard hospital laboratory analysis at the time of blister sampling (Figure 2).
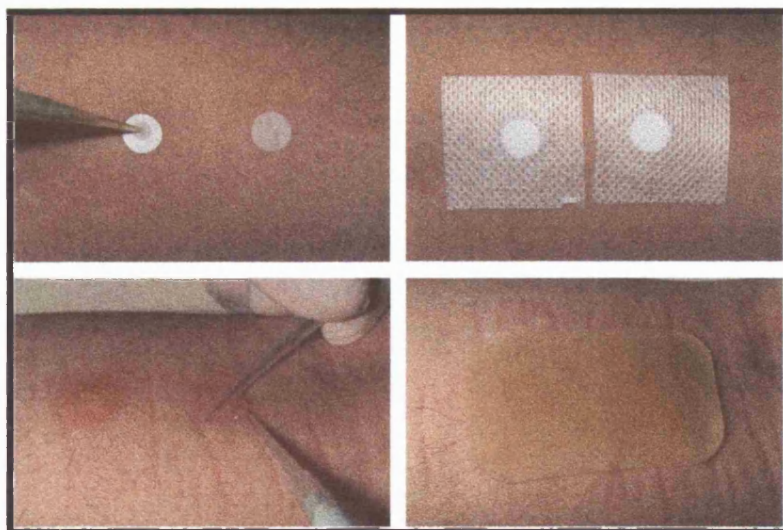
Figure 2: Blister production. 25 μl cantharidin solution is applied to each disc. The discs are covered for 24 h.. Blister fluid is aspirated from each blister using a pipettor. The blisters are covered with an adhesive dressing for 4 days. After 4 weeks the area of blistering is fully healed.

Analysis of cantharidin blister constituents

The volume of blister fluid was determined by weight. The cell number and viability within each blister was determined in duplicate by manually counting cells stained with

0.4 % trypan blue (BDH chemicals) in PBS using a Neubauer counting slide (Hawksley, England). The blister fluid was centrifuged (500xg; 5 min; 4 °C), the supernatant removed and stored at -70 °C in 50 µl aliquots. The pellet was re-suspended in 200 µl FACS buffer on ice. 10 µl of FITC-conjugated and 5 µl of phyco-erythrin (PE)-conjugated antibody to a variety of cell surface antigens (delineated below) were added to appropriate tubes containing 200 µl of a cell suspension together with at least 2×10^5 cells. An additional tube contained an isotype-matched control antibody. The cells were incubated with the antibodies for 30 min at 4 °C in the dark. Subsequently, they were washed twice in FACS buffer (500xg; 5 min; 4 °C), then incubated in the dark with 200 µl of 1 % paraformaldehyde (BDH Chemicals) in PBS, which had been freshly prepared by dissolving at 70 °C for 30 min.

For the main analysis FACS after FITC-conjugated anti-CD16 (Fleit et al. 1982) (Becton Dickinson, San Diego, U.S.A.) and PE-conjugated anti-CD14 (Wright et al. 1990) (Becton Dickinson) monoclonal antibody labelling was used to quantify neutrophils and monocytes/macrophages respectively. In order to verify blister cell types and cell surface receptors, monoclonal antibodies to HLA-DR, CD1a, CD3, CD19, CD20, CD40, CD80 & CD86 (Becton Dickinson Immunocytometry Systems, California, USA) and CD49d (Pharmingen, California, USA) were also used. Viable, antibody-labelled cells were identified according to their forward and right angle scatter, electronically gated, and analysed for surface fluorescence on a FACScan flow-cytometer (Becton Dickinson). Fluorescent data were collected and analysed using CellQuest software (Becton Dickinson).

Assay of chemokine and cytokine blister fluid composition

The blister fluid supernatant was analysed for GROα, epithelial neutrophil activating peptide 78 (ENA-78), IL-1β, IL-4, IL-5, IL-8, IL-12, TNFα, macrophage inflammatory protein-1α (MIP-1α), monocyte chemotactic protein-1 (MCP-1), interferon-γ (R&D Systems, Abingdon, Oxford, UK), C3a and C5a (Pharmingen, New Jersey, U.S.A.), histamine (Immunotech, Marseille, France) and bradykinin (Peninsula Laboratories, Merseyside, England) using commercial kits according to the manufacturers' instructions. The appropriate dilution of blister fluid was established for each assay.

Neutrophil preparation from peripheral blood

This has been described (Boyum 1968; Segal & Jones 1980). Briefly, preservative free venous blood was collected into a plastic 50 ml syringe containing 5 u/ml heparin (Leo Laboratories). 10% of the blood volume of pre-autoclaved 10% dextran was added and the erythrocytes allowed to sediment over 45 min. The supernatant was layered over Lymphoprep (Nycomed), centrifuged (1000xg; 10 min; 20 °C), the pellet re-suspended in distilled water for 10 sec and then an equivalent volume of twice normal saline (1.8 g/dl NaCl) added, the cells recovered by centrifugation (500xg; 10 min; 20 °C) and re-suspend in PBS containing 5 u/ml heparin and 5 mM glucose.

Chemotaxis assay

Venous neutrophils were purified from a healthy control and resuspended in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, U.S.A.) containing 1% BSA. Chemotaxis through a membrane containing 3 µm pores of 10^4 neutrophils to 10% (v/v) blister fluid was measured in a 96-well chemotaxis chamber (Neuroprobe, Cabin John, MD, USA) as described (Harvath et al. 1980). Briefly, migrated cells were stained with 5 µl 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue (MTT) for 3 hr at 37 °C and the absorbance measured at 570 nm using an automatic plate reader (Dynatech MR600). The assay could only be performed in a subset of subjects where sufficient blister fluid was available (Table III).

Safety and acceptability

The concentration of cantharidin used in this study equates to 25 µg per blister, a seven-fold dilution of the pharmaceutical preparation used for the removal of plantar verrucae. More than 1 million prescriptions of cantharidin have been dispensed (Tromovitch 1971) without serious adverse effects. There are three case reports associating cantharidin and lymphangitis (Dilaimy 1975; Roth 1976; Stazzone et al. 1998) therefore susceptible patients were not recruited. The technique did not interfere with the subjects' lifestyle and there were no adverse effects. Hyperaesthesia was present in the skin underlying the disc at six hours. A minority of subjects reported discomfort overnight when pressure was inadvertently applied during sleep. The skin re-epithelialised by 7 days. Hyperpigmentation persisted for no more than one to six months.

Table III: Subject characteristics for the assay of neutrophil chemotaxis to cantharidin blister fluid (mean ± standard deviation)

	CD	UC	Control
Number	14	6	9
Age (years)	43.1+/-13.2	42.7 +/- 6.8	29.9 +/- 11.2
Male	9	5	6
Smoking ^"	5	0	2
Body mass index	24.8 +/- 4.3	28.7 +/- 5.6	23.5 +/- 1.9
Objective activity index	1.4 +/-1.2	0.3 +/- 0.4	-
ESR (mm/hr) *"	16.8 +/- 11.8	12.4 +/- 7.3	3.7 +/- 1.6
CRP (mg/l)	9.6 +/- 7.9	12.4 +/-7.3	2.5+/- 1.7
Leukocyte count (x10^9/l)	8.4 +/- 2.1	5.8 +/- 1.3	6.7 +/- 1.3
Neutrophil count (x10^9/l)^	5.7 +/- 2.0	3.2 +/- 0.8	3.9 +/-0.9
Monocyte count (x10^9/l)	0.7 +/- 0.2	0.5 +/- 0.1	0.6 +/- 0.2
Haemoglobin (g/dl)	13.7 +/- 1.4	15.2 +/- 0.7	14.3 +/- 0.6

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein
Significant difference ($p<0.05$) by the unpaired 2-tailed t test
*CD : control; ^CD : UC; "UC : control

Statistics

A p-value of < 0.05 was taken as significant. Results are expressed as mean ± standard error unless specified. Technical errors required blinded data adjustment as stated: three blisters formed in five patients, attributed to disc slippage; the smallest two by volume were combined and the cell number was calculated in proportion to the volume. In two subjects it was obvious the blisters had joined; the blister volume was halved. In one subject a blister had obviously burst and in three subjects not developed; the results for the second blister were assumed to be identical to the first.

Logarithmic transformations to normalise the distributions and obtain equality of variance were applied to disease duration; body mass index; leukocyte, platelet and neutrophil counts; erythrocyte sedimentation rate (ESR); C-reactive protein (CRP); vitamin B$_{12}$; folate; ENA-78; GROα; blister volume, cell concentration, CD14$^+$ and CD16$^+$ cell concentration.

One way ANOVA was used to determine differences in blister cell and mediator composition and blister fluid chemotactic potential, with significance compared to control subjects quantitated by the unpaired two-tailed *t* test.

Analysis of covariance (ANCOVA) was performed using Intercooled Stata (version 6) (Stata Corp., Texas, U.S.A.), to adjust for effects of potential prospectively recorded confounding variables (season blistered; age; gender; ethnicity; disease duration; disease site; subjective and objective (Kozarek et al. 1989) activity indices; body mass index; smoking status; history of previous intestinal resection; extra-intestinal IBD manifestations; topical steroid use; oral steroid use; azathioprine use; NSAID use; mesalazine use; use of no medication; full blood count; ESR; serum iron, vitamin B_{12}, folic acid, creatinine, albumin and CRP) on the outcome variables (chemokine, cytokine and histamine concentrations; blister volume and cell concentration; blister $CD14^+$ and $CD16^+$ cell concentration). The final model was obtained using stepwise elimination. Variables were removed from the model if the p-value corresponding to the coefficient was not significant (after adjusting for other variables in the model). In borderline significance, the alteration of the coefficients relating to the other variables before and after removal was considered. Partial R^2 was calculated by dividing partial sum of squares for the relevant variable by the total sum of squares.

Associations between blister cell and mediator composition or between chemotactic potential and blister mediators was assessed using pairwise (Pearson) correlation with adjustment for multiple comparisons using the Bonferroni all-pair-wise multiple comparison.

RESULTS

Blister volume

Cantharidin-induced blisters allowed the simultaneous analysis of both the cellular infiltrate and inflammatory mediators present at sites of acute inflammation. At 24 h a blister approximately 1-2 cm in diameter had formed. The volume of blister fluid and the number of cells collected varied between the subjects studied. Cellular viability remained high (92-100%). Blister volume was similar in CD, UC and control subjects (ANOVA p = 0.52; CD 258 ± 39 µl (p = 0.46); UC 304 ± 41 µl (p = 0.72); control 294 ± 42 µl).

Cellular composition of infiltrate

Granulocytes were the predominant cell population within the infiltrate, showing high forward scatter values on the light scatter plot due to their dense granularity. The leukocyte infiltrate was characterised with antibodies to CD16 (FITC) and CD14 (PE); CD19 (FITC) and CD20 (FITC) and CD3 (PE); CD16 (FITC) and CD49d (PE). Staining of the cellular infiltrate for CD16 and CD14 subdivided the neutrophil and monocyte/macrophage populations, respectively, confirmed by back gating onto the light scatter plot. Staining of cellular infiltrate for CD3 and CD19/20 identified T lymphocyte and B lymphocyte cell populations, respectively; the latter rare in the blister infiltrate. Eosinophils were identified by the co-expression of CD16 and CD49d and their relatively high FSC values. The proportion of cell sub-types were similar when viewed microscopically (Figure 3).

Paired duplicate blisters were analysed in one normal individual at 24 h and 48 h and demonstrated similar volume (253 µl & 199 µl), cell concentration (2.3 x 10^6/ml & 2.6 x 10^6/ml) and CD14$^+$ cell concentration (58% & 58%) but a marginal reduction in CD16$^+$ cell concentration (23% to 16%).
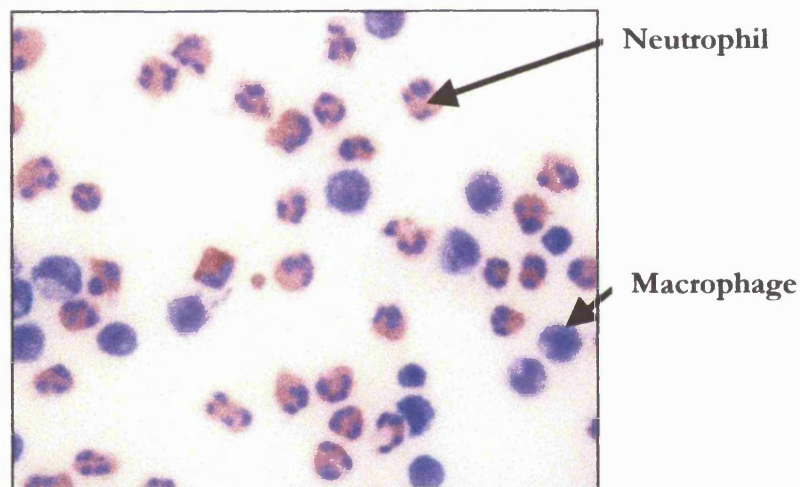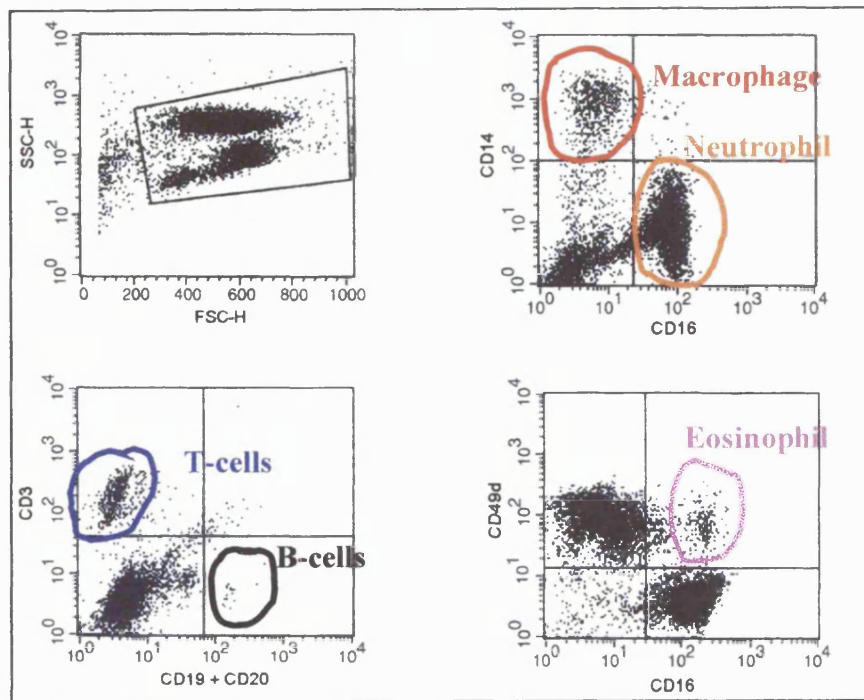
Figure 3: Composition of the cellular infiltrate from a typical blister. FACS scatter plots reveal the majority of cells to be granulocytes. The next most common are monocytes/macrophages. Immunophenotyping the cellular infiltrate provides an estimate of the proportion of CD16+ neutrophils and CD14+ monocytes/macrophages; CD3+ T cells and CD19/20+ B cells; and CD49d+/CD16+ eosinophils. Giemsa staining of a blister fluid cytospin preparation demonstrates the high proportion of granulocytes within the blister infiltrate.

In healthy subjects, the majority of cells collected from each subject were neutrophils (CD16$^+$) (63 $\pm$ 11%). Monocytes and macrophages, identified by CD14$^+$ labelling were the second largest cell population found in the blister (14 $\pm$ 6%). There was a significant reduction in total cell concentration (ANOVA p < 0.05) in the blisters from patients with CD compared to controls (CD 2.27 $\pm$ 0.56 x 10^6 cells/ml blister fluid (p = 0.01); UC 2.85 $\pm$ 0.85 x 10^6 (p = 0.07); control 4.55 $\pm$ 1.09 x 10^6). This was due to a

reduction in CD16$^+$ neutrophils (ANOVA p < 0.05) (CD 0.95 ± 0.30 x 10^6 cells/ml blister fluid (p = 0.005); UC 1.95 ± 0.68 x 10^6 (p = 0.09); control 3.55 ± 1.09 x 10^6). There was no difference in mean CD14$^+$ macrophages compared to controls in either UC or CD (ANOVA p = 0.21) (CD 0.48 ± 0.15 x 10^6 cells/ml blister fluid (p = 0.16); UC 0.35 ± 0.10 x 10^6 (p = 0.08); control 0.59 ± 0.18 x 10^6) (Figure 4). There was no relationship between blister volume and the cellular composition of the blister.
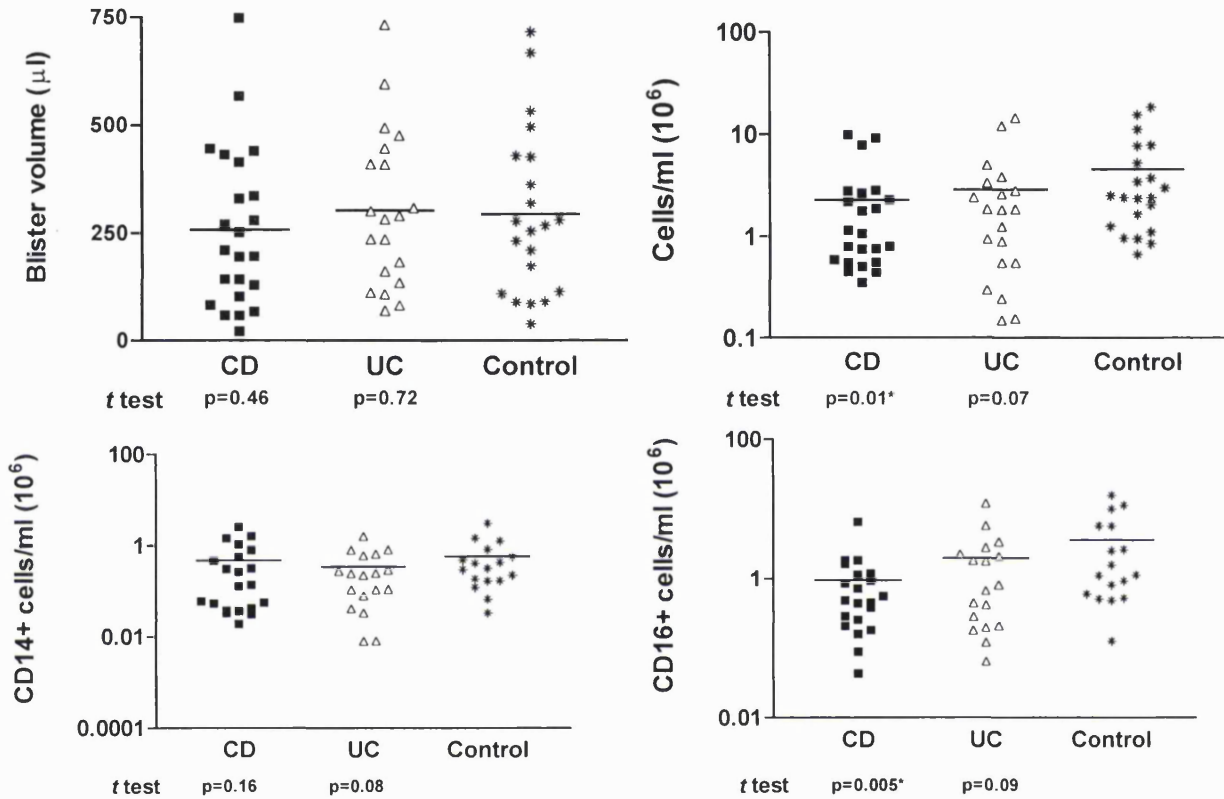
Figure 4: Blister volumes, total cell concentration, CD14$^+$ cell concentration and CD16$^+$ cell concentration in CD, UC and control subjects. There is a reduction in CD16$^+$ cell concentration in CD blisters. Horizontal bars demonstrate mean values. Significant differences determined by ANOVA and delineated as shown compared to the control group by the unpaired two-tailed *t* test. Note the logarithmic scale on the y-axis.

Blister cytokines and chemokines

Measurable blister fluid levels were obtained for the chemokines GROα, ENA-78, IL-8, MCP-1, C3a and C5a (Figure 5 & Table IV); for the cytokines IL-1β, IL-4, IL-12, TNFα; and for histamine (Figure 6 & Table IV). MIP-1α, interferon-γ and bradykinin levels were below the sensitivity of the assay (10 pg/ml, 8 pg/ml and 40 pg/ml respectively). There was no difference for either CD or UC compared to control subjects, except for GROα (ANOVA p < 0.05), which was raised in UC (*t* test

compared to controls p = 0.04) and a borderline significant reduction in IL-5 (ANOVA p = 0.16) in CD (*t* test compared to controls p = 0.05), although 31 samples (equally distributed between IBD and control subjects) were below the assay sensitivity (8 pg/ml). Importantly, there was no relationship between any chemokine or cytokine concentration and blister phenotype.
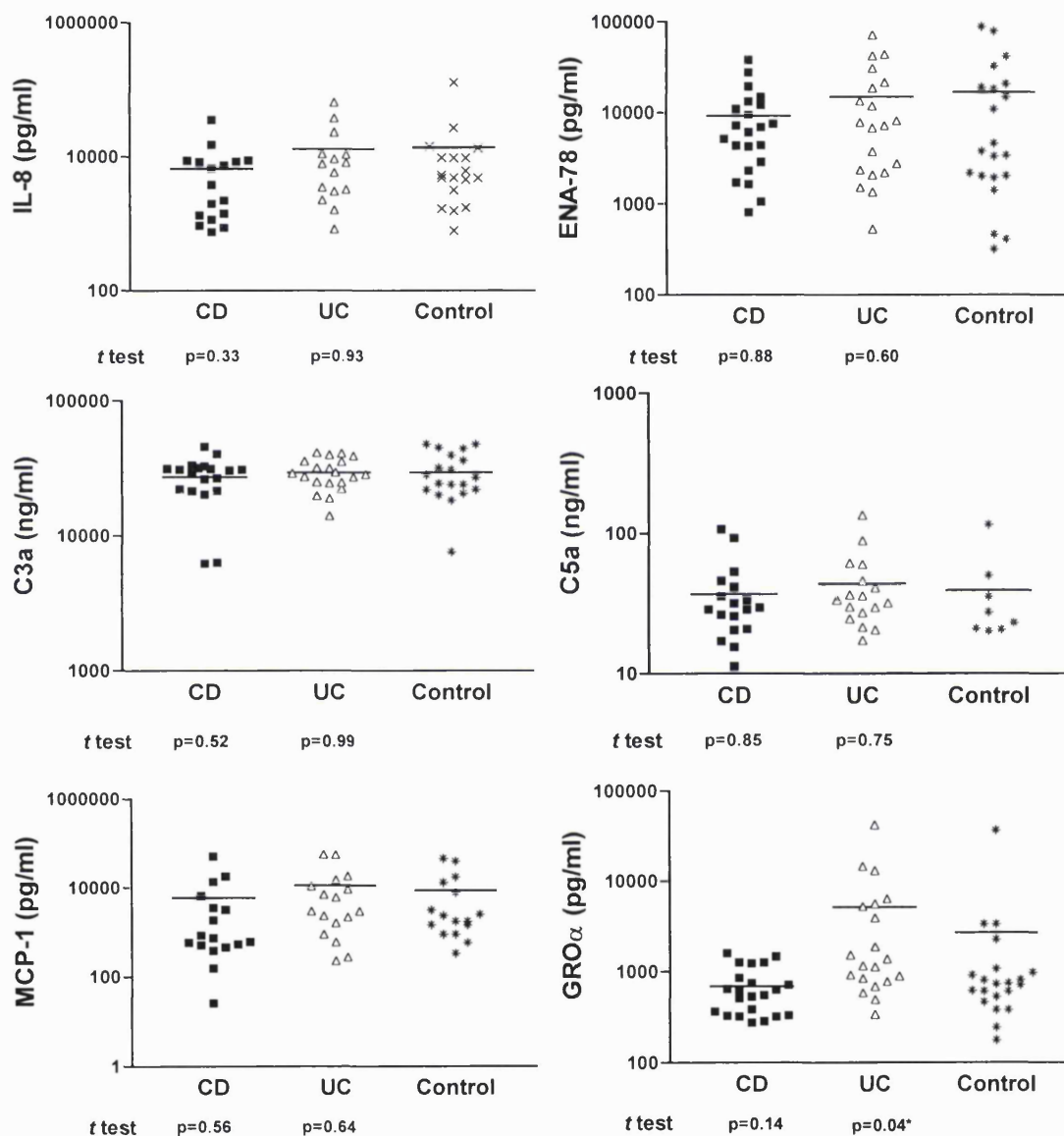
Figure 5: There is no significant difference between either CD or UC and control subjects in the concentrations of ENA-78, IL-8, MCP-1, C3a and C5a. GROα is raised in UC (ANOVA p<0.01; *t* test compared to controls p=0.04). Horizontal bars demonstrate mean values. Significant differences determined by ANOVA and delineated compared to the control group as shown by the unpaired two-tailed *t* test.
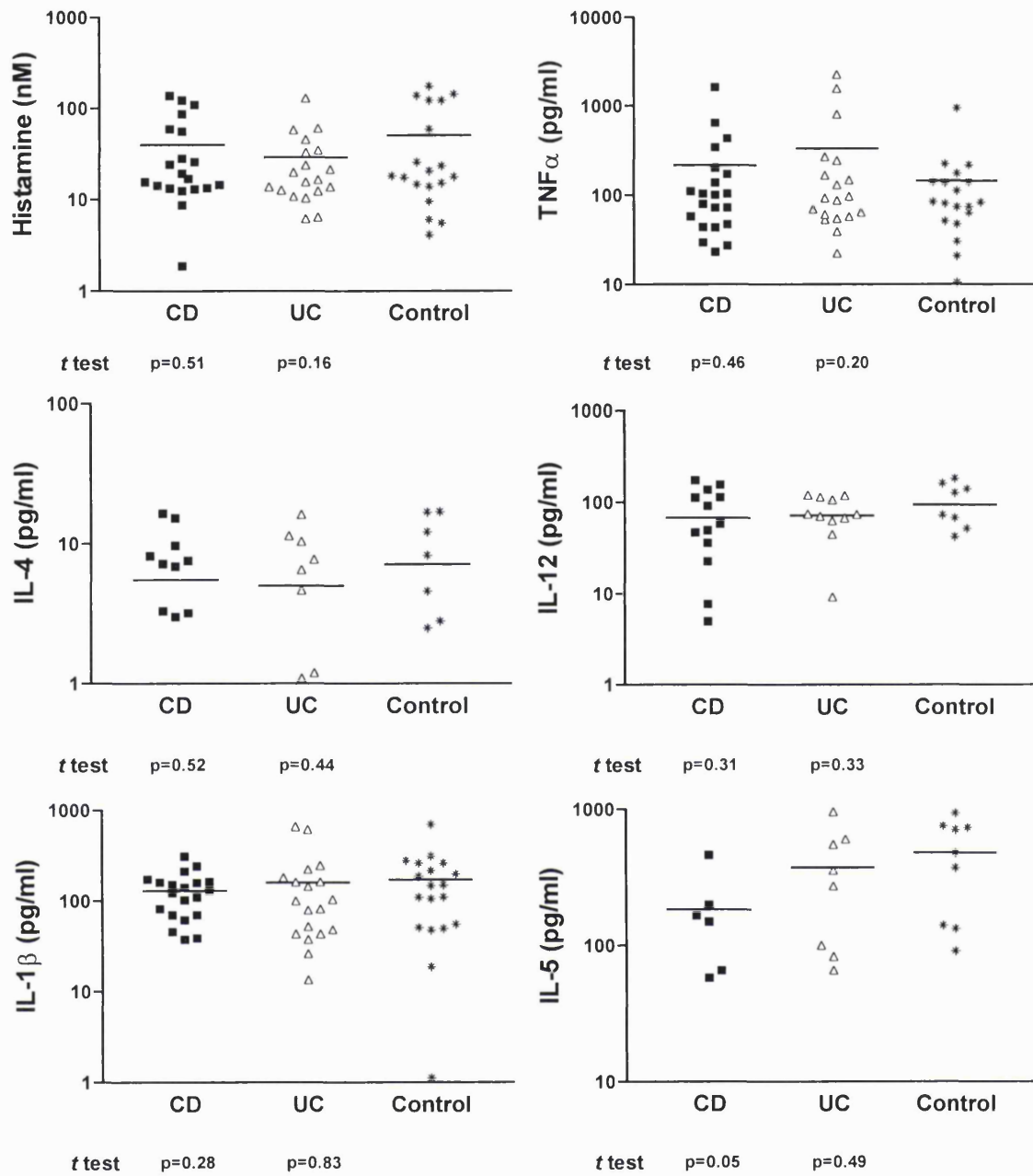
Figure 6: Blister cytokine and histamine concentrations. There is no significant difference between CD and UC and control subjects in the concentrations of Histamine, TNFα, IL-4, IL-12, IL-1β and IL-5. Horizontal bars demonstrate mean values. Significant differences determined by ANOVA and delineated compared to the control group as shown by the unpaired two-tailed *t* test.

Table IV: Chemokine and cytokine concentrations in cantharidin blisters (mean ± SE)

	IL-8 pg/ml	ENA78 pg/ml	GROa pg/ml	Histamine nM
All	11090+/-2844	13530+/-2355	2770+/-901.4	39.80+/-5.875
CD	6641+/-2035(n=17)	9189+/-1963(n=22)	689.6+/-87.70(n=22)	39.81+/-9.340(n=20)
UC	12980+/-4501(n=15)	14940+/-4171(n=20)	5137+/-2129(n=20)*	29.01+/-6.822(n=19)
Control	13710+/-6747(n=18)	16740+/-5477(n=21)	2695+/-1718(n=21)	50.57+/-13.32(n=19)

	MCP-1 pg/ml	TNF pg/ml	IL-4 pg/ml	IL-5 pg/ml
All	8734+/-2095	230.1+/-54.57	5.772+/-0.9438	364.4+/-64.70
CD	6011+/-3049(n=17)	215.2+/-79.32(n=21)	5.527+/-1.377(n=15)	128+/-28.16(n=5)
UC	11420+/-4297(n=17)	333+/-137.3(n=19)	5.050+/-1.585(n=12)	376.8+/-112.1(n=8)
Control	8774+/-3510(n=16)	143.7+/-47.04(n=19)	7.144+/-2.261(n=9)	484.8+/-105.6(n=9)

	C3a pg/ml	C5a pg/ml	IL-12 pg/ml	IL-IB pg/ml
All	30930+/-1986	40.11+/-4.274	75.84+/-8.994	154.2+/-18.93
CD	24940+/-3560(n=10)	37.09+/-5.996(n=18)	67.83+/-15.41(n=15)	129.8+/-16.15(n=20)
UC	32260+/-2553(n=8)	43.66+/-7.203(n=17)	71.98+/-11.51(n=12)	160.9+/-42.13(n=19)
Control	35410+/-3167(n=11)	39.38+/-11.57(n=8)	94.33+/-20.53(n=9)	173.2+/-36.63(n=19)

CD, Crohn's disease; UC, ulcerative colitis
SE, standard error; *p=0.04 by the unpaired 2-tailed t test

Confounding factors

Within disease groups no confounding effect was observed, except for oral steroid use associated with increased GROα levels in UC patients. Specifically, there was no significant effect due to inter-current use of steroids; mesalazine or NSAID's. When all the subjects were analysed, potential confounding effects were found (Table V & Figure 7).

Table V: Potential confounding variables on cantharidin blister phenotype

Outcome Factor	Confounding variable	Coefficient (x1000)	Std. Error	Partial R^2 (x100)	p
GROa (in UC)[1]	Oral steroid use	0.711	0.314	0.06	0.027
ENA-78[2]	Age	-0.014	0.005	0.145	0.002
GROa[2]	Oral steroid use[a]	0.458	0.169	0.107	0.009
CD14+ cells / ml[2]	5-ASA use	-0.394	0.197	0.101	0.015
Cells / ml[2]	5-ASA use[b]	-0.264	1.22	0.07	0.035
Blister volume[2]	Gender[c]	0.306	0.078	0.197	<0.001
Blister volume[2]	Intestinal resection	-0.312	0.111	0.114	0.006

[1] Analysed for each disease category
[2] Analysed independent of disease status
[a] A significant interaction was present between oral steroid use and age (p=0.007)
[b] A significant interaction was present between 5-ASA use and the subjective activity index (p=0.023)
[c] A significant interaction was present between gender and ESR (p=0.015) and the subjective activity index (p=0.030)
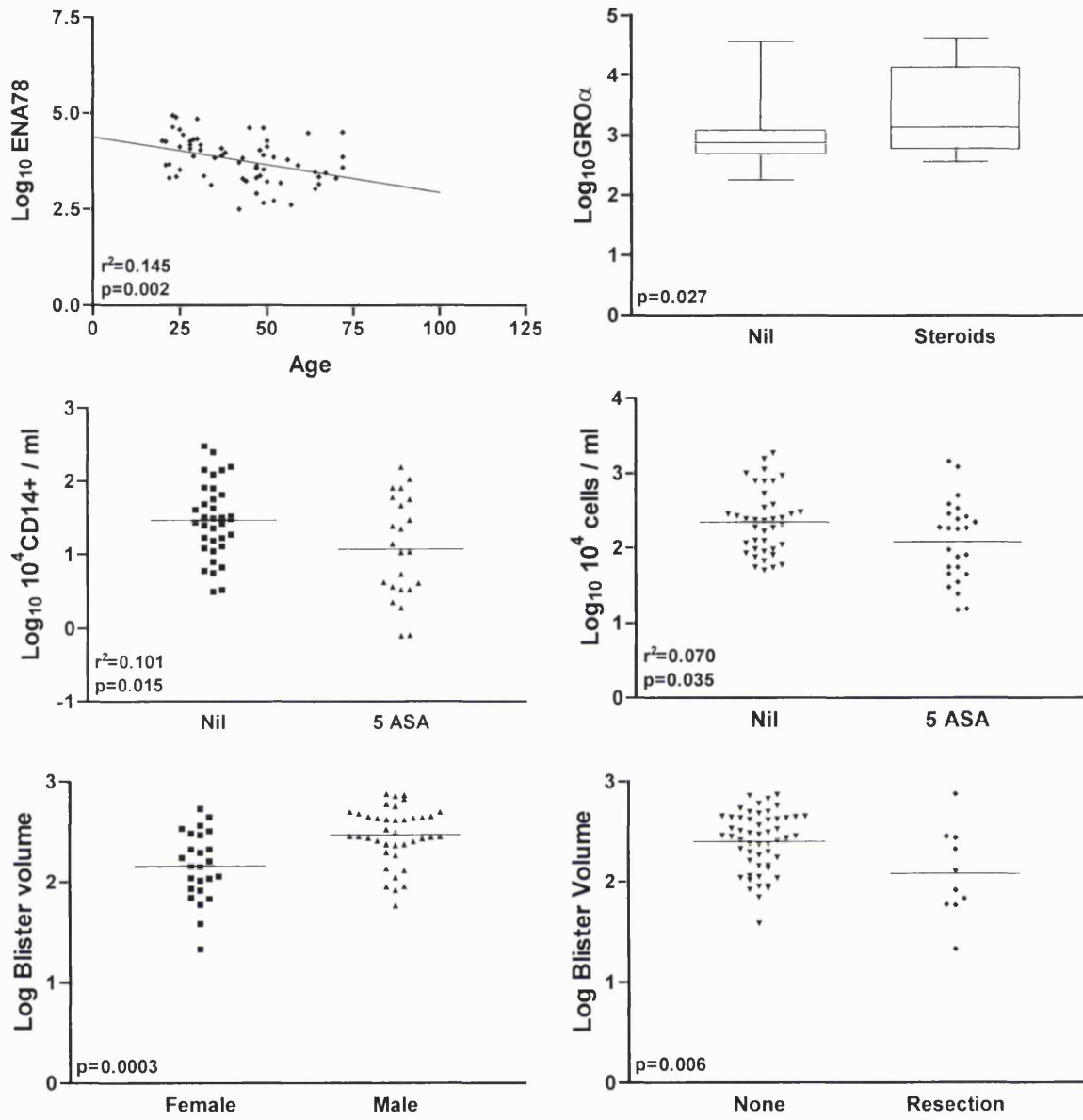
Figure 7: Relationship between outcome factor and potential confounding variable in all subjects studied. Oral steroid use affecting GROα was the only disease specific relationship; the findings above were disease-independent.

There was no relationship between venous and blister neutrophil concentrations; there was a non-significant, positive association between venous monocyte and blister macrophage concentrations counts, most pronounced in the patients with CD (Figure 8).
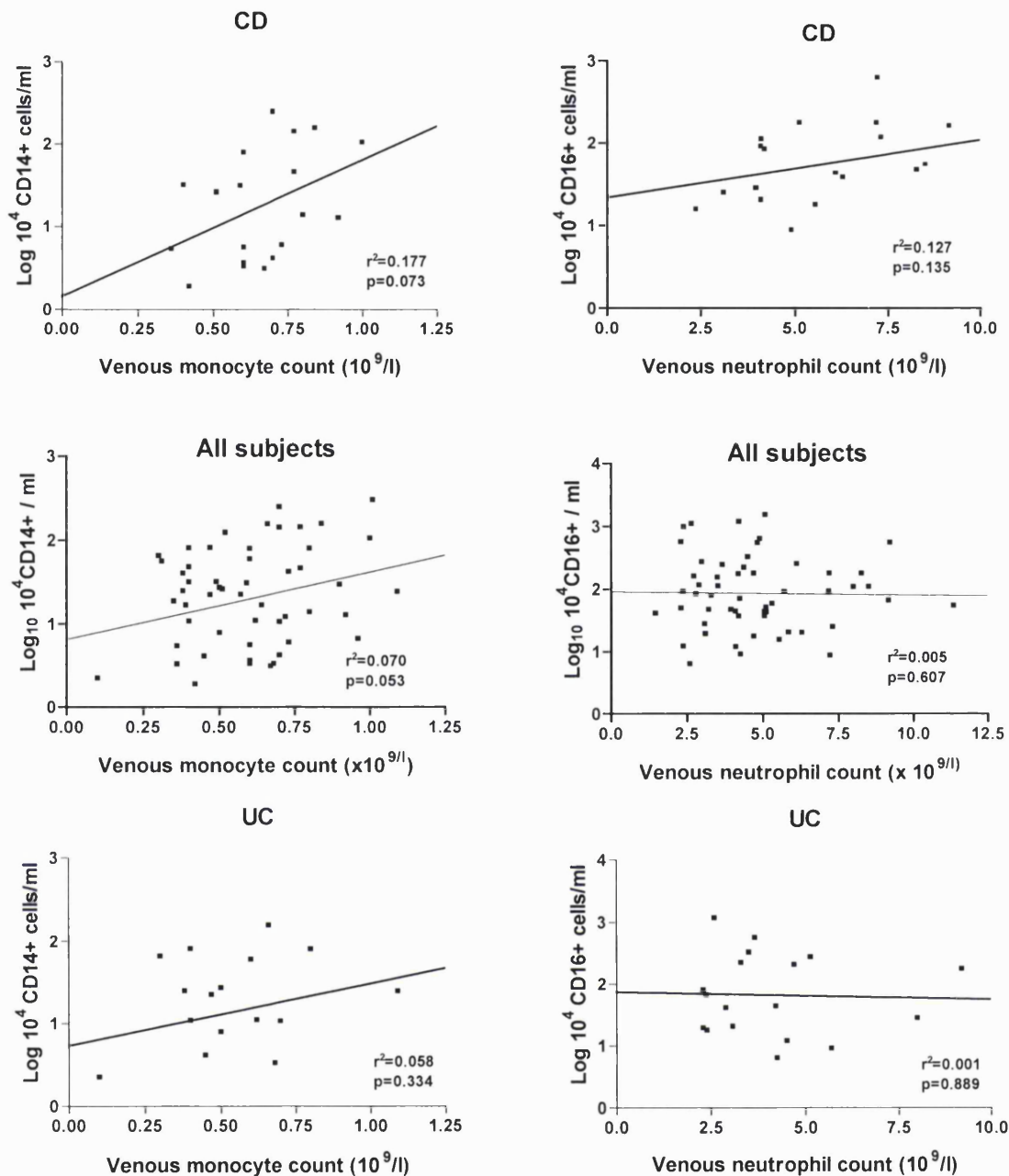
Figure 8: The correlation between venous and blister cell subtype concentration is strongest for CD.

Validity

Duplicate cantharidin blisters were induced on two occasions in 14 subjects (12 with CD). Intra-subject correlation was strongly positive for blister volume ($r^2 = 0.58$, $p < 0.01$) but there was no relationship for cell concentration ($r^2 = -0.008$, $p = 0.75$), $CD14^+$ cell concentration ($r^2 = 0$, $p = 0.98$) or $CD16^+$ cell concentration ($r^2 = -0.14$, $p = 0.31$). There was a significant correlation in the blister phenotype between synchronous blisters in all control and all CD subjects studied but not subjects with UC (Table VI).

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Table VI: Correlation co-efficient r^2 (p value) for synchronous blisters

	CD	UC	Control	All
Volume	0.301 (0.010)	0.022 (0.534)	0.229 (0.033)	0.102 (0.013)
Cells / blister	0.233 (0.027)	0.030 (0.465)	0.743 (<0.001)	0.344 (<0.001)

Chemotaxis assay

There was no inhibition or stimulation of normal neutrophil chemotaxis by CD or UC blister fluid, compared to control blister fluid (ANOVA p = 0.61) (CD (19500 ± 831 cells/well) (p = 0.53); UC (21820 ± 2260 cells/well) (p = 0.77); control (20810 ± 2260 cells/well)) (Figure 9). There was no correlation between chemotaxis and blister mediators when comparing within CD, UC or control subjects. However, when all the subjects (n = 29) are analysed, chemotaxis positively correlated with blister IL-8 (r^2 = 0.52; p = 0.02) and GROα (r^2 = 0.38; p = 0.04).
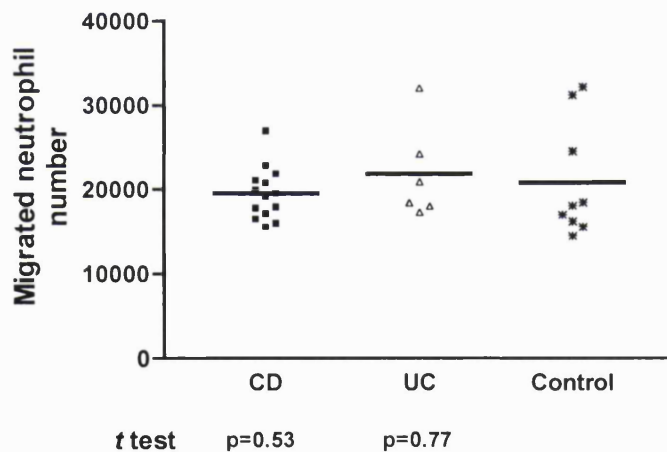
Figure 9: Blister fluid from CD or UC does not modulate normal neutrophil chemotaxis *in vitro*. Horizontal bars demonstrate mean values. Significant differences determined by ANOVA and delineated compared to the control group as shown by the unpaired two-tailed *t* test.

DISCUSSION

Little is known about the acute inflammatory response in CD *in vivo*. No experimental techniques are available to investigate acute inflammation in the human colonic mucosa and there is no animal model available that sufficiently resembles either CD or UC. Therefore cantharidin blisters were employed to examine the composition and function of exudate cells and inflammatory mediators during the first 24 h of the inflammatory response in CD and UC.

A similar skin vesiculation technique has been used to investigate the presence of basophils and eosinophils in normal subjects and patients with bronchial asthma (Kimura et al. 1974). In this study basophils accounted for 0.3% and eosinophils 3% of the cellular elements present in the vesicular fluid obtained from sites of skin injected with various allergens. This is the first time cantharidin has been used to quantify neutrophils and chemokines in cutaneous vesicular exudates. Clinically it has been used as a vesicant in the treatment of plantar verrucae (Miller & Brodell 1996) and experimentally to study the pharmacokinetics of drugs within the interstitial space (Klimowicz et al. 1992; Brunner et al. 1998) and once in order to obtain acute inflammatory cells (Hess et al. 1999).

The procedure was easy to standardise therefore allowed valid comparisons between individuals. The skin covering the blister provided a suitable collection chamber avoiding the need to attach a separate chamber that could have leaked or influenced experimental results. Therefore unlike other skin window techniques, this method allowed the measurement of acute inflammatory mediator concentration within endogenous tissue fluid. The cells were undisturbed at the time of blister sampling, therefore functional assays will approximate *in vivo* cellular kinetics. There are two important points to consider when using cantharidin blisters to investigate IBD.

Firstly, is it relevant to investigate inflammation in the skin? Unknown cellular defects that predispose to IBD might be present at extra-intestinal sites and therefore investigation of inflammation at a surrogate site, for example the skin, may reveal abnormalities that are aetiopathogenic in the gut. Indeed, although there is differential homing of memory lymphocyte subsets between skin and gastrointestinal tissue, regulated by the chemokine receptor CCR4 (Campbell et al. 1999), this is unlikely to

occur in neutrophils and monocytes which are naïve cells that do not respond to the CCR4 ligand TARC (Imai et al. 1997).

Secondly, to what part of the inflammatory response do cantharidin blisters apply? Cantharidin blisters were used to investigate <u>acute</u> inflammation in IBD.   This is important since they are more likely to enable measurement of factors that initiate dysregulated inflammation rather than factors that perpetuate inflammation.   This differs from most assays of inflammation performed in patients with IBD, in which the findings reflect chronic inflammation.   Therefore, even if assays such as white cell labelling (Saverymuttu et al. 1985) were sufficiently sensitive to detect the differences demonstrated here, the test would be performed too late in almost all patients studied.

There were less neutrophils in the blisters of patients with CD at 24 hours, confirming the experiments using skin windows.   Most of the patients studied had quiescent disease and therefore the reduction in CD blister neutrophil number is unlikely to be due to neutrophil sequestration at alternative sites of inflammation.   In support of this, standard measures of disease activity were not associated with blister phenotype when analysed by multivariate analysis.   There was no reduction in blister neutrophil number in UC, which excludes a generalised effect due to colonic disease.   There was no difference in body mass index amongst the patient groups; body mass index correlates with nutritional status (Krondl et al. 1999) and therefore it is unlikely that a reduction in nutrition accounts for the findings.

In vitro studies have shown chemotaxis to be normal (Rhodes & Jewell 1983; Solis et al. 1993) or enhanced *in vitro* (Curran & Keighley 1991), although in one study that used complement C5a as the chemoattractant, it was reduced (Elmgreen 1984).   This study demonstrated no difference in control neutrophil chemotaxis in the presence of IBD blister fluid.   Nonetheless, it is possible that neutrophils from a subset of CD patients contain defective chemokine receptors that cause dysfunctional responses to chemotactic signals at an inflammatory site.   This was not assessed here but has been addressed by an *in vitro* study by O'Morain et al (Morain et al. 1981), who showed that zymosan activated serum increases neutrophil migration equally in CD and control skin windows, demonstrating that the cellular response to chemokines is normal and implicating a defective inflammatory response.   In addition this last study suggested that the neutrophil deficit observed *in vivo* is not due to the presence of serum inhibitors

of migration; a relative impermeability of the extra-cellular matrix; or indeed the early loss of blister neutrophils through apoptosis or necrosis.

Pro-inflammatory cytokines such as TNFα and IL-1 secreted by leukocytes within the mucosa activate endothelial cells, macrophages and fibroblasts to secrete chemokines that have been shown to be elevated in active IBD, such as IL-8 (Mahida et al. 1992; Uguccioni et al. 1999), MCP-1 (Reinecker et al. 1995; Uguccioni et al. 1999) and ENA-78 (Scammell & Keighley 1986; Z'Graggen et al. 1997). The combined action of the early, rapidly acting chemoattraction of IL-8 and the late, long-acting chemoattraction of ENA-78 has been proposed to contribute to the continued attraction of granulocytes into the mucosa of IBD patients (MacDermott 1999). Also, venous neutrophils from patients with intestinal inflammation secrete increased amounts of pro-inflammatory cytokines, such as IL-1β, IL-1ra and TNFα (Nikolaus et al. 1998) and therefore they may contribute to the chemotactic milieu during inflammation.

These studies have been performed in patients with active chronic inflammation and therefore may not represent the cytokine milieu early during inflammation. This is supported by the fact that CD is thought to be TH1 mediated although early in the disease TH2 responses may predominate (Desreumaux et al. 1997). In addition, early recurrent ileal lesions in CD have low neutrophil counts and low IL-8 mRNA and protein production compared with chronic ileal lesions, suggesting that the tissue chemokine profile may differ considerably between acute and chronic phases of CD (Brandt-E et al. 2000). Indeed, very early during inflammation, as measured in skin window chambers, serum chemotactic inhibitory factors have been demonstrated in both CD and UC, which were thought to account for the diminished chemotaxis of neutrophils observed (Rhodes et al. 1982). Therefore it was necessary to define chemokine and cytokine concentrations during acute inflammation in IBD.

There was an increase in GROα in UC. GROα mRNA expression is increased in active UC (Isaacs et al. 1992). However, there was no correlation between this mediator and disease activity. None of the other mediators were abnormal in UC or CD. There was no correlation between any of the mediators and the concentration of cells within the blisters.

There are several explanations. The reduced neutrophil number in CD blisters may not be due to abnormalities in inflammatory mediators. There may be defects in mediators that were not measured. The mediators measured or their receptors may be biologically inactive. There may be differences in mediator concentration earlier than 24 hours. Alternatively, mediator interactions within the inflammatory milieu may be too complex to be deduced by quantification individually. Finally, there may be abnormalities in prostaglandins or leukotrienes, although these were not measured since these compounds are unstable and the quantity of available blister fluid too limited for the assays available.

OXYGEN CONSUMPTION AND DIGESTION IN CROHN'S DISEASE

AIM

To quantify oxygen consumption by CD neutrophils following faecal flora challenge. To measure O_2^- production by CD neutrophils and macrophages derived from cantharidin blisters.

INTRODUCTION

There is evidence that there may be a respiratory burst defect in (at least a subset) of patients with CD. If so this could reduce the digestive capacity of CD neutrophils and macrophages, allowing microbial antigens to persist within the gastrointestinal mucosa and resulting in granuloma formation. However, this defect has only been measured by indirect methods, using O_2^--induced luminol chemiluminescence; O_2^- reduction of ferricytochrome C to the ferrous form; and hydrogen peroxide formation. This has resulted in a confusing and at times contradictory literature, probably biased by the under-reporting of negative studies.

In only one report was a typical gastrointestinal organism used as a stimulus, IgG-opsonised *E. coli* (Morain et al. 1981), in which oxygen consumption was measured using an oxygen electrode, which provides a more physiological assessment of the respiratory burst. No difference between CD and normal patients was found. A similar study is described here, in which autologously opsonised faecal flora or PMA have been used as the stimulus.

More sensitive techniques are available now to measure the respiratory burst, that require only small numbers of cells. These use flow cytometry, which measures and analyses optical properties of single cells passing through a focused laser beam. The cells disrupt and scatter laser light, detected as forward and side scattered light. Forward scatter is related to cell size, side scatter an indicator of cellular granularity. Consequently, cells of different size or internal structure can be differentiated.

Dihydrorhodamine 123 (DHR) is a non-polarised, non-fluorescent, non-toxic cytometric indicator for respiratory burst activity in neutrophil granulocytes (Rothe et al.

1988). It is oxidised intracellularly by O_2^- to brightly fluorescent rhodamine 123. This can be measured in a flow cytometer and when repeated many times can provide a histogram of O_2^- production for each cell type analysed. In this way, the respiratory burst of cantharidin blister derived, non-manipulated, neutrophils and macrophages was measured in CD.

A recent observation in mice with one mutated p47^{phox} allele (p47^{phox} +/-) showed that O_2^- production of peritoneal macrophages (but not neutrophils) was reduced compared to normal mice (N.A. Mitchison, FRS – personal communication). Therefore, O_2^- production was also measured in parents of patients with CGD, to see whether a similar phenomenon occurred.

The high frequency of granuloma formation in CD suggests that persistent antigens may remain to stimulate macrophages. This suggests that poor digestion of antigenic material *in vivo* may be a causal factor. Digestion within the phagolysosome and the fate of the products of digestion has not been investigated in CD. Using radiolabelled *S. aureus*, digestion of bacteria by CD neutrophils has been assessed.

METHODS

Oxygen consumption

Human enteric flora was obtained as described (chapter 2). The flora was filtered through 60 µM gauze, re-suspended in PBS containing 4 mM sodium azide at an OD of 0.365 at 600 nm (equivalent to 5 x 10^9 *E. coli*/ml – Dr. J. Roes, personal communication) and stored in 1 ml aliquots at –20 °C until required. Aliquots were opsonised at 37 °C for 1 h with 1 g Human Normal Ig (Bioproducts Labs., Elstree, Herts, UK) in 30 mM Tris-HCl, pH 8.6 or with autologous serum, then washed twice in PBS (4000x*g*, 20 min; 25 °C) before use.

Neutrophil oxygen consumption was measured in an oxygen electrode (Rank Brothers Ltd., Canterbury, England) (Root et al. 1975). As oxygen is consumed, the oxygen tension adjacent to the sensor elements falls, which reduces the current across the electrode. Initially, sodium dithionate was added to 1 ml fresh water at 37 °C, which contains 217 n.mol of oxygen, to calibrate the electrode. The semi-permeable membrane was replaced and the electrode re-calibrated at the beginning and half way through each experiment in order to avoid debris accumulation.

Initial experiments were performed measuring oxygen consumption of (i) an increasing concentration of buffy-coat neutrophils (Blood Transfusion Service, UK) in response to 1 x 10^9/ml human IgG-opsonised or non-opsonised faecal flora, in duplicate; and (ii) maintaining a neutrophil concentration of 2 x 10^7/ml and increasing the bacteria to neutrophil ratio.

Subsequent experiments were performed in triplicate or quadruplet, using 1 x 10^7 neutrophils from subjects whose characteristics are shown (Table I), and 1 x 10^9 faecal flora in a final volume of 1 ml, opsonised with autologous sera. The maximum rate of O_2 consumption (nmol O_2/10^7 neutrophils/min) and the delay until the maximum oxygen consumption (sec) were measured. 1 µg PMA in 1 µl DMSO was used as a stimulus instead of faecal flora for the second measurement.

Table I: Characteristics of subjects donating blood for assay of neutrophil oxygen consumption (mean ± standard deviation)

	Control	CD	UC
Number	20	18	6
Age (years)	52.8+/-16.2	44.4+/-16.8	44.2+/-20.2
Male	13	9	3
Smoking	6	6	0
Mesalazine	0	11	4
Oral steroids	0	4	2
Active disease	0	6	2
ESR (mm/hr)	14.1+/-10.2	26.2+/-18.1	26.0+/-35.8
CRP (mg/l)	4.4+/-4.2	13.0+/-21.5	27.1+/-61.7
Leukocyte count (x10^9/l)	7.7+/-1.7	8.8+/-3.5	10.1+/-3.3
Neutrophil count (x10^9/l)	4.5+/-3.3	6.1+/-3.6	7.1+/-3.6

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein

Respiratory burst activity of blister cells

13 Caucasian patients with CD and 12 Caucasian non-inflammatory control patients were recruited (Table II). Data from 5 individuals was excluded, due to insufficient cell number (1) and technical error (2) in CD; and insufficient cell number (1) and indistinct cell populations (1) in control patients.

Table II: Subject characteristics for the assay of blister cell respiratory burst (mean ± standard deviation)

	CD	Control
Number	10	10
Age (years)	44 +/- 13	46 +/- 11
Male	6	4
Smoking	4	2
Body mass index*	22.8 +/- 3.1	26.4 +/- 2.0
Objective activity index	2.8 +/- 2.1	-
ESR (mm/hr)	14.0 +/- 10.5	9.5 +/- 4.5
CRP (mg/l)	5.9 +/- 6.7	2.6 +/- 1.5
Leukocyte count (x10^9/l)	8.1 +/- 2.5	6.3 +/- 0.8
Neutrophil count (x10^9/l)*	5.5 +/- 2.5	3.5 +/- 0.6
Monocyte count (x10^9/l)*	0.6 +/- 0.1	0.4 +/- 0.1
Haemoglobin (g/dl)	13.7 +/- 1.7	13.9 +/- 0.7

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein
Significant difference (p<0.05) by the unpaired 2-tailed *t* test

S. aureus (National Collection of Tissue Culture #12981) was grown in 200 ml LB broth (Gibco) at 37 °C for 18 h, concentrated (4000x*g*; 20 min; 25 °C), opsonised with Human Normal IgG, re-suspended in 5 x 10^{10} *S. aureus*/ml aliquots and stored at -20 °C (OD of 0.165 at 600 nm equivalent to 1 x 10^8 cells – Dr. Jürgen Roes, personal communication).

Duplicate cantharidin blisters were induced as described previously. The cells were maintained at room temperature after blister sampling. Within 30 min, 2 - 5 x 10^5 cells were incubated at 37 °C in a final volume of 600 µl of AIM V medium (Life Technologies) containing 1 µM Dihydrorhodamine (Sigma) in 0.1% DMSO and either 0.5 µg PMA (Sigma); or 5 x 10^8 colony forming units of *S. aureus*; or AIM V medium alone.

3000 events were recorded and neutrophil and macrophage populations identified by characteristic forward and side scatter properties (Figure 1). The fluorescence produced was measured at 0, 15 and 30 min, using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, California). Geometric mean fluorescence was calculated using CellQuest software (Becton Dickinson).
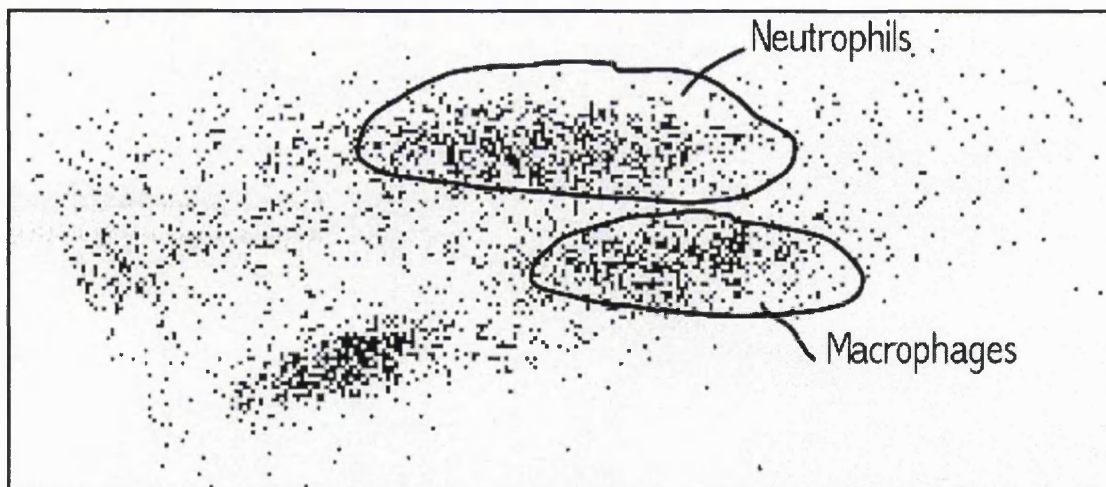
Figure 1: Scatter plot of blister fluid. Neutrophils were the predominant population, characterised by increased granularity and therefore side scatter. The unlabelled population in the lower left quadrant are lymphocytes.

In some of the patients, erythrocytes were removed from 200 µl of heparinised (5 u / ml) blood by 10 s osmotic lysis in water. The leukocytes were retrieved (500x*g*; 5 min;

°C) and re-suspended in AIM V medium and analysed in parallel with blister fluid. 20,000 events were recorded and neutrophil and macrophage populations identified as above.

Respiratory burst activity of p47^{phox} and p67^{phox} heterozygotes

Parents of CGD patients deficient in p47^{phox} and p67^{phox} were recruited and the respiratory burst of cells from cantharidin blisters analysed. Characteristics of the subjects are shown (Table III).

Table III: Subject characteristics for the assay of blister cell respiratory burst (mean ± standard deviation)

	p47^{phox}+/-	p67^{phox}+/-
Number	3	2
Age (years)	54 +/- 12	63 +/- 2
Male	1	1
Smoking	2	1
Body mass index	29 +/- 5.1	31.9 +/- 2.5
CRP (mg/l)	3.9 +/- 3.5	15.0 +/- 15.7
Leukocyte count (x10^9/l)	7.8 +/- 1.0	7.0 +/- 0.8
Neutrophil count (x10^9/l)	4.6 +/- 1.0	4.4 +/- 1.0
Monocyte count (x10^9/l)	0.6 +/- 0.2	0.5 +/- 0.2
Haemoglobin (g/dl)	14.4 +/- 1.3	13.9 +/- 0.3

CRP, C-reactive protein

Neutrophil digestion in CD

S. aureus was grown in 50 ml LB broth (Gibco) containing 250 µCi ^{35}S-methionine (Amersham) for 18 h at 37 °C, pasteurised at 60 °C for 15 min, washed three times in water and pelleted at 4000x*g* for 20 min at 4 °C. This was opsonised with 1.5 g Normal Human IgG in RPMI, in a final volume of 5 ml, at 37 °C for 1 h, washed in RPMI, centrifuged at 4000x*g* for 20 min at 4 °C, resuspended in RPMI containing 5 u/ml heparin and stored at 4 °C. The concentration was determined from the OD as before.

Initially pilot experiments were performed with aliquots of 1 x 10^8 human neutrophils purified from buffy coat residues, incubated in a vigorously stirred chamber with a 2 to 5000-fold excess of IgG-opsonised ^{35}S-methionine labelled *S. aureus*. Subsequently, aliquots containing 1 x 10^8 venous neutrophils, purified from 5 patients with quiescent CD (2 male, aged 43 ± 21 years (mean ± standard deviation)) and 5 control patients (1 male, aged 62 ± 15 years), were incubated in a final volume of 500 µl at 37 °C with a

large excess of the IgG-opsonised ^{35}S-methionine labelled *S. aureus* (range 600 to 6000-fold). Three 25 µl aliquots were transferred into 225 µl iced tri-chloro-acetic acid (TCA) at 0, 10, 20, 40 and 60 min and centrifuged at 13000x*g* for 15 min at 4 °C. The pellet was washed once in 150 µl of ice cold TCA and the supernatants pooled and mixed with 2 ml scintillant (UltimaGold, Packard Bioscience Co., CT, USA). After 2 h, the radioactivity was measured in a Tri-Carb Liquid Scintillation Analyzer (Packard Bioscience, Berkshire, UK). In addition, radioactivity of six 12.5 µl aliquots containing ^{35}S-methionine labelled *S. aureus* were also measured. The results are expressed as the % of bacterial radioactivity solubilised by the neutrophils, with comparisons between CD and control neutrophils that had been assayed synchronously. The variability in baseline radioactivity analysed on the same day was only 0.11% (range 0.04 % - 0.28%).

Statistics

A p-value of < 0.05 was taken as significant. Results are expressed as mean ± standard error unless specified. Inter-individual differences were assessed by the unpaired, 2-tailed *t* test. ANCOVA was performed to adjust for age, gender, disease location, subjective disease activity, smoking, medication and blood analyses. Association between maximum venous neutrophil oxygen consumption and delay to the maximum response was assessed using Pearson correlation.

RESULTS

Optimisation of the assay for venous neutrophil oxygen consumption

This study was undertaken to determine oxygen consumption by CD venous neutrophils following the administration of autologously opsonised faecal flora. 5×10^6 neutrophils / assay were sufficient to record the respiratory burst.   Oxygen consumption increased with increasing bacterial number. 1×10^7 neutrophils and a 100-fold concentration of bacteria produced a large oxygen consumption and these concentrations were used for further studies (Figure 2).   The delay to maximum oxygen consumption decreased with both increasing bacterial concentration but also increasing neutrophil concentration, suggesting a paracrine effect.
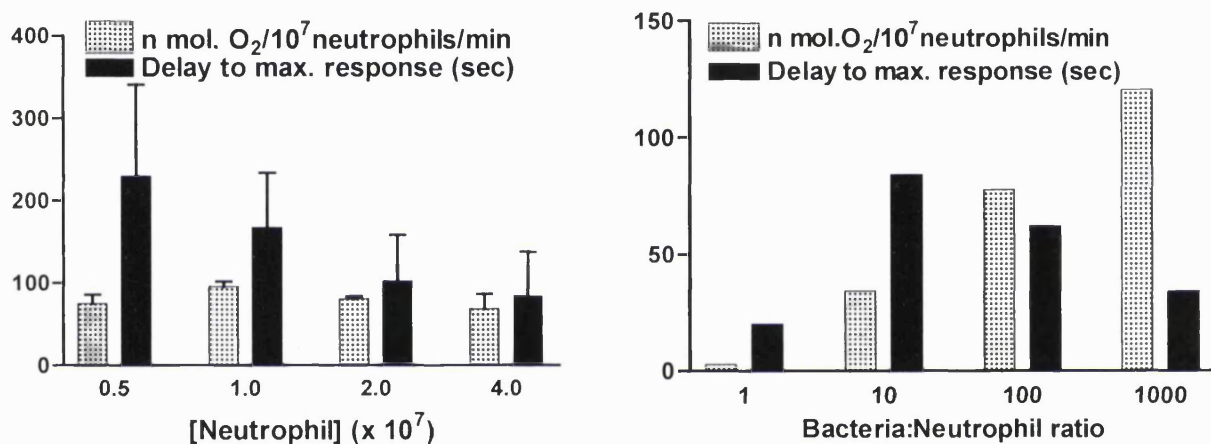
Figure 2: Buffy coat neutrophil oxygen consumption in response to opsonised faecal flora.   The effect of increasing concentrations of neutrophils or opsonised faecal flora on the rate and delay in oxygen consumption is shown.

There was no difference in rate or delay in oxygen utilisation using opsonised or non-opsonised flora (98 $\pm$ 21 cf. 106 $\pm$ 20 n mol. O_2 consumed/10^7 buffy-coat neutrophils/min and 271 $\pm$ 40 seconds cf. 234 $\pm$ 24 respectively).   Nonetheless, future experiments were performed following opsonisation with autologous sera in order to control for sera factors that might affect the respiratory burst *in vivo*.   Spontaneous oxygen consumption by faecal (but not ileal) flora was negligible and minimal by unstimulated neutrophils (data not shown).

Venous neutrophil oxygen consumption is normal in CD

There was no difference in oxygen consumption nor in the delay to the maximum response between CD, UC and controls after faecal flora or PMA challenge (Table IV).

Table IV: Neutrophil oxygen consumption following faecal flora or PMA administration (mean ± standard error)

	Control	CD	UC
Fecal oxygen consumption (n mol.O_2/10^7 PMN/min)	84.8+/-7.8	80.8+/-5.1 p=0.67	80.6+/-25.2 p=0.83
Fecal delay (sec)	222.4+/-20.4	224.0+/-15.2 p=0.95	233.3+/-38.5 p=0.80
PMA oxygen consumption (n mol.O_2/10^7 PMN/min)	45.1+/-7.0	41.2+/-8.0 p=0.71	56.5+/-14.9 p=0.45
PMA delay (sec)	274.3+/-31.5	364.0+/-45.8 p=0.11	331.7+/-33.3 p=0.32
PMA:fecal oxygen consumption	71.3+/-6.1	60.1+/-7.8 p=0.26	74.6+/-10.8 p=0.78

Significance comparing to control subjects determined by the 2-tailed *t* test

Specifically, rate of oxygen consumption was normal in CD subjects (Figure 3).
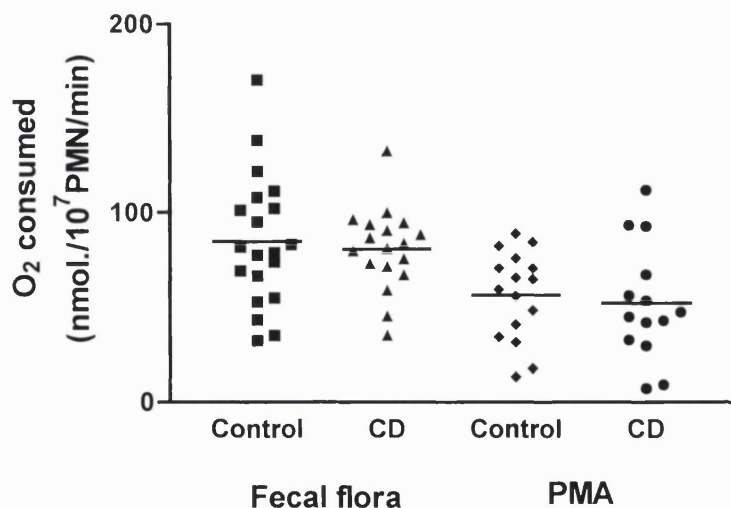
Figure 3: Oxygen consumption by venous neutrophils measured in an oxygen electrode after addition of autologously opsonised faecal flora or PMA. Horizantal bars show the mean values.

However, low oxygen consumption after PMA was noted in two patients with CD that had normal faecal flora responses. The PMA to faecal flora oxygen consumption

for these patients was 9% and 21% (and in the latter, 26% on a separate day), which compares with 60% for all the CD patients or 71% for the controls (Figure 4). It is possible that these patients represent a sub-set of CD patients in whom the pathways involved in PMA activation of the respiratory burst is aberrant.
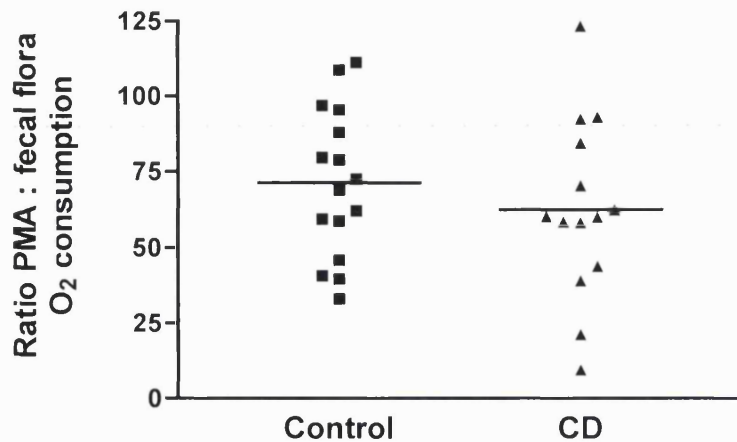
Figure 4: Ratio of oxygen consumption after administration of faecal flora compared to PMA. Two patients with CD had a markedly reduced response to PMA.

Oxygen consumption after the addition of faecal flora correlated with oxygen consumption due to PMA (all subjects $r^2=0.67$, p<0.001; control $r^2=0.69$, p=0.003; CD $r^2=0.72$, p=0.003) but inversely correlated with the delay to maximum oxygen consumption (all subjects $r^2=-0.45$, p=0.02; control $r^2=-0.51$, p=0.02; CD $r^2=-0.55$, p=0.02). This could be explained by cell priming in those subjects that have a larger rate of oxygen consumption.

Multivariate analysis revealed that oxygen consumption following faecal flora or PMA administration was decreased in smokers. There was an increase in the delay to maximum response after faecal flora challenge in patients with raised white cell and neutrophil counts and raised CRP; and after PMA administration in patients with a high haemoglobin concentration and in those receiving mesalazine (Figure 5).
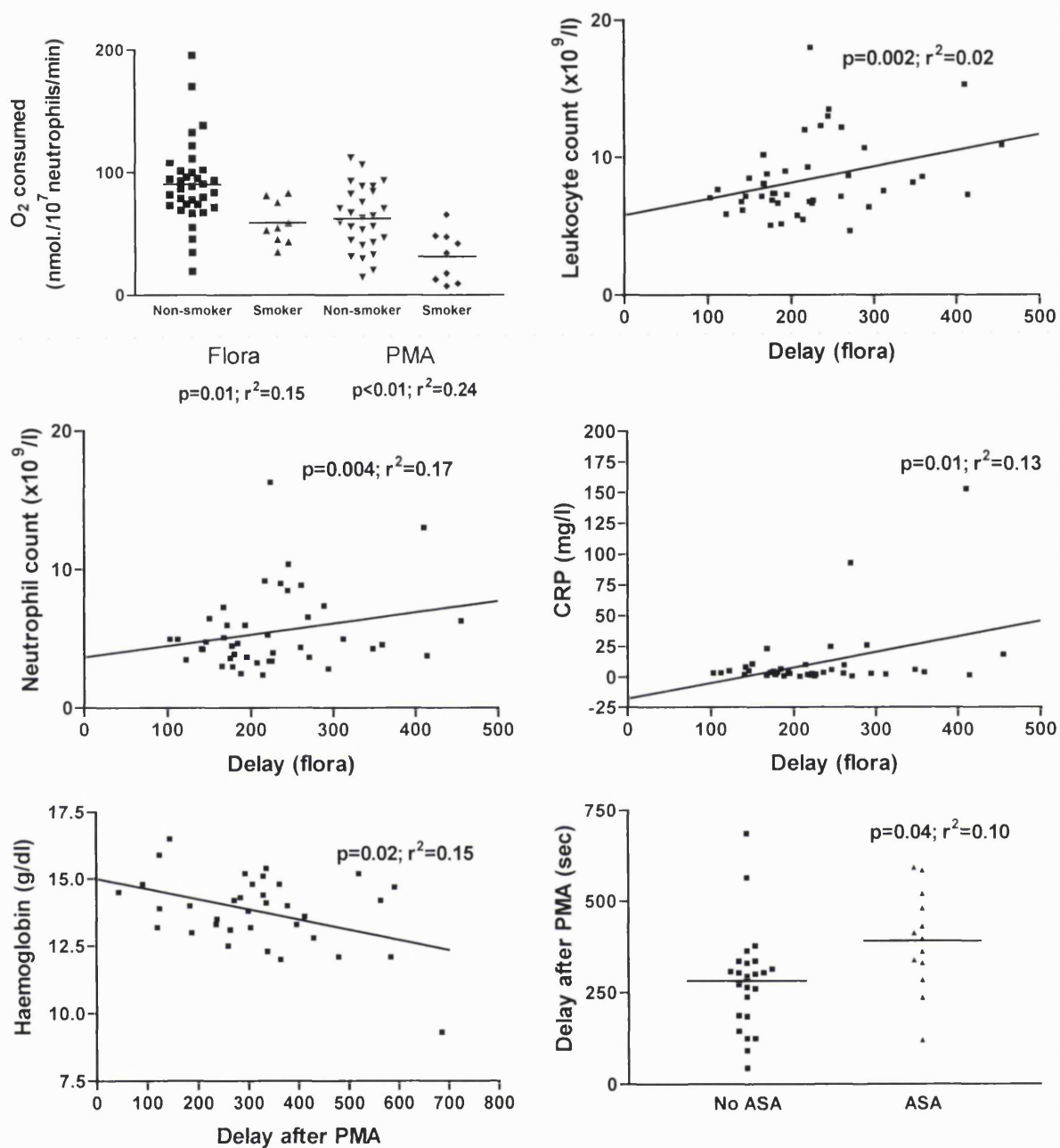
Figure 5: Modifiers of neutrophil maximum oxygen consumption and time to maximum oxygen consumption. The former was negatively associated with smoking status. The latter was positively associated with venous leukocyte and neutrophil concentrations, CRP and ASA use; but inversely associated with venous haemoglobin concentration.

Respiratory burst of cantharidin blister neutrophils and macrophages in CD

There was no difference in blister volume (CD (n = 10) 196 ± 60 μl; control (n = 10) 155 ± 43 μl) or cell concentration (CD 2.4 ± 0.7 x 10^6/ml; control 2.7 ± 0.8 x 10^6/ml). Some unstimulated cells demonstrated a spontaneous respiratory burst; this is likely to be indicative not just of cell priming but also of cell activation during sampling despite

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cell collection designed to minimise *ex vivo* activation (e.g. use of LPS-free siliconised plastic ware; avoidance of major temperature change; analysis within 15 minutes of cell harvest).

There was no difference between CD and control subjects in the respiratory burst activity of neutrophils upon stimulation with either PMA or *S. aureus* or of macrophages upon stimulation with PMA. However, there was a significant increase in the CD macrophage respiratory burst activity 30 minutes after stimulation with *S. aureus* (CD 47 $\pm$ 11 u; control 24 $\pm$ 3 u; p = 0.04) (Figure 6 & Table V). Lymphocyte populations demonstrated a minimal respiratory burst with or without stimulation (data not shown).
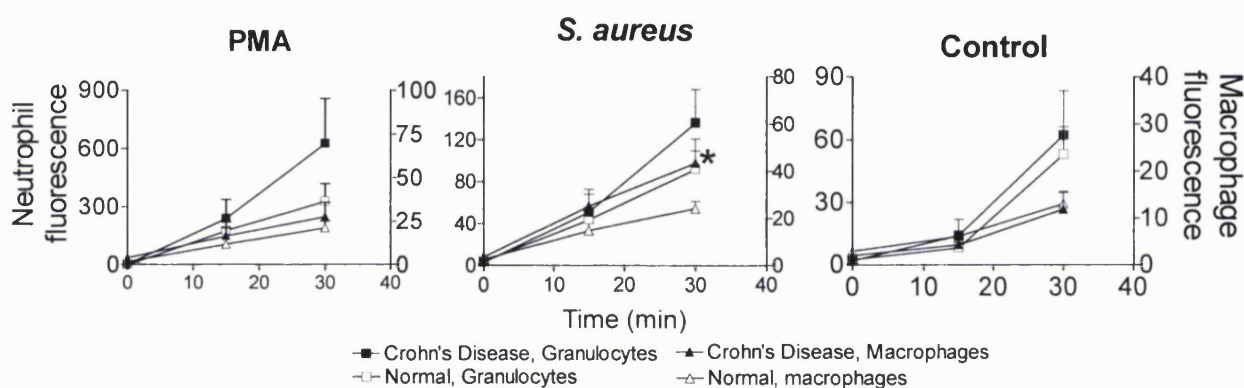
Figure 6: Fluorescence from blister cells during challenge with PMA, *S. aureus* or AIM V medium alone over 30 min at 37 °C. Fluorescence was significantly increased in CD macrophages at 30 min after *S. aureus* challenge. *(p=0.04) determined by the unpaired 2-tailed t test.

Table V: Blister cell mean fluorescence (min^{-1}) (95% confidence intervals); n=8-10

	PMA		S. aureus		AIM V	
	CD	Control	CD	Control	CD	Control
Neutrophil	20.9 (7.2-34.5)	12.1 (6.7-17.5)	4.4 (2.5-6.4)	3.0 (1.9-4.0)	2.0 (0.8-3.3)	1.7 (0.9-2.5)
Macrophage	0.8 (0.3-1.4)	0.7 (0.4-0.9)	1.4 (0.7-2.2)*	0.7 (0.5-0.9)	0.4 (0.1-0.6)	0.3 (0.2-0.5)

*Significant difference at 30 minutes (p=0.04) by the unpaired 2-tailed *t* test

Within disease categories, respiratory burst was independent of confounding variables. Assessing all subjects, there was an association between macrophage respiratory burst activity and age in response to both PMA (partial R^2 (x100) 31.5; p=0.004) and *S. aureus* (partial R^2 (x100) 23.3; p=0.02).

In general, the blister granulocyte response to PMA was greater than the response to opsonised bacteria, in contrast to the venous neutrophil respiratory burst as measured in the oxygen electrode. The macrophage response was broadly similar with both stimuli, but less than the granulocyte response. The stimuli were used in high concentration, therefore it is unlikely that this accounts for the differences seen.

The respiratory burst of venous leukocytes was analysed in parallel in some of the subjects. Surprisingly, the venous granulocyte response was consistently greater, perhaps in part due to stimulation of venous neutrophils during osmotic lysis or due to the relatively greater age of blister neutrophils. The responses of venous monocytes and blister macrophages were broadly similar, although the small number of subjects studied limits interpretation of these results. These findings did not appear to be different in CD compared to controls and therefore further studies were not undertaken

Within each sample of macrophages and neutrophils, there was a bimodal increase in respiratory burst activity, most marked at 30 minutes. When this sub-population was analysed, no significant differences in individual cell fluorescence were found. This suggests that the enhanced O_2^- production in CD found 30 minutes after *S. aureus* challenge did not occur in a subset of highly active cells (Figure 7).
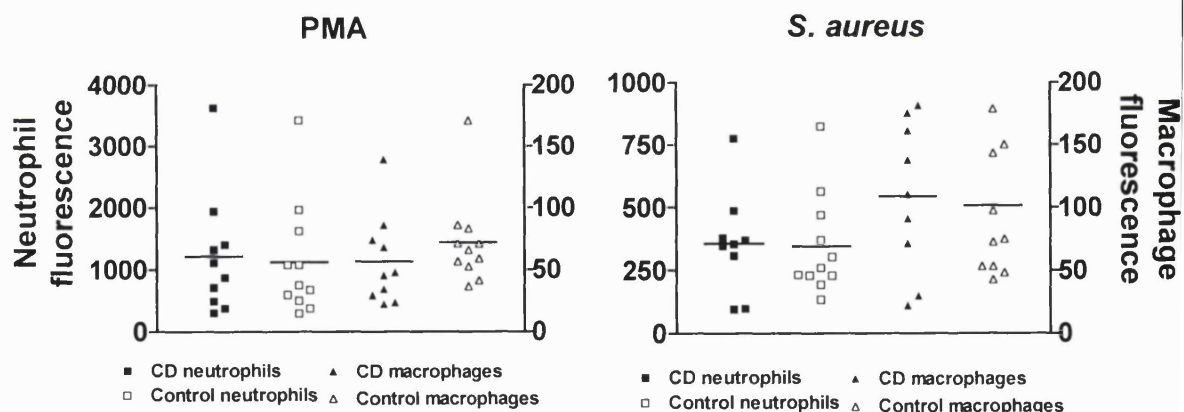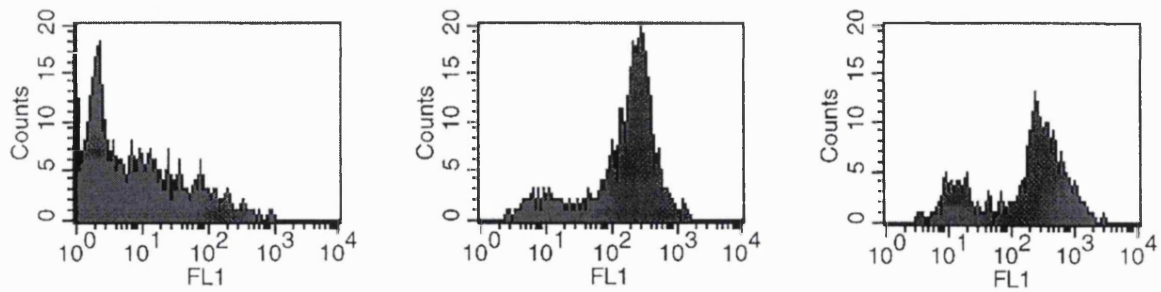
Figure 7: Maximally activated cell populations at 30 min are normal in CD. Histograms depicting typical neutrophil fluorescence intensity are shown in the lower panel (top of this page); left, 0 min; middle 15 min; right, 30 min. At 30 min, two cell populations are seen clearly, with low and high fluorescence intensity. Analysis of the actively fluorescent population is depicted in the top panel (bottom of previous page) and demonstrates no difference between CD and control subjects. Horizontal bars depict mean geometric mean fluorescence.

By back gating onto the flow cytometric scatter plot, the maximally fluorescent macrophages were noted to have reduced cell granularity (i.e. appear lower on the y-axis). In order to characterise this population, naïve CD blister cells were incubated with antibodies reactive with HLA-DR of antigen presenting cells; CD1a (T cell ligand for Natural Killer cells); CD3 (TCR); CD14 (monocyte LPS receptor); CD19 (B cell activator); CD40 (B cell ligand for T-cell CD40L); CD80 and CD86 (macrophage ligands for T cell CD28). Cells expressing high amounts of CD14 (but none of the other cell surface receptors) co-localised with the sub-population of macrophages that had high fluorescence. This suggests that activated macrophages express the LPS receptor at a site of cutaneous inflammation.

Respiratory burst activity of p47^{phox} and p67^{phox} heterozygotes

Cantharidin blister cell O_2^- production was analysed in both parents of a p67^{phox}-deficient CGD patient and in three parents of two p47^{phox}-deficient CGD patients. The parents were presumed to be heterozygous at *NCF1*. There was no reduction in the rate of macrophage or granulocyte respiratory burst compared to control subjects (Figure 8).
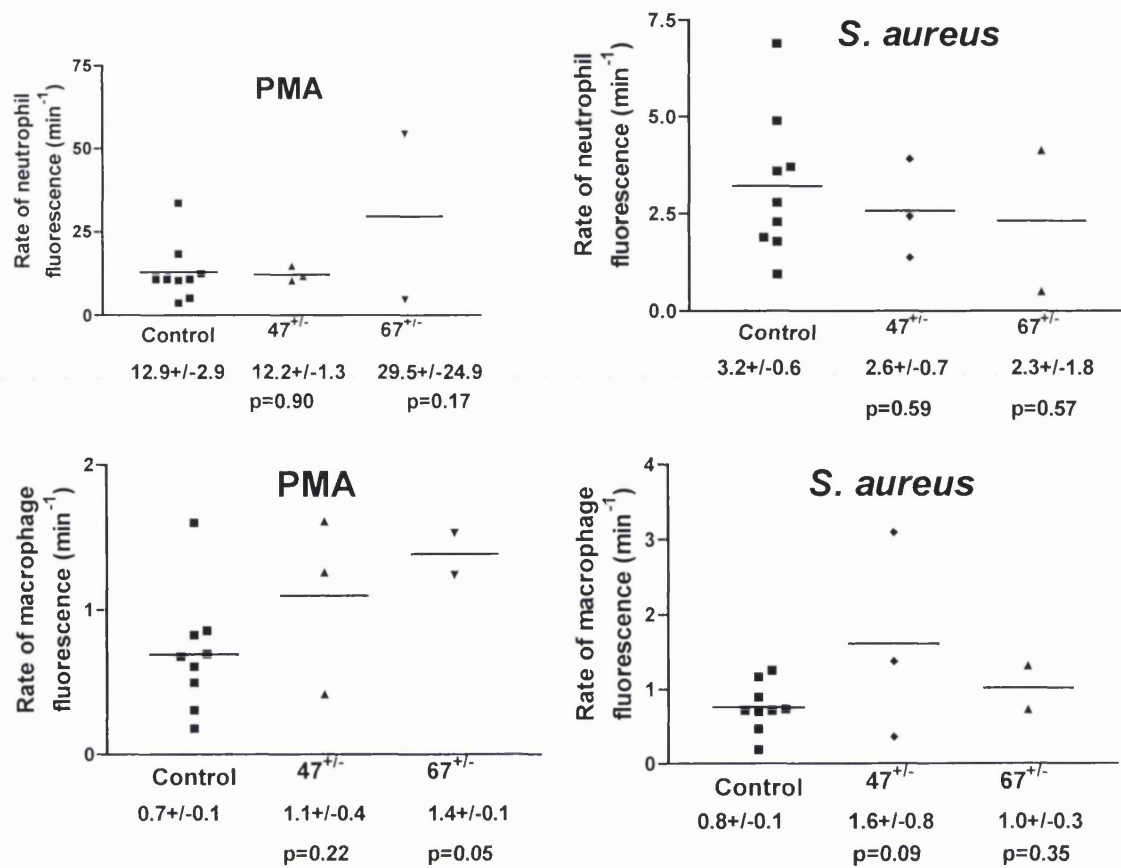
Figure 8: Blister cell rate of change of geometric mean fluorescence. No reduction was observed in patients presumed to be heterozygotes for p67phox & p47phox.

Digestion Assay

S. aureus ^{35}S-labelled protein that has been digested by neutrophils will not be precipitated by TCA and therefore digestion can be measured by the increase in soluble radioactivity after TCA precipitation. Preliminary experiments demonstrated that proportional neutrophil digestion capacity reduced on incubation with more than a 500-fold excess of opsonised *S. aureus*, suggesting this is the threshold for maximal digestion capacity (Figure 9).
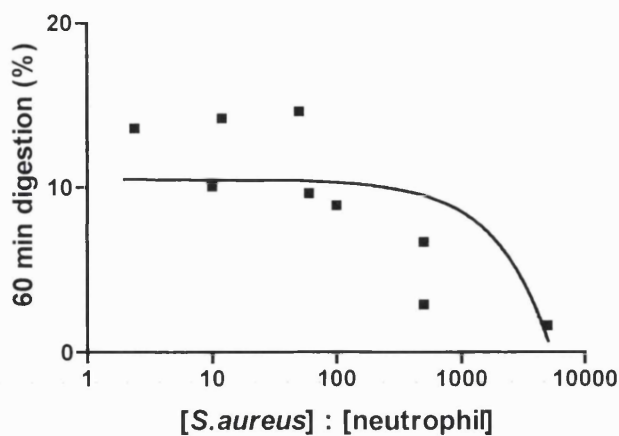
Figure 9: Digestion of ³⁵S-methionine *S. aureus*. Solubilisation of radio-labelled bacteria by neutrophils following incubation for 60 min at 37 °C is saturated when the bacterial concentration is greater than 100-fold the neutrophil concentration.

Therefore, experiments with CD and control patients were performed with at least a 500-fold *S. aureus* excess, in case any deficit was rate limiting only on maximal stimulation. CD and control supernatant radio-activity were similar at 60 min (6.9% ± 2.2 % (mean ± standard error) and 7.0% ± 2.0% respectively), which suggested there was no deficit in maximal digestion capacity in the 5 CD patients studied (Figure 10).
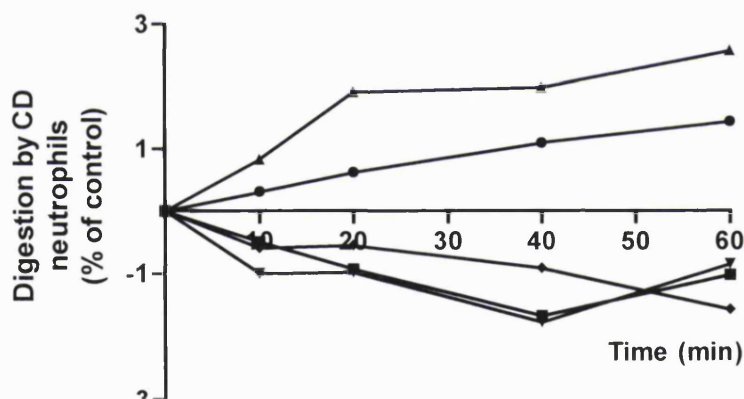
Figure 10: CD neutrophils digest ³⁵S-methionine labelled *S. aureus* normally. Each patient was studied concurrently with a control subject. Each data point represents the mean of triplicate assays. Results are expressed as the % difference in digestive capacity between CD and control subject neutrophils.

DISCUSSION

Due to the granulomatous nature of CD, it was hypothesised that tissue neutrophils or macrophages may have a reduced respiratory burst in CD, resulting in antigen persistence and chronic inflammation. Oxygen consumption of venous neutrophils from CD patients was found to be normal; as was O_2^- production of tissue neutrophils. However, O_2^- production by tissue macrophages following *S. aureus* administration was increased.

There are single reports of respiratory burst activity in CD tissue macrophages (Mahida et al. 1989) and blood monocytes (Baldassano et al. 1993) which describe enhanced activity, probably due to monocyte priming by LPS (Baldassano et al. 1993). This is supported by the association of a high macrophage O_2^- production with enhanced CD14 (LPS receptor) expression. This suggests that LPS-sensing is not rate limiting for O_2^- production in the majority of patients with established CD. This is important since mutations in NOD2 are believed to interfere with intracellular LPS recognition and therefore attenuate NF-κB signalling. Of the 10 CD patients studied here, 5 had ileo-caecal CD and 1 patient ileal disease, therefore this analysis does not refute the hypothesis that NOD2 mutations per se interact with LPS sensing.

It is unclear from the literature whether there is a respiratory burst defect in CD neutrophils. Previous investigators have demonstrated normal oxygen consumption to opsonised latex and to opsonised *E. coli* in the oxygen electrode (Morain et al. 1981); a reduced chemilumenescent response to *S. aureus* (Worsaae et al. 1982); and reduced O_2^- mediated reduction of ferricytochrome C after PMA (Verspaget et al. 1988; Curran et al. 1991; Solis et al. 1993; Gionchetti et al. 1994). In all these studies, the cells were purified from peripheral blood. The study reported here is unique since it provides the first measurement of the respiratory burst from non-manipulated tissue-derived neutrophils and macrophages, largely avoiding *ex vivo* cell stimulation (Fornas et al. 2000). Tissue derived cells may be analogous to the cells present within the gastrointestinal mucosa during acute inflammation.

The number of cells in each assay varied, which can be criticised since it has been reported that cell number is inversely related to rhodamine fluorescence (van Pelt et al.

1996). In addition, it is possible that some of the cells became activated on blister sampling if they came into contact with the skin.

It was striking that there was markedly reduced venous neutrophil oxygen consumption in response to PMA in a sub-set of CD patients. A similar finding has been reported in $p47^{phox}$ heterozygote subjects (Verhoeven et al. 1988) and one report of reduced PMA-induced ferricytochrome C reduction in CD (Solis et al. 1993). The reason for these findings is unknown. Could CRP mediate this effect? CRP has been reported to cause greater inhibition of neutrophil O_2^- production after PMA compared to fMLP stimulation, associated with reduced PKC-β2, $p47^{phox}$ and Rac2 translocation to the membrane (Mortensen & Zhong 2000). However, there was no correlation in serum CRP concentration and the PMA to faecal flora oxygen consumption ratio ($r^2 = 0.002$; $p=0.80$) This does not exclude an effect from tissue CRP or tissue CRP-derived peptides.

The reduced oxygen consumption by neutrophils from subjects that smoke contrasts with previous reports of enhanced O_2^- production in smokers (Anderson et al. 1987; Kalra et al. 1991). It would have been interesting to have measured O_2^- production in the same patients but unfortunately this was not done. The correlation between CRP and delay to maximum oxygen consumption is consistent with reports of inhibition of the neutrophil respiratory burst by this molecule (Mortensen & Zhong 2000). However, there are no reports associating haematocrit and the neutrophil respiratory burst; and sulphsalazine but not mesalazine has been shown to reduce neutrophil O_2^- production (Wandall 1991).

There was no abnormality in protein digestion by neutrophils from CD patients in the pilot study performed. This was a small study however, so does not provide conclusive proof that digestion is normal in CD. Further studies should include assessment of CD neutrophil (and macrophage) digestion of carbohydrate and fat, particularly since the latter has been implicated in the pathogenesis of CD (Roediger 1991).

p47$^{\text{PHOX}}$ HETEROZYGOSITY IN IBD

AIM

To quantify the number of patients with IBD that have a mutation in one allele of *NCF1*, the gene that encodes p47$^{\text{phox}}$.

INTRODUCTION

Mutations in *NCF1*, the gene encoding p47$^{\text{phox}}$, account for one quarter of cases of CGD. Interestingly, in 90% of p47$^{\text{phox}}$-deficient patients a 2 basepair (bp) deletion (ΔGT) at the beginning of exon 2 of *NCF1* (GTGT$\rightarrow$$\Delta$GT) occurs, causing a frameshift mutation leading to a stopcodon (TAA) at amino-acid position 51 (Gorlach et al. 1997). In contrast, deficiency in gp91$^{\text{phox}}$, p67$^{\text{phox}}$ and p22$^{\text{phox}}$ occurs due to sporadic mutations only. The high incidence of this single mutation is thought to be caused by recombination events during meiosis between the proximal part of *NCF1* and one of at least two highly homologous pseudogenes (termed *ΨNCF1*), which contain ΔGT. Indeed, the GTGT locus forms the focal point for recombination events, with common but less frequent mutations comprising conversion of CG$\rightarrow$TG 122 bp upstream of the 5' end of exon 2, a 30 bp deletion in intron 1 and a 20 bp insertion in intron 2 (Roesler et al. 2000b) (Vazquez et al. 2001). The repetitive DNA structure has prevented elucidation of the genomic sequence of 7q11.23. Specifically, the location and number of *ΨNCF1* is unknown.

It is now recognised that 20% of patients with CGD have CD-like chronic inflammation in the bowel (Mitomi et al. 1999) (Winkelstein et al. 2000), believed to be caused by incompletely digested bowel bacteria or food debris stimulating chronic inflammation. This raises the possibility that aberrant neutrophil or macrophage NADPH oxidase function may be a susceptibility factor for IBD.

It was hypothesised that one such factor was p47$^{\text{phox}}$ protein haploinsufficiency. Several pieces of evidence supported this hypothesis. Firstly, primary defects in innate immunity have been described in CD (Hugot et al. 2001) (Ogura et al. 2001) (Segal & Loewi 1976) therefore p47$^{\text{phox}}$, a component of the innate immune system, is a plausible candidate protein. Secondly, the frequency of p47$^{\text{phox}}$ heterozygosity at 1:700 (Roesler

et al. 2000b) is similar to the prevalence of CD at 1:1000 (Sandler & Eisen 2000). Thirdly, in a British genome wide screen for susceptibility loci in IBD, significant linkage was demonstrated for both CD and UC with microsatellite markers spanning a 22 cM region of chromosome 7 (Satsangi et al. 1996) in the middle of which lies the *NCF1* locus. Fourthly, p47^{phox} heterozygotes have reduced neutrophil oxygen consumption in response to PMA but a normal response to opsonised zymosan (Verhoeven et al. 1988). I have noted the same phenomenon in a minority of CD patients. Finally, peritoneal macrophages from mice heterozygous at the p47^{phox} locus produce less O_2^- following PMA administration compared to wild type mice (Prof. NA Mitchison FRS – personal communication). A similar phenomenon in humans could account for the granulomata of CD.

Standard molecular biological techniques can not distinguish p47^{phox} heterozygotes from normal individuals since both have the GTGT and ΔGT sequences. However, since in about 90% of heterozygotes partial gene conversion has occurred from Ψ*NCF1* to *NCF1*, heterozygotes will have an increase in the ΔGT to GTGT ratio. Therefore, assuming the normal genomic ratio of Ψ*NCF1* to *NCF1* is 4 : 2, the haplotype ratio will be 5 : 1. A gene-scan technique (Dekker et al. 2001) was modified and applied following PCR around the GTGT/ΔGT sequence, to quantify this ratio in order to determine whether p47^{phox} haploinsufficiency is a risk factor for IBD.

METHODS

Patients

Venous blood samples were collected in the IBD clinic of University College London Hospitals from patients with CD (n = 76; 39 male; 42 ± 14 years); UC (n = 64; 35 male; 48 ± 18 years); indeterminate colitis (n = 4; 4 male; 31 ± 8 years); from three parents of patients with confirmed p47^{phox}-deficient CGD; and normal controls (n = 37; 22 male; 41 ± 16 years; comprising 22 normal individuals from the University College London faculty and 15 patients with a variety of gastrointestinal conditions (6 with fore-gut irritable bowel disease, 3 with peptic ulcer disease, 2 with gastro-oesophageal reflux disease, 2 with colonic adenoma, 1 with Barretts oesophagus and 1 with chronic pancreatitis) and stored in EDTA at -20 °C. Age, gender, diagnosis, operative history and immuno-suppressant use were recorded.

DNA extraction and purification

5 ml whole blood was added to 25 ml ice-cold sucrose lysis buffer (0.32 M sucrose, 5 mM MgCl$_2$, 1% Triton X100, 10 mM TRIS-HCl pH 7.5) in a polycarbonate 50 ml falcon tube. After centrifugation (20,000xg, 15 min, 4 °C) the pellet was washed vigorously in 30 ml sucrose lysis buffer, re-suspended in 2 ml nuclei lysis buffer (400 mM NaCl, 2 mM EDTA, 1 % SDS, 10 mM TRIS-HCl pH 8.2) and incubated in a shaking water bath for 60 min at 65 °C after the addition of 1 ml 5 M sodium perchlorate. The sample was agitated with 1 ml of ice cold chloroform in a 15 ml falcon tube for 30 min at 25 °C, centrifuged (2000xg, 3 min; 25 °C) and the aqueous phase aspirated into a 15 ml tube. 2 vol of ice cold ethanol were added, the tube inverted several times and the DNA removed and purified as follows.

Each sample was emulsified with an equal volume of phenol : chloroform, centrifuged at 13000xg for 5 min at 25 °C, the aqueous layer aspirated and the extraction of DNA repeated once more with phenol : chloroform and once with chloroform alone. Sodium acetate (final volume 0.3 M) and 2 vol of 100 % ethanol were added and the DNA allowed to precipitate on ice for 1 h. It was removed and washed in 70 % ethanol. For long term storage at 4 °C it was centrifuged (13000xg, 10 min; 25 °C), the pellet air dried for 20-60 min and 100 μl TE Buffer (1 mM EDTA, 10 mM TRIS-HCl pH 8) added to each DNA sample which were left to dissolve (18 h at 25 °C or 1 h

at 65 °C). Sample purity and concentration were determined by electrophoresis through 0.7 % agarose containing ethidium bromide.

PCR of NCF1 and ΨNCF1

DNA from a patient with confirmed p47^{phox}-deficient CGD and from her mother (presumed to be a p47^{phox} heterozygote) were used in every reaction as control samples. Nested PCR primers were designed and produced commercially. The PCR reaction conditions were optimised by co-varying temperature and MgCl$_2$ concentration.

The first reaction yielded a 368 bp gene product and a 386 bp pseudogene product. The reaction components were 60 ng DNA; 25 ng primers (Roesler et al. 2000) ("2LA" (5'-ttc ctc cag tgg gta gtg gga t-3') & "2RC" (5'-aga gaa cat gag gtg ttc aga gt-3')) (MWG-biotech); 1 mM MgCl$_2$, 3 mM dNTP's, 0.1 µl Taq polymerase (Gibco Brl); in a final reaction volume of 15 µl. The reaction temperatures were 94 °C for 1.5 min, 60 °C for 35 sec and 72 °C for 15 sec; then 30 cycles of 94 °C for 35 sec, 60 °C for 30 sec and 72 °C for 15 sec; then 94 °C for 35 sec, 60 °C for 30 sec and 72 °C for 2 min.

The second reaction yielded a 142 and 144 bp product. The reaction components were 0.5 µl PCR product from the first reaction; 25 ng primers ("forward FAM" (5'-tgcac acagcaaagc ctctttgga -3' with 5-carboxyfluorescein attached to 5' end)) & "short reverse primer" (5'- caccg agatctacga gttccatgt -3')) (MWG-Biotech); 1 mM MgCl$_2$, 3 mM dNTP's, 0.1 µl Taq polymerase in a final reaction volume of 12.5 µl. The reaction temperatures were 94 °C for 1.5 min, 59.2 °C for 35 sec and 72 °C for 15 sec; then 30 cycles of 94 °C for 35 sec, 59.2 °C for 30 sec and 72 sec for 15 sec; then 94 °C for 35 sec, 59.2 °C for 30 sec and 72 °C for 2 min. PCR product was confirmed by 8% PAGE. PCR product from a subset of patients was purified following 2% agarose gel electrophoresis and the DNA sequence confirmed by direct DNA sequencing using fluorescent TAQ cycle sequencing run on an ABI 373 sequencer. The primers and sequence amplified is shown (Table I).

Genescan

The product from the second PCR reaction was denatured by heating at 94 °C for 3 min and immediately placed on ice. 0.2 µl aliquots or 0.5 µl vortexed Genescan-500 Tamra size standards (Applied Biosystems, Warrington, England) were added to 4 µl loading dye (1 part 50 mM EDTA pH 8, 30 mg/ml blue dextran, 5 parts deionised

formamide; stored at − 20 °C). 3 µl was resolved by 8% PAGE (using 32 ml Sequagel-8, 8 ml Sequagel buffer (National Diagnostics, Hull) and 300 µl 10% ammonium persulfate for each gel) for 2 h in 1 x TBE buffer (2 mM EDTA pH 8, 5.5 g/l boric acid, 10.8 g/l TRIS base), using an ABI 373XL sequencer. PCR product was quantified by measuring the maximum fluorescence present (peak height) and analysed using Genescan 672 software, version 1.2 (Applied Biosystems). All subjects were analysed at least in duplicate, with additional analyses (range 1-8) performed in 22 CD, 11 UC and 3 normal individuals.

Table I: Partial genomic structure of the human p47-phox (NCF1) gene[1] with primer sites, complimentary DNA and pseudogene anomalies annotated.

```
    1981 tcacatcgcc ctgctgggct ttgagaagcg cttcgtaccc agccagcact
at²gtgagtag
    2041 ctggtggagg gcatccccgt gggggggaata cgggagggac agcacggcca
..............................
    4621 .............................. cttcctgccc ctcccgtccc ctcccctctc ctcctgtccc
ctccct³tccc
    4681 ctcccctctc ctcctgtccc ctcccttccc ctcccctccc cacccaagct
ggagtgcagt
    4741 ggtgcaatca tagctcacta aagccttgac ctccaagtct caagcaattc
tcctgcctca
    4801 cctggggcca caggtgtgcg gcaccacacc cggacaattt ttgtgttttt
agtagatatg
    4861 ggggtctcgc tatgttgccc aggctggtct caaactcttg gactcaagcg
atcttcccac
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    5281 cgcttcaccg agatctacga gttccat⁸gt⁹g agtgtgggga cggaggaggg
acagggaccc
    5341 accgttccag ctccaccctt tgggaaggac cttagcccag gtgatgggga
aactgcagaa
    5401 cccagaatcc ccttccagac cacagttaaa ggggatttat ttatttatat
aaatttttgt
    5461 gacagggtct tgctctgtca cc¹⁰actctgaa cacctcatgt tctct¹¹gatta
caggcatgag
    5521 cccccacgct cggccttta ggtggttttg agaggtattt aggtttgcag
tgcaggggcg
```

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[1] Accession number AF184614

[2] End exon 1

[3] 30 base pair sequence duplication found in the normal gene but absent in the pseudogene

[4] tg in pseudogene

[5] Sequence identical to the external primer "2LA"

[6] Sequence identical to the internal primer "Forward FAM", 5'- terminus conjugated to FAM (fluorescent label)

[7] Beginning of both exon 2 and site of gt deletion in the pseudogene

[8] End of exon 2

[9] Sequence complimentary to the internal primer "Short Reverse Primer", acatggaac tcgtagatctcggtg

[10] 20 base pair sequence which is duplicated in the pseudogene

[11] Sequence complimentary to the external primer "2RC", agagaacatgaggtgttcagagt

RESULTS

Quantification of ΨNCF1 to NCF1 ratio

No DNA band was visible from the first PCR reaction. However, three bands were seen after the second reaction, consistent with product from the second primer pair, Forward FAM and Short Reverse Primer (142 & 144 bp), and a faint band from the first primer pair, 2LA and 2RC (368 & 386 bp), now just visible following a total of 60 PCR cycles (see Figure 1). Following agarose gel electrophoresis, only one band was seen at about 140 bp. This band was sequenced and shown to contain PCR product derived from both *NCF1* and *ΨNCF1*.
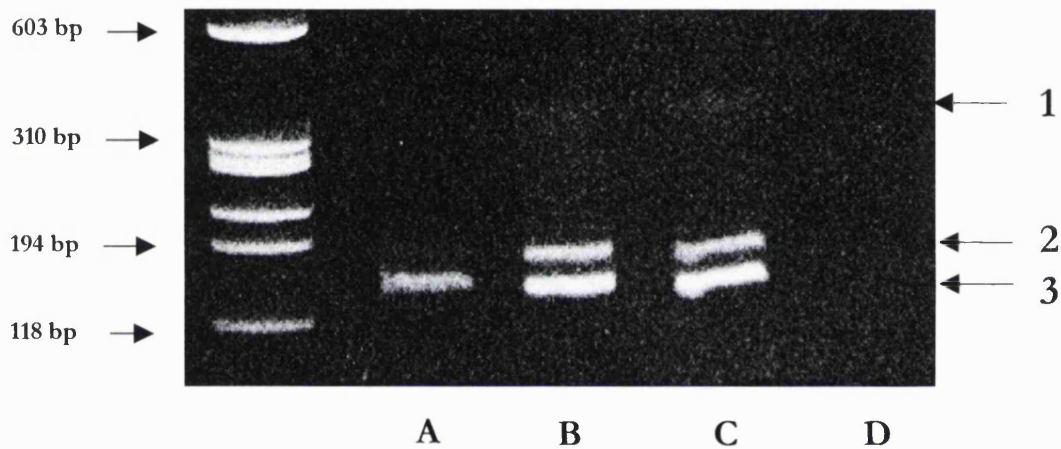
Figure 1: PAGE of product after nested PCR demonstrates three bands; the very faint band 1 shows product from the first PCR reaction; bands 2 & 3 show product from the second PCR reaction. Band 2 represents product derived from *NCF1* whereas band 3 is derived from *ΨNCF1*. Only bands 2 & 3 were fluorescent. The marker ΦX174 is shown in the left hand lane; lane A was derived from DNA from a patient with p47phox-deficiency; lane B and C from a patient with CD and UC respectively; lane D contained no DNA.

The relative quantity of fluorescence, present only in the products of 142 & 144 bp, determined the ratio of 142 and 144 bp product and therefore the ratio of *ΨNCF1* to *NCF1*, assuming that the PCR reaction for both proceeds with equal efficiency. In general pseudogene product was greater than gene product, approximately in the ratio 2 : 1, suggesting that there are two pseudogenes for each gene.

In 16 subjects, three or more genescans were performed in which discordant genescan ratios were present (i.e. 2 : 1 and 1 : 1 in the same subject). However, if peak heights exceeding 4050 units were excluded, discordant ratios were present in 2 of the 16 subjects only. Therefore, peak heights greater than 4050 were excluded from the analysis.

ΨNCF1 to NCF1 ratio can identify p47^{phox} heterozygotes, which are not over-represented in IBD

Although there were a range of values of the *ΨNCF1 : NCF1* ratio, two distinct populations existed, in which the ratio approximated to 1:1 or 2:1, defined as those in which the ratio was less or greater than 1.2 respectively. The *ΨNCF1* to *NCF1* ratio was about 1:1 in a minority of subjects. PCR product derived from the p47^{phox}-deficient control only demonstrated a pseudogene product, whereas her mother demonstrated a ratio between 3 and 4 (Figure 2).
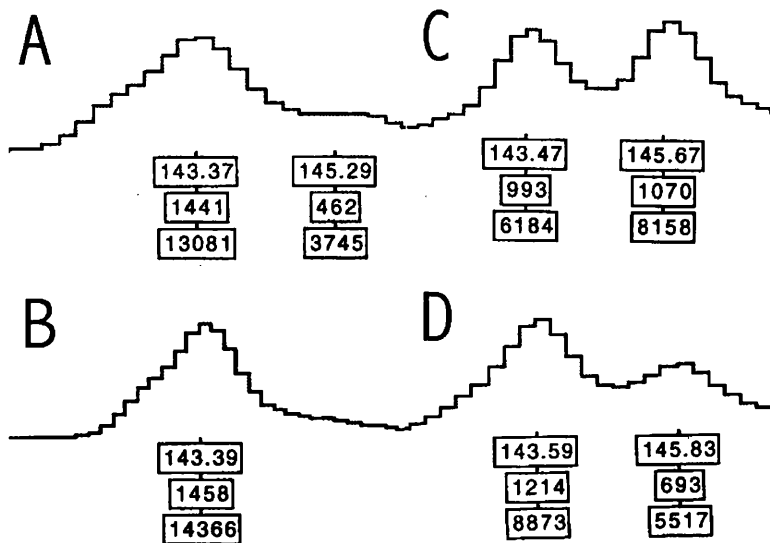
Figure 2: Sample gene-scan histograms after nested PCR around exon 2 of *NCF1* and ΨNCF1. Gene-scan plots from the mother of a patient with p47^{phox}-deficiency (A), the patient (B), a normal subject with pseudogene : gene ratio of 1 : 1 (C) and 2 : 1 (D). The numbers represent (from the top) product size (bp), peak height and peak area.

Two parents of confirmed p47^{phox}-deficient CGD patients had high ratios (3.4 and 3.2), which suggested at least one *NCF1* allele was absent. Since this ratio is close to 4, it suggests that a single gene conversion had occurred, causing GTGT→ΔGT in one *NCF1* gene with the original *ΨNCF1* to *NCF1* ratio having been 1:1. Another parent tested had a ratio of 1.6, which was within the normal range, suggesting that her daughter had developed CGD consequent to a new mutation at this locus. This is estimated to occur in about 10% of cases of p47^{phox}-deficient CGD (Roesler et al. 2000a). None of the IBD patients or controls had a *ΨNCF1* to *NCF1* ratio greater than 2.5. This suggests that there were no occult p47^{phox} heterozygotes within the 181 subjects tested.

An equal pseudogene : gene ratio is associated with IBD

There appeared to be an excess of IBD patients with a *ΨNCF1 : NCF1* ratio of 1:1 (IBD 18.6%; control 8.1%; (x^2=2.3, degrees of freedom 1, p≈0.1)). This excess was particular pronounced in the CD patients (CD 22.4% (x^2=4.1, degrees of freedom 2, p≈0.1); rather than those with UC (14.1%) (Figure 3). In four patients with indeterminate colitis, the mean ratio was 1.67 (range 1.57 – 1.77).
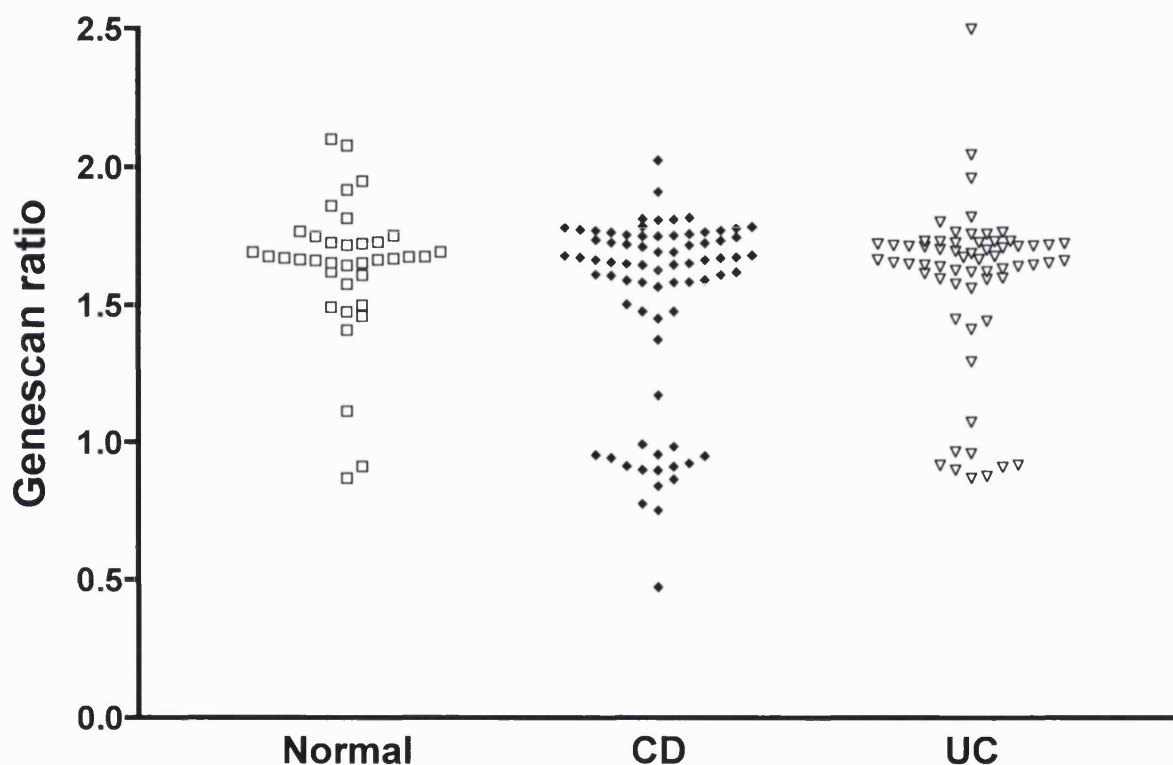
Figure 3: Ratio of *ΨNCF1* to *NCF1* in IBD. There appeared to be two populations with a predominance of the population with approximate parity in *ΨNCF1* and *NCF1* in patients with CD.

DISCUSSION

Identification of the genomic sequence of chromosome 7 at 7q11.23 has been hampered by the high density of genes and replicated sequence present. The genes include *NCF1*, which encodes p47^{phox}, a component of the leukocyte NADPH oxidase, together with *ΨNCF1*, which encodes a p47^{phox} pseudogene. The sequence of *ΨNCF1* is 99% homologous to *NCF1* (Gorlach et al. 1997) and both contain multiple sequences that predispose to recombination events (e.g. Alu repeats (Britten et al. 1988), Chi sequence (Smith 1994)) . This is believed to allow meiotic recombination events between *ΨNCF1* and *NCF1* and therefore the transfer of the highly conserved ΔGT mutation found at the start of exon 2 in 90% of p47^{phox}-deficient patients.

The location and number of pseudogenes is unknown, although it has been suggested that *ΨNCF1* and *NCF1* are separated by a 1.5 Mb chromosomal segment containing 17 genes (DeSilva et al. 1999). Deletion of this region on one chromosome by unequal recombination events is believed to cause Williams syndrome, resulting in haploinsufficiency, where one normal allele in a diploid cell results in insufficient protein to fulfil normal cellular function (Wilkie 1994)) and the clinical phenotype of infantile hypercalcaemia, cardiac abnormalities, developmental retardation and a distinctive facial appearance (Donnai & Karmiloff-Smith 2000). A fluorescent in-situ hybridisation (FISH) study has demonstrated 4 possible loci at 7q11.23 and further possible loci at 7p22 and 7q22 with homology to *ΨNCF1* or *NCF1* (DeSilva et al. 1999). Thus, it seems that there are between three and six loci on each chromosome available for gene conversion.

A genescan method was designed to identify p47^{phox} heterozygotes based on a previous report (Dekker et al. 2001). In the majority of individuals, two pseudogenes were present for each p47^{phox} gene. This suggests that there are three (or six) chromosomal loci and therefore one (or two) *NCF1* and two (or four) *ΨNCF1* loci. However, in a minority of individuals, there was parity between p47^{phox} gene and pseudogene.

How could this occur? If three chromosomal loci exist, this could arise if nucleotide exchange had occurred in one chromosome from *NCF1* to *ΨNCF1* resulting in ΔGT→GTGT and the production of a novel NCF1 gene and gene product (termed ν-

NCF1 and *v*-p47^{phox} respectively). This is in contrast to the CGD haplotype, where nucleotide exchange is thought to occur from *ΨNCF1* to *NCF1*. It is less likely that deletion of one and then a second *ΨNCF1* locus accounts for the ratio of 1 to 1. If six loci exist, as has been suggested by FISH, a single ΔGT→GTGT would produce a *NCF1* to *ΨNCF1* ratio of 1.4 and double gene conversion a ratio of one. A few individuals had a ratio near 1.4 and therefore our results cannot exclude this possibility.

Indeed, the concept of partial gene shuffling from *ΨNCF1* to *NCF1* may account for the finding of C345→T, considered to be *ΨNCF1* specific (Gorlach et al. 1997), associated with the GTGT containing allele in four of twenty one individuals (Noack et al. 2001). This was assumed to be a silent polymorphism, however if these four patients were shown to have *ΨNCF1* to *NCF1* parity, then partial gene transfer is more likely. It is unlikely that the findings are due to *ΨNCF1* and *NCF1* undergoing PCR at a different rate, since polymorphisms have not been described in 33 normal subjects, 6 *ΨNCF1* and 2 *NCF1* clones in the regions covered by our PCR primers (Gorlach et al. 1997); and in addition, variable PCR efficiency would produce a range of product ratios, not distinct populations as seen here.

A ratio of one *ΨNCF1* to one *NCF1* in 1 of 15 normal individuals was reported in a prior gene-scan study (Dekker et al. 2001) and has been alluded to by separate workers (Noack et al. 2001). Our finding a sub-population with equal amounts of *ΨNCF1* and *NCF1* means that the ratio between these two genes may not reliably screen for p47^{phox} heterozygosity, as has been suggested (Dekker et al. 2001), since these patients could have a ratio between 3 : 1 and 5 : 1.

The frequency of parity between *ΨNCF1* and *NCF1* was significantly increased in patients with IBD, especially in patients with CD. This suggests that partial gene conversion from *NCF1* to *ΨNCF1* may be a susceptibility factor for CD. How might this mediate an effect? If allele conversion is analogous to the CGD haplotype, with the transfer of a region surrounding exon 2 but not including exon 6 and 9, *v*-p47^{phox} will differ from p47^{phox} at just three amino acids (R→H^{90} (conserved); S→G^{99} (homologous); and N→D^{166} (non-homologous)) (Vazquez et al. 2001). The first two mutations occur within the PHOX domain. The third mutation occurs in the first SH3-domain, as such could effect intra-molecular binding of p47^{phox}, and interfer with

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the interaction with p22^{phox}. It is perhaps telling that p47^{phox} mutated at the next residue (Y167L) has been shown to have reduced oxidase activity (de Mendez, I et al. 1997).

To speculate, if p47^{phox} were rate limiting and v-p47^{phox} functional, it could mediate the enhanced macrophage respiratory burst described in CD and therefore increase gastrointestinal inflammation. Alternatively, it is possible that v-p47^{phox} were non-functioning and competitively inhibited normal p47^{phox} leading to haploinsufficiency; or since p47^{phox} is found in lymphocytes in excess of the flavocytochrome {Chetty, Thrasher, et al. 1995 196 /id} it is possible that this is the cell affected in IBD. Also it is possible that IBD susceptibility is conferred by a change in a gene or gene promoter adjacent to *NCF1* or *ΨNCF1* following gene conversion (at 7q11.23, or possibly 7p22 or 7q22 (DeSilva et al. 1999)). Such a gene is unlikely to be contained within the region deleted in Williams's syndrome, which is not associated with IBD. IBD susceptibility is unlikely to be due to a reduction in *ΨNCF1*-derived protein following gene conversion, since *ΨNCF1* only encodes the first 20 bp of p47^{phox} in a 51 bp polypeptide that is unlikely to be functional. Finally, it is interesting that *ΨNCF1* is also found in non-human primates but absent in mice (DeSilva et al. 1999); could this account in part for the lack of spontaneous colitis reported in the latter (Scott & Keymer 1975)?

The lack of replication of significant linkage in IBD with chromosome 7 in other genome wide scans (Ma et al. 1999) (Hugot et al. 1996) (Hampe et al. 1999) (Rioux et al. 2000) (Duerr et al. 2000) (Cho et al. 2000), except for nominal linkage for the marker D7S820 @ 7q21.11, 21.5 mb telomeric to p47^{phox} (Cho et al. 1998), does not exclude the presence of an IBD susceptibility gene on this chromosome. It is recognised that most genome wide scans are under-powered due to the genetic and phenotypic heterogeneity of CD. This problem can be lessened by studying individuals with specific clinical sub-types, which has demonstrated for example that the IBD1 locus is associated with severe, early onset CD (Brant et al. 2000).

G-CSF IN CROHN'S DISEASE

AIM

To analyse the effect of G-CSF on neutrophil function in CD. To assess G-CSF treatment in active CD.

INTRODUCTION

Administration of G-CSF, a human granulopoetic cytokine (Souza et al. 1986), causes neutrophil mobilisation from the bone marrow into the circulation and consequently a rise in venous neutrophil concentration. G-CSF is used in clinical practice to increase tissue neutrophil number (Weiss et al. 1999), although the only study to investigate tissue penetration found it to be reduced (Price et al. 1996). G-CSF therapy increases neutrophil O_2^- and H_2O_2 production, both markers for the NADPH oxidase, in patients with a spectrum of disease (Ohsaka et al. 1989; Lindemann et al. 1989; Vecchiarelli et al. 1995; Wiltschke et al. 1995; Weiss et al. 1995; Aslan et al. 1998; Pitrak 1999; Gerber et al. 2000), except in one study (Hustinx et al. 1998). However, the effect of G-CSF in normal subjects is less clear, since enhanced (Turzanski et al. 1997; Liles et al. 1997; Hoglund et al. 1997), static (Chatta et al. 1994; Vecchiarelli et al. 1995; Hoglund et al. 1997) or variable (Leavey et al. 1998) effects on O_2^- production have been described (Table I).

Neutrophil penetration of skin windows (Segal & Loewi 1976; Morain et al. 1981; Wandall & Binder 1982a) and cantharidin blisters is reduced in CD. Therefore, inducing an increase in the concentration of circulating neutrophils by administering G-CSF may prevent or treat the disease. In addition to direct effects on the neutrophil, G-CSF attenuates pro-inflammatory cytokine responses (Weiss et al. 1999) and increases TH2 (immuno-suppressive) cytokine concentration (Hartung et al. 1995), which could be of benefit in CD.

The considerable cost of G-CSF has limited the number of studies of this granulopoetic factor in CD. One study evaluating very high prophylactic G-CSF doses (weight : weight 10 and 40 times the human dose) in experimentally induced rabbit colitis (Hommes et al. 1996) demonstrated a reduction in pro-inflammatory mediators

Leukotriene B4 and Thromboxane B2, but without histological improvement. During the course of this work, two open label human studies of G-CSF therapy for CD have been reported, in abstract form only. In 5 patients, 3 times weekly G-CSF was associated with resolution of neo-terminal ileitis in one patient and recto-vaginal fistula closure in another (Dejaco et al. 2000). More recently, improvement was seen in 10 of 17 patients given daily G-CSF for 12 weeks (Korzenik & Dieckgraefe 2001), particularly in fistulous disease.

G-CSF was assessed as a therapy for CD following single case reports of its benefit in the colitis of GSD-Ib (Roe et al. 1992); CGD (Myrup et al. 1998) and fistulating CD (Vaughan & Drumm 1999). Serum G-CSF concentrations were determined in patients with IBD. Neutrophil migration into an acute inflammatory lesion and neutrophil oxygen consumption in patients with CD during G-CSF induced neutrophilia were investigated. Finally, an open-label pilot study was performed, designed to assess whether G-CSF can be used to treat different manifestations of active CD.

Table I: Summary of effect of G-CSF *in vivo* on O_2^- production

Subjects	n	Day assayed	Daily G-CSF dose (duration in days)	Method	Stimuli	Result (for stimuli respectively)
Normal (Chatta et al. 1994)	40	0, 5, 9	0, 30 & 300ug (14)	DBA^{++} & luminol CL	PMA (3ng/ml) , op.Z	Unchanged; increased
Normal (Chatta et al. 1994; Turzanski et al. 1997)	12	0, 1 & 4	2.5, 5 & 7.5ug/kg (5)	Lucigenin CL	FMLP, op. *S.epidermidis*	Increased
Normal (Chatta et al. 1994; Leavey et al. 1998)	14	0 & 5	10ug/kg (7)	CytC+/-SOD	PMA (200ng/ml), op.Z, FMLP	Reduced; unchanged; increased
Normal (Chatta et al. 1994; Liles et al. 1997)	3	0, 5 & 6	300ug (5)	Luminol CL	*Aspergillus, Rhizopus, Candida*	Increased
Normal (Chatta et al. 1994; Hoglund et al. 1997)	12	0, 1, 2 & 5	7.5 & 10ug/kg (6)	Luminol & lucigenin CL	PMA (10ng/ml) , op.Z	Increased (luminol only)
Neutropenic AIDS/Normal (Chatta et al. 1994; Vecchiarelli et al. 1995)	18	0-4	5ug/kg (1)	CytC+/-SOD	op.Z	Increased (AIDS only)
Testicular cancer + chemotherapy (Chatta et al. 1994; Wiltschke et al. 1995)	20	12	20mg/m^2 (10-19)	CytC+/-SOD	FMLP	Increased
Lymphoma (Ohsaka et al. 1989; Chatta et al. 1994)	7	3 & 15	50-800ug/m^2 (14)	CytC+/-SOD	PMA (100ng/ml), FMLP	Increased (FMLP only)
Advanced cancer (Lindemann et al. 1989; Chatta et al. 1994)	18	1, 5	1-60ug/kg (14)	Luminol CL	FMLP	Increased
Post surgical poor wound healing (Chatta et al. 1994; Gerber et al. 2000)	30	0, 1 & 5	2ug/kg (7-10)	Luminol & lucigenin CL	op.Z	Increased
Pneumonia (Chatta et al. 1994; Hustinx et al. 1998)	10	0 & 3	300 & 480ug	Luminol & lucigenin CL	C5a, PAF, FMLP; then op.Z	Unchanged
Solid tumours (Chatta et al. 1994; Aslan et al. 1998)	6	Variable	5ug/kg	Luminol CL	FMLP, Z activated serum	Increased
Post surgical at risk of sepsis (Chatta et al. 1994; Weiss et al. 1996)	20	0-16	1ug/kg (7)	Luminol CL	FMLP, op.Z	Increased (FMLP only)
AIDS (Chatta et al. 1994; Pitrak 1999)	78	0, 5, 8 & 10	300ug / daily (8)	CL	C5a, PAF, FMLP; then op.Z	Increased

CytC, cytochrome C; DBA, diaminobenzoate; CL, chemilumenescence; op.Z, opsonised zymosan; MPO, myeloperoxidase; PMA, phobol ester; FMLP, f-Met-Leu-Phe; C5a, complement 5a; PAF, platelet activating factor

METHODS

G-CSF sera levels in IBD

Serum samples were collected from 28 patients with CD (16 male; mean age 38 years); 28 patients with UC (13 male; mean age 45 years); and 18 control patients (9 male; mean age 34 years) attending the IBD clinic of St. Mark's Hospital, London and frozen at −70 °C. None of the patients had inter-current infection at the time of collection. Disease activity was assessed by the Harvey-Bradshaw index (Harvey & Bradshaw 1980) (CD) and the Mayo Clinic Score (Schroeder et al. 1987) (UC). G-CSF concentrations were assayed in duplicate using an ELISA kit (R&D systems).

Effect of G-CSF on cantharidin blister phenotype and venous neutrophil oxygen consumption in quiescent CD

9 Caucasian patients with histologically confirmed CD (4 with colitis; 3 with ilio-colitis; 2 with ileal disease; 7 with inactive disease, and 2 with active disease) and 9 healthy Caucasian controls were recruited from the outpatients department and faculty of University College London. A controlled acute inflammatory lesion was created by inducing duplicate skin blisters using topical cantharidin, sampled at 24 h as described in Chapter 4 (Day et al. 2001). This allowed quantification of the proportion of neutrophils within each blister using cell surface expression of CD16, which has been described as remaining static during G-CSF therapy (Hoglund et al. 1997). After sampling the first pair of blisters, 5 µg/kg G-CSF (Lenograstim, Chugai) was administered subcutaneously, repeated 24 h later when 2 further cantharidin blisters were induced and sampled as above. This is demonstrated below (Figure 1).
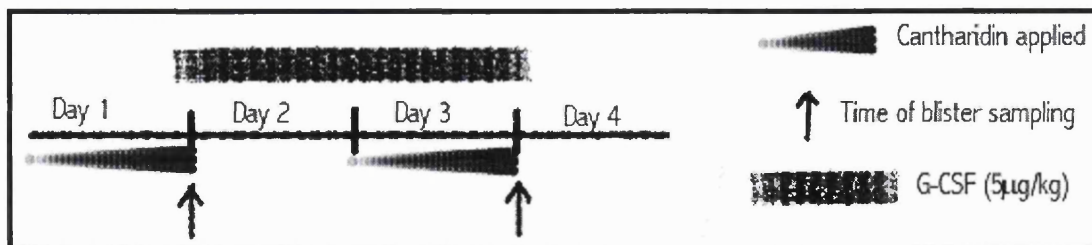
Figure 1: Schematic representing protocol for investigation of the effect of subcutaneous G-CSF on cellular migration into inflammatory cantharidin blisters.

At the time of blister sampling, venous blood samples were taken for standard analysis, and in five CD and five control subjects for the measurement of the neutrophil oxidative burst using the oxygen electrode. In addition, a FBC was performed at the time of the second G-CSF injection. No new medication except for simple analgesia was allowed during the study. 12 subjects reported bone pain on the second and/or third study day, which required co-proxamol (2), neurofen (2) or aspirin (1).

One subject with CD, in whom blisters failed to form, was withdrawn from the study on the second day; and one control subject withdrawn at the end of the study due to cannabis ingestion on day 2 and 3. Cannabinnoids are known to inhibit leukocyte migration (Schwartzfarb et al. 1974) which may account for the marked reduction in blister neutrophil number in the second pair of blisters in this subject (3.68×10^6/ml $\rightarrow$ 0.96×10^6/ml). The characteristics of the patients completing the study are shown below (Table II).

Table II: Subject characteristics (mean ± standard deviation) for G-CSF modulation of cantharidin blister phenotype

	CD	Control
Number	8	8
Age (years)	45.3 +/- 8.5	41.5 +/- 7.1
Male	3	7
Smoking	1	2
Medication	5-ASA (5); Azathioprine (1)	Nil
Body mass index	25.6 +/- 4.6	26.2 +/- 5.1
Harvey Bradshaw Index	1.9 +/- 2.2	Not done
ESR (mm/hr)	25.0 +/- 7.7	9.8 +/- 10.8
CRP (mg/l)	7.2 +/- 5.6	2.6 +/- 1.8
Leukocyte count ($\times 10^9$/l)	6.6 +/- 1.7	6.9 +/- 1.0
Neutrophil count ($\times 10^9$/l)	4.3 +/- 0.6	3.7 +/- 0.8
Monocyte count ($\times 10^9$/l)	0.5 +/- 0.1	0.6 +/- 0.2
Albumin (g/l)*	43.4 +/- 1.9	48.1 +/- 2.7
Haemoglobin (g/dl)*	13.2 +/- 0.9	15.1 +/- 0.7

ESR, erythrocyte sedimentation rate
ASA, aminosalicylic acid; CRP, C-reactive protein
Significant difference by the unpaired 2-tailed t test (*$p < 0.01$)

Pilot study assessing G-CSF for the treatment of acute Crohn's disease

Seven patients with manifestations of CD, previously refractory to systemic steroid therapy, were recruited from the outpatients clinic between January and July 1999. Subcutaneous G-CSF (Lenograstim (Chugai Pharma UK Ltd., London) (5 patients at University College London) or Filgrastim (Amgen Ltd., Cambridge) (2 patients at St Mark's Hospital)) was administered on an outpatient basis for 28 days.

The initial dose of G-CSF was 0.5 µg/Kg/d. Blood counts were performed three times a week. The dose of G-CSF was titrated to maintain an absolute neutrophil count of 20-30 x 10^9/l. G-CSF was not provided when the neutrophil count was greater than 50 x 10^9/l or if a serious adverse event occurred. Pre-trial medication was maintained but new treatments were not allowed.

The dominant symptom at trial entry determined the specific treatment goal, a previously utilised approach in CD (Present et al. 1980). Complete response was defined as absence and partial response as improvement in the dominant symptom, assessed by both patient and physician three weeks after G-CSF cessation.

In addition, objective assessments were performed, including endoscopic or radiological studies in 4 patients, and the Crohn's Disease Activity Index (CDAI) (Best et al. 1979) recorded. Patients attending University College London completed the EuroQol questionnaire (Anonymous 1990) and Inflammatory Bowel Disease Questionnaire (Irvine et al. 1994) (IBDQ), validated measures of life quality, with a scale 3 - 15 (good to bad) and 224 - 32 (good to bad) respectively.

Statistics

A p-value of < 0.05 was taken as significant. Results are expressed as mean $\pm$ standard error unless specified. Intra-individual differences were assessed by the paired, 1-tailed *t* test and inter-individual differences by the unpaired, 2-tailed *t* test.

ANCOVA was performed to adjust for age, gender and disease activity on serum G-CSF concentration after logarithmic transformations to normalise the distributions and obtain equality of variance.

Association between blister cell composition and age, BMI, Harvey-Bradshaw index, mesalazine use, haemoglobin, albumin, ESR, CRP and serum iron was assessed using Pearson correlation.

RESULTS

G-CSF sera levels are raised in CD

G-CSF sera levels were found to be mildly raised in patients with CD (36.5 ± 3.7 pg/ml) (ANOVA p = 0.04) but not in patients with UC (33.6 ± 3.3 pg/ml) compared to control subjects (24.0 ± 1.4 pg/ml) (Figure 2). There was no difference within each group when analysed by multiple regression for age (control p = 0.068; CD p = 0.52; UC p = 0.6); gender (control p = 0.196; CD p = 0.689; UC p = 0.478); or disease activity (CD p = 0.72; UC p = 0.13).
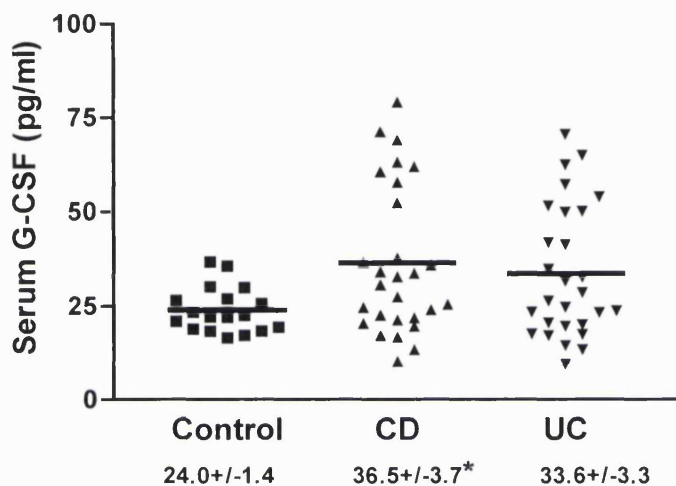
Figure 2: Serum G-CSF concentrations, measured in duplicate by ELISA, were significantly raised in CD compared to control subjects (ANOVA p = 0.04). Results shown are the average of two assays.

During G-CSF therapy, CD tissue cell mobilisation is normal

Raised G-CSF sera levels in CD suggested either end-organ resistance or a physiological requirement for this cytokine. The former was assessed using cantharidin blisters. There was a large increase in venous neutrophil concentration and a small increase in venous monocyte concentration in both CD and control subjects after G-CSF, although the increase in monocytes was significantly lower in CD (Figure 3). Blister volume was unchanged after G-CSF, however there was a significant increase in blister cell concentration in both CD and control subjects, mainly caused by a significant increase in blister neutrophils (CD16^{+} cells). There was also a borderline significant increase in blister macrophages (CD14^{+} cells) in both CD subjects and controls. There was no significant difference between CD and control subjects in blister volume and cellular concentrations comparing before or after G-CSF (Figure 4).
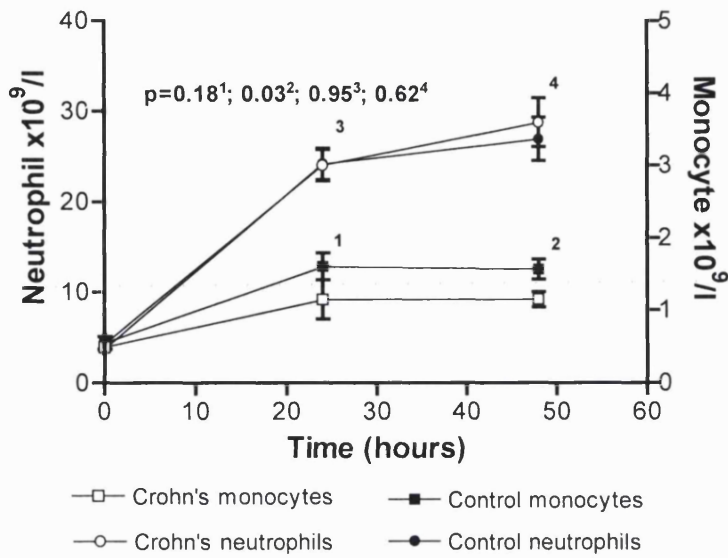
Figure 3: The increase in venous neutrophil and monocyte concentrations due to G-CSF administration (5 μg/Kg) at 0 and 24 h. At 48 h the monocyte count was significantly reduced in CD compared to control subjects (*p=0.03).

The number of cells in the blisters was lower than the number of cells in the blood, more so after G-CSF. Specifically, blister macrophage concentration was lower than venous monocyte concentration in CD (73 ± 33%) and controls (89 ± 65%) and the proportion further reduced after G-CSF in both CD (50 ± 15%) (p = 0.18) and controls (70 ± 42%) (p = 0.41). Blister neutrophil concentration was markedly lower than venous neutrophil concentration in CD (21 ± 10%) and controls (14 ± 5%) and the proportion further reduced after G-CSF in CD (12 ± 5%) (p = 0.11) but was unchanged in controls (15 ± 6%) (p = 0.25).
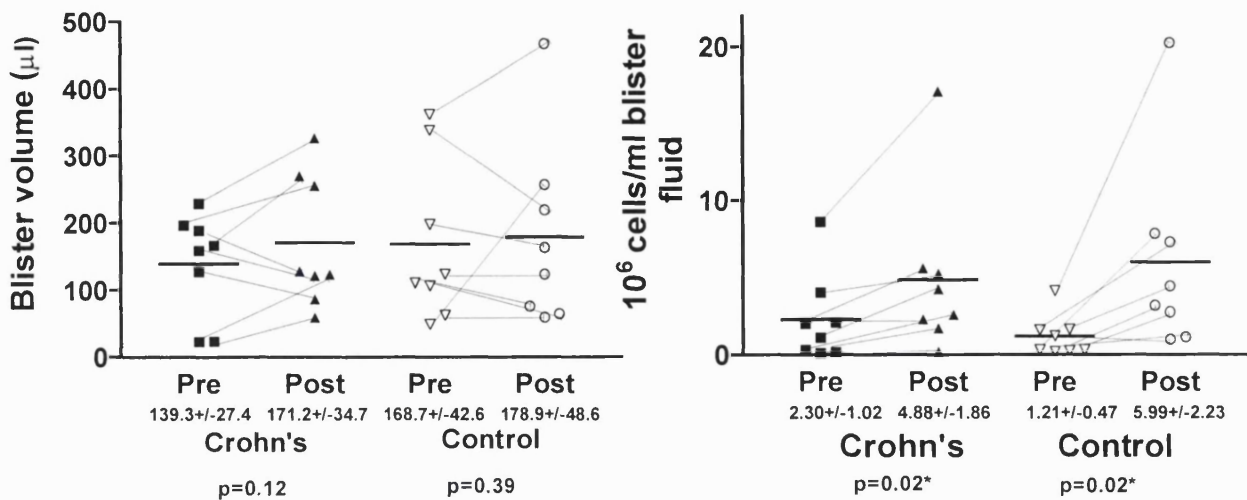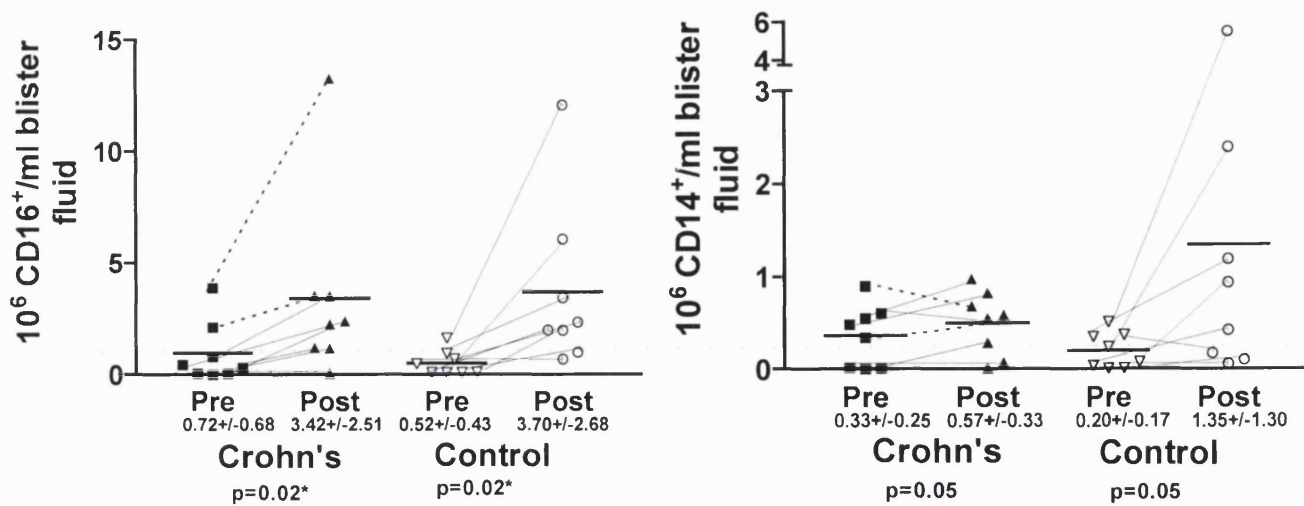
Figure 4: The effects of G-CSF administration (5 µg/Kg) at 0 and 24 h on cantharidin blister volume, cell concentration and cell phenotype at 48 h are shown for CD and control subjects (mean ± standard error and p values are shown). The concentration of neutrophils (CD16+ cells) increased significantly. There were no differences between CD and control subjects. A dashed line links the cell sub-types from the two subjects with moderately active CD.

More active CD was associated with higher blister neutrophil concentrations both before ($r^2 = 0.86$, p < 0.01) and after ($r^2 = 0.68$, p = 0.04) G-CSF. Blister neutrophil concentration was highest in the two CD patients with active disease. CRP levels were inversely associated with CD blister macrophage concentration, but only following the administration of G-CSF ($r^2 = 0.78$, p = 0.01). Serum albumin was positively associated with control subjects' blister macrophage concentration before G-CSF (Figure 5).
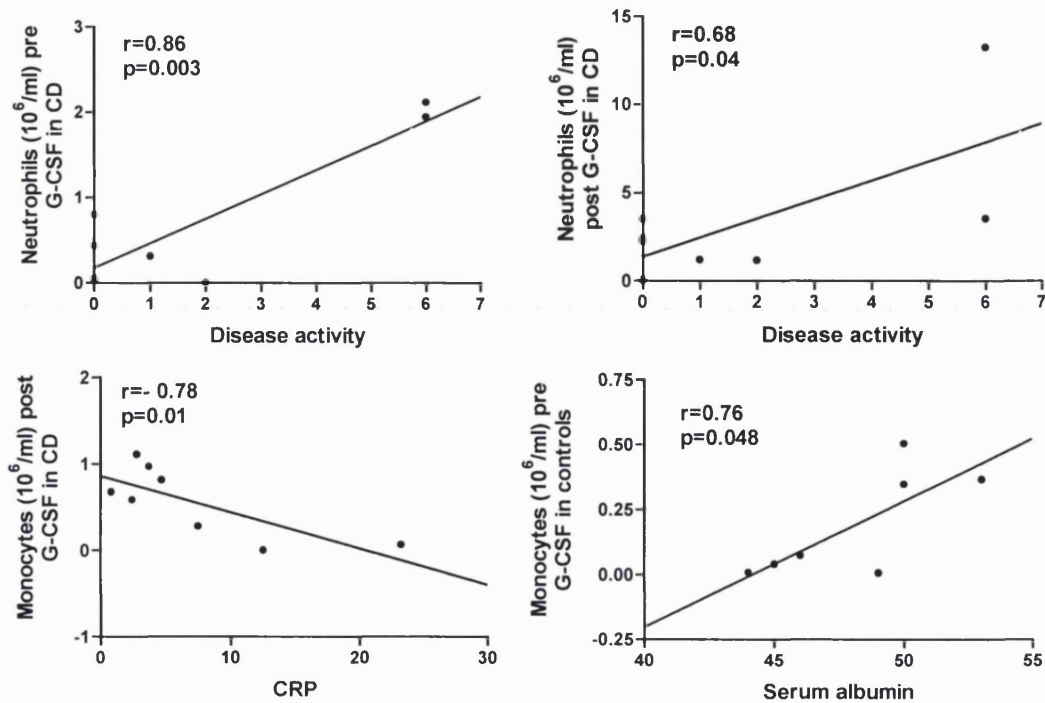
Figure 5: Modifiers of blister cell concentration. There were significant relationships between disease activity and CRP and blister cell phenotype in CD; and between serum albumin and blister cell phenotype in controls.

G-CSF reduces peripheral blood neutrophil oxygen consumption equally in both CD and controls

Although the proportion of venous cells entering the blisters was reduced with G-CSF, the absolute cell number increased, suggesting that G-CSF therapy maybe effective in enhancing the acute inflammatory response in CD. Therefore it was important to establish whether G-CSF induced neutrophils in CD patients had a normal respiratory burst in response to physiological challenge with faecal antigens. Delay to maximum response and maximum oxygen consumption after administration of faecal flora (a receptor stimulus) or PMA (an intracellular stimulus) were the same in CD and controls before and after G-CSF. However, unexpectedly, G-CSF administration was associated with a significant reduction in maximum oxygen consumption with both stimuli and a reduction in delay to maximum response after PMA (Table III).

Table III: Effect of G-CSF on maximum O2 consumption (n.mol
$O_2/10^7$neutrophils/min) and delay to maximum response (sec)

| | CD | | | Control | | |
	Pre-G-CSF	Post-G-CSF	p	Pre-G-CSF	Post-G-CSF	p
Fecal flora Q	85.7+/-14.3	42.7+/-9.4	0.05	88.5+/-11.1	45.7+/-11.2	0.03'
Fecal flora D	216.8+/-27.1	221.9+/-42.3	0.42	222.1+/-60.7	195.9+/-19.9	0.35
PMA Q	68.9+/-14.1	22.7+/-4.4	0.02*	60.7+/-8.8	30.8+/-4.9	<0.01
PMA D	212.4+/-44.3	148.6+/-48.0	0.08	199.9+/-49.5	85.9+/-17.3	0.07
Fecal flora:PMA Q	81.7+/-11.8	63.5+/-14.3	0.12	69.5+/-7.2	107.1+/-41.3	0.19

D, delay until Q; Q, maximum O_2 consumption
Significance (*p<0.05) comparing pre- and post-G-CSF shown, by the paired, 1-tailed t test
There was no difference between CD and control subjects, by the unpaired, 2-tailed t test

G-CSF therapy is ineffective in active CD

Having established that G-CSF can increase the absolute number of tissue cells, albeit cells with reduced respiratory burst activity, its effect was assessed in various different manifestations of treatment-resistant CD.

None of the patients studied achieved a complete response, defined as a subjective or objective resolution of the primary symptom (Table IV). Quality of life improved in only one of five patients (female aged 36, IBDQ index 140→189). There was a reduction in pain, accompanied by a moderate reduction in the CDAI in two of the three patients with ileal CD. In addition, there was a reduction in fistula flow in both patients with ilio-cutaneous fistulae, although failure to heal required alternative therapy.

In both patients aged 36, neutrophil migration into cantharidin blisters was measured at the time of moderately active CD. It is noteworthy that the female patient, who improved on G-CSF, had 0.95 ± 0.15 cells/ml blister fluid whereas the male patient who did not benefit had 3.50 ± 1.78 cells/ml blister fluid.

Table IV: G-CSF in active CD, patient details and outcomes (mean ± standard deviation)

Age / gender	36 / M	47 / M	37 / M	41 / F	36 / F	[St.M]30 / F	[St.M]43 / M
Disease location	Ileum	Ileum & sigmoid	PG & rectum	Pancolitis	Ileum	Ileum	Ileum
CDAI at entry	266	34	76	147	242	327	224
Dominant symptom	Pain	F1	PG	F2	Pain	Pain	Pain & F1
Medication	Nil	Metronidazole*	Azathioprine	Nil	Nil	Codeine	Azathioprine
G-CSF µg/d/kg	1.3+/-1.0	1.3+/-0.5	2.4+/-1.0	1.9+/-0.2	1.7+/-0.6	1.0+/-0.0	1.0+/-0.0
ANC (10^9/l)	21.2+/-8.4	36.3+/-5.5	16.9+/-3.6	19.8+/-4.0	32.5+/-6.4	25+/-6.0	24+/-0.0
G-CSF (days)	28	28	28	21	28	28	28
End-point	Nil	Partial response	Nil	Rectal bleeding	Partial response	Partial response	Partial response
Clinic assessment	CDAI = 270	Output reduced 14 ml/d to 1 ml/d	PG reduced 168mm^2-23mm^2	New fistulous tract	CDAI = 176	CDAI = 273	CDAI = 179 Output reduced
Investigations	Ileal ulcers unchanged	CT reduction in fistula size	Sigmoidoscopy unchanged	MRI collection 3.6 cm^2 to 6.0 cm^2	Not done	Not done	Not done
Adverse effects	Bone pain Acne	Bone pain Rectal bleed	Acne	Thrombocytopenia	Nil	Nil	Bone pain
Future therapy	Prednisone	Surgery	Azathioprine dose increased	Surgery	G-CSF	Azathioprine	Anti-TNFα

[St.M]Attended St. Mark's Hospital   [F2]Perineal fistula   ANC, absolute neutrophil count

[F1]Ileal-cutaneous fistula   * 5 day course for dental abscess day 7 of trial

[PG]Peri-stomal pyoderma gangreno S.D., standard deviation

DISCUSSION

G-CSF stimulates granulopoesis, increases neutrophil CD64 (FcγR1) expression (Gericke et al. 1995), primes neutrophil O_2^- production (Turzanski et al. 1997) and reduces neutrophil chemotaxis and apoptosis (Leavey et al. 1998). It has been used successfully to prevent serious infections in patients with HIV (Kuritzkes et al. 1998), community acquired pneumonia (Weiss et al. 1999) and diabetic foot ulceration (Gough et al. 1997). More recently its use has been advocated in acute liver failure (Rolando et al. 2000). However, relatively little is known about the effects of exogenous G-CSF on tissue neutrophil number and function. Indeed, the effect of G-CSF on neutrophil migration *in vivo* into inflamed human tissues is unpredictable. For example, G-CSF increases the adhesive receptor CD11b/CD18 but decreases L-selectin expression (Yong 1996).

Initially, G-CSF levels were measured in IBD sera and found to be significantly raised in CD. This is the first report of G-CSF sera levels in IBD. Although there was no relationship with measurable disease activity, suggesting a primary defect, activity indices are poor at assessing sub-clinical disease (Nielsen et al. 2000). It is more likely that the increased level reflects on-going, sub-clinical inflammation since the concentration of G-CSF was almost as high in UC. This is supported by the finding that lamina propria mononuclear cells from patients with active CD produce more G-CSF in culture (Pullman et al. 1992), which has been shown to prevent neutrophil apoptosis (Ina et al. 1999) and by the finding of increased serum G-CSF levels during infection (Kawakami et al. 1990).

This is the first study to quantify the proportion of venous monocytes and neutrophils that migrate into the tissue space in CD during G-CSF administration. This was assessed for two reasons. Firstly, G-CSF may be used as a treatment for CD and therefore it is important to know its effect on neutrophil tissue penetration in this disease. Secondly, it was hypothesised that reduced neutrophil migration into cantharidin blisters would be exaggerated during neutrophilia.

The absolute number of migrating cells increased, providing a rationale for its use *in vivo*. The concentration of neutrophils in cantharidin blisters was similar in CD and control patients during G-CSF. This suggested that their reduced penetration of cantharidin blisters in CD is due to a defective local inflammatory response, rather than

a relative impermeability of the tissues to neutrophils, which is in agreement with observations in the skin window model (Morain et al. 1981). However, it is acknowledged that the numbers of patients studied was small.

Previously, granulocyte-macrophage colony-stimulating factor infusion has been shown to cause a reduction in the number and migration distance of neutrophils into skin windows at two hours (Addison et al. 1989). The only study to quantify neutrophil tissue penetration during G-CSF therapy investigated cell mobilisation into skin windows in healthy volunteers after subcutaneous administration of 300 μg/d G-CSF (Filgrastim) (Price et al. 1996). Leukocyte migration at 24 h was similar to basal levels but after 5 days the number migrating was reduced by 60%, despite a neutrophil count greater than 20 x 10^9/l. Conversely, a five-fold increase in the neutrophil concentration in cantharidin blisters was seen following administration of G-CSF for two days. There are three possible explanations for this disparity. Firstly, cantharidin blisters produce a greater acute inflammatory response than skin windows, perhaps more representative of microbial invasion of the gastrointestinal mucosa. Secondly, cells that migrate during the first 48 hours will be derived from the marrow storage pool but after this time comprise newly synthesised cells (Price et al. 1996). It is possible that these cells migrate poorly into inflamed tissues. Finally, there may be enhanced migration of neutrophils induced by glycosylated G-CSF (Lenograstim), the human isoform, as compared to non-glycosylated G-CSF (Filgrastim), which is analogous to the enhanced priming of O_2^- production by glycosylated G-CSF (Decleva et al. 1995).

Two CD patients had more active disease, both with a Harvey-Bradshaw score of 6, one requiring surgery six weeks after study completion. Both patients had high blister neutrophil concentrations pre-GCSF but normal venous neutrophil concentrations, suggesting that once more active disease is present, neutrophil tissue penetration is increased. Conversely CRP, a standard disease activity marker, was associated with reduced blister macrophage concentrations in CD after G-CSF, suggesting either that CRP has a physiological role in limiting macrophage influx during leukocytosis, in contrast to stimulating weak chemotaxis *in vitro* (Mortensen & Zhong 2000), or that those CD patients with high CRP concentrations have defective macrophage tissue penetration. The increase in venous monocytes and tissue macrophages was less in CD subjects compared to control subjects after G-CSF. This also raises the possibility that migration of monocytes and macrophages may be rate limiting in CD at times of peak

myeloid cell mobilisation, as could occur during bacterial invasion of the gastrointestinal mucosa.

It was surprising to find that neutrophil oxygen consumption decreased in all subjects during G-CSF therapy. In normal subjects administered G-CSF, there is only one report (Leavey et al. 1998) of reduced O_2^- production but several demonstrating unchanged (Chatta et al. 1994) (Hoglund et al. 1997) (Leavey et al. 1998) (Vecchiarelli et al. 1995) or enhanced (Chatta et al. 1994) (Hoglund et al. 1997) (Leavey et al. 1998) (Liles et al. 1997) (Turzanski et al. 1997) production. All of these studies used indirect methods (Babior et al. 1973) (Gyllenhammar 1987) (Allred et al. 1980) to measure the respiratory burst, for example lucigenin chemiluminescence (Hoglund et al. 1997) (Turzanski et al. 1997) or superoxide dismutase inhibition of ferricytochrome C reduction (Leavey et al. 1998) (Vecchiarelli et al. 1995), both which only measure extra-cellular O_2^-; and luminol chemilumenescence (Chatta et al. 1994) (Hoglund et al. 1997) (Liles et al. 1997), which measures intracellular H_2O_2 / O_2^- production in the presence of MPO (Figure 6). However, luminol chemilumenescence is limited, since it will only measure extra cellular release of H_2O_2 / O_2^- when excessive cell stimulation causes MPO secretion (Chatta et al. 1994) or when saturating amounts of peroxidase are added to the cell medium (Edwards 1994b), which was not done in the studies with G-CSF. Moreover, it can not be used in MPO-deficient individuals (Kutter 1998) and for a quantitative measure assumes that MPO and luminol co-localise in the same sub-cellular compartment, which is unknown (Dahlgren & Karlsson 1999).

In contrast, this study used the oxygen electrode (Segal & Jones 1980), which provides a direct assay of the NADPH oxidase respiratory burst by measuring the substrate oxygen. It does not depend on the presence of other cellular molecules (unlike luminol) and measures total oxidase activity, occurring in the specific granules, phagosomes and plasma membrane (unlike lucigenin, ferricytochrome or luminol). Therefore it provides a more accurate measure of oxidase activity and has demonstrated that after two days G-CSF therapy, neutrophil NADPH oxidase activity is reduced.

None of the manifestations of CD completely responded to G-CSF. A partial symptomatic response occurred in the majority of patients, although all but one required alternative definitive treatment. The number of patients treated was small, limited by the cost of G-CSF. CD is a polygenic disease, therefore, the partial response

seen here does not rule out efficacy in a subset of CD patients. It is tempting to speculate that patients with fistulous CD may benefit, as demonstrated both here in 2 of 3 patients and as reported in previous studies (Vaughan & Drumm 1999; Dejaco et al. 2000; Korzenik & Dieckgraefe 2001), supporting the call for further trials (Shanahan 2001). Certainly, in this pilot study G-CSF appeared reasonably safe and therefore could have a role when therapeutic alternatives are limited. Theoretically, the optimal time to administer G-CSF would be early in the inflammatory process, assuming that a neutrophil deficit causes the relapse of CD in some patients. However, identifying these patients and predicting when this occurs is not yet possible.
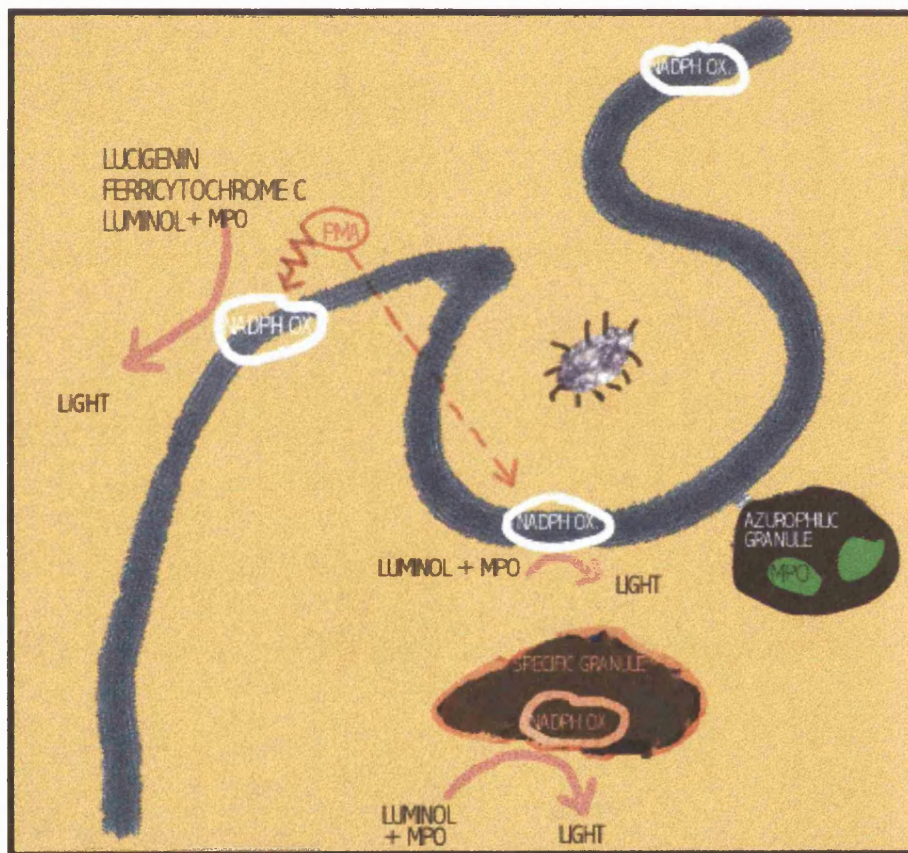
Figure 6: Measurement of the neutrophil respiratory burst. Luminol can diffuse (to an unknown degree) into the intracellular compartment and measures phagolysosomal NADPH oxidase during bacterial ingestion. PMA preferentially activates the NADPH oxidase at the plasma membrane. Only a high PMA concentration (e.g. 5 n.mol./l c.f. 10 p.mol./l) causes extra-cellular MPO release, thereby enabling extra-cellular luminol chemilumenescence. High bacterial concentrations (e.g. more than 100 bacteria per neutrophil) will cause frustrated phagocytosis where NADPH oxidase activity occurs only at the plasma membrane. Derived from (Chilvers et al. 2000) (Dahlgren & Karlsson 1999) (Edwards 1994a).

CONCLUSION

CD and CGD have very similar appearances in the colon. This suggests that CD may be caused, at least in part, by a failure of the innate immune response. This is supported by the recent discovery that mutations in monocyte *NOD2* predispose to CD. However, *NOD2* mutations are absent in more than half of the patients with ileal CD (Ahmad et al. 2002) and therefore it is likely that other CD-susceptibility mutations exist in genes that regulate innate immunity.

The vast majority of individuals heterozygous for *NOD2* do not develop CD. Assuming that the populations that have been examined are representative of a normal population, and that the prevalence of CD is 1 in 1000, the population prevalence of reduced and absent normal NOD2 are shown (Table I).

Table I: Prevalence of *NOD2* mutations / 100,000 of the population
(Mutation frequency (%) in a CD population shown in brackets)

Reference Number	CD present			No CD present		
	1	2	3	1	2	3
Simple Heterozygote	39 (39%)	28 (28%)	28 (28%)	15684 (16%)	13586 (13.6%)	14545 (15%)
Compound Heterozygote / Homozygote	12 (12%)	8 (8%)	15 (15%)	572 (0.6%)	0 (0%)	0 (0%)

[1]Ahmad et al. 2002
(CD, n = 244; control, n = 349; 1 compound heterozygote or homozygote expected per 615 control subjects)
[2]Cuthbert et al. 2002
(CD, n = 688; control, n = 250; 1 compound heterozygote or homozygote expected per 461 control subjects)
[3]Hugot et al. 2001
(CD, n = 468; control, n = 103; 1 compound heterozygote or homozygote expected per 529 control subjects)

It is clear that haplo-insufficiency of a major susceptibility gene (as in *NOD2*) increases the risk of CD. An important question is whether absence of normal NOD2 protein can exist without the CD phenotype. As shown, the studies performed so far do not have sufficient power to answer this question. Therefore it is still not known whether CD is caused by:

➤ Homozygous mutations alone in a single major susceptibility gene;

➤ Mutations in a single major susceptibility gene (e.g. *NOD2*) followed by an abnormal environmental stimulus;

> Interaction between more than one mutated major susceptibility gene, with or without an abnormal environmental stimulus.

Following the characterisation of NOD2 in CD, three things are clear. Firstly, that CD is likely caused by mutations in one or more of just a few critical genes. As such, it could be re-classified as an oligogenic disease (on a population basis), and perhaps as a monogenic disease in the individual patient.

Secondly, the sensitivity and specificity of any investigation of CD aetiopathogenesis will be limited unless patients are stratified according to genotype. For this reason, it is likely that the causes of IBD will be deduced by a genetic approach. Thereafter, cellular mechanisms can be studied and subsequently the physical abnormalities clarified. In summary, as genes are discovered, patient segregation will simplify. One day it is likely that complete genotype subsets will be available which will then allow more directed analysis of the role of environmental factors.

Thirdly, that a normal innate immune response is very important. It is tempting to speculate that this is macrophage mediated in ileal disease, as in CD, and neutrophil mediated in colonic disease, as in CGD.

How could malfunction in innate immunity predispose to inflammation? Bacterial antigens stimulate inflammation in CD (Harper et al. 1985; D'Haens et al. 1998) and the concentration of bacteria is very high adjacent to the mucosal cells of the terminal ileum and colon (Onderdonk 2000). Bacterial invasion provokes an acute inflammatory response, with the release of cytokines and vasoactive factors, neutrophil and macrophage influx, phagocytosis and digestion of bacteria (Wright 2001). If this process is relatively deficient at times of maximal invasion, bacterial products and/or food antigens will remain and stimulate further inflammatory cell influx, causing damage to the local environment and allowing further bacterial invasion (Sartor 2000).

In examining abnormal inflammatory responses in the bowel, it is important to always consider that factors that initiate inflammation may be distinct from the (measurable) factors that perpetuate inflammation. For example, an unknown factor allows bacterial invasion of the intestinal mucosa. Subsequently, appropriate inflammatory responses contain the bacteria but in so doing cause local tissue destruction, allowing further bacterial influx (Rath et al. 2001). This would not have occurred in the absence of the

initial bacterial invasion, which is the aetiopathogenic factor. Therefore, the chronic inflammatory response may occur in consequence to a relative failure of acute inflammation and not itself initiate inflammation.

For the reasons out-lined above, the role of the neutrophil during acute inflammation was examined in CD. Cantharidin blisters provided a safe and reliable technique to investigate acute inflammation, hopefully avoiding the complication of assaying a secondary inflammatory response. A failure of neutrophil migration into the blisters was demonstrated in CD. The reasons for this remain unknown. There was no polarisation of cytokine or chemokine responses in the blister fluid from patients with IBD, suggesting that the TH1 cytokine phenotype of CD may be a secondary phenomenon. The reduction in blister fluid neutrophils in CD was unrelated to disease activity, and therefore perhaps a primary aetiopathogenic factor, although this was not reproducible in individual patients. This suggests that neutrophil tissue penetration maybe mediated by several factors, which may vary with time.

It was hypothesised that the reduction in neutrophil tissue penetration in CD would be exaggerated during G-CSF therapy. However, this did not occur. This suggested that a relative impermeability of neutrophils to the tissues was not present in CD, compared to patients without CD. This implicates a defective tissue inflammatory response in CD. Only a small number of patients were studied which limits the power of this observation. The small pilot study performed using G-CSF for active CD suggested it might have a role in fistulous CD.

The respiratory burst of neutrophils and macrophages was assessed. Macrophages are long-lived cells and therefore the finding of increased O_2^- production in CD blister macrophages may represent prior priming by LPS. Nonetheless, it is possible that this is a primary abnormality in CD. There was no abnormality in oxygen consumption in venous or tissue neutrophils in CD; therefore this is unlikely to be an aetiopathogenic factor. The pilot study that investigated digestion in CD demonstrated no abnormality, but only analysed five patients. Since abnormalities in digestion by neutrophils and macrophages are hypothesised to cause granulomata, this area deserves further study.

The association study reported here has demonstrated possible linkage dysequilibrium with IBD, particularly CD. This agrees with Satsangi's finding of linkage with this region (Satsangi et al. 1996). Therefore it is possible that in this population the ratio

between *NCF1* and *ΨNCF1* is a CD susceptibility gene or in close linkage dysequilibrium with a CD susceptibility gene. Further studies are required to extend the experimental findings in this cohort, which then would require replication in a family based study. $p47^{phox}$ is an interesting candidate protein since it is present in leukocytes, which are a cell type implicit in causing CD. In addition, it is a rate-determining enzyme, as such small changes in activity could have a profound effect on cellular function. How parity between *NCF1* and *ΨNCF1* could mediate IBD is unknown. It is unlikely that $p47^{phox}$ haplo-insufficiency per se is associated with IBD, as originally hypothesised, in view of the absence of reports of IBD predisposition in parents of CGD patients.

Unlike CD, the genetic causes of CGD have been defined. However, the genetic and environmental factors that precipitate enteritis in at least 20% of these patients are largely unknown. Therefore it was interesting that $p47^{phox}$ -/- mice were susceptible to diclofenac-induced colitis, which suggests that NSAID's could precipitate colitis onset or relapse in CGD patients. Enteritis often presents insidiously and NSAID's are used so commonly that an association between the two may not have been noticed. Studies of the effects of NSAID's on gut permeability, as performed in CD (Hilsden et al. 1996), should be undertaken in CGD. In addition, the model demonstrates that chronic NSAID colitis is independent of reactive oxygen intermediates, suggesting that treatment strategies for chronic colitis must target cell types other than just the neutrophil.

The discovery of crystals at inflammatory sites in the CGD mouse allowed the identification of Ym1 as a neutrophil granule protein. The function of Ym1 is still unclear but by defining the cellular location as neutrophil granules and gastric epithelium, it is plausible to suggest that it has a major role in digestion during acute inflammation. Although knockout mice may help determine the function of Ym1, redundancy within this family of proteins may mean that a specific deficiency can be tolerated without a phenotypic change. This is known to be the case with human chitotriosidase deficiency (Boot et al. 1998). Since chitinase-like proteins are found highly expressed during chronic inflammation in mammals, their role in diseases such as CD and CGD should be evaluated further.

The studies performed have raised as many questions as answers they have provided. Science then is similar to philosophy. "Philosophy, if it can not answer so many questions as we could wish, has at least the power of asking questions which increase the interest of the world, and show the strangeness and wonder lying just below the surface even in the commonest things of daily life" (Bertrand Russell, 1912).

NOVEL FINDINGS GENERATED BY THIS WORK:

CGD mice are more susceptible to colitis induction but do not develop chronic colitis

129Sv mice are susceptible to Diclofenac colitis; this is especially true for p47^{phox}-/- mice

Ym1 crystallises in the CGD murine lung and is also expressed in the distal stomach

Ym1 is a murine neutrophil granule protein with weak β-hexosaminidase activity at pH4.5

Cantharidin blisters can be used to study acute inflammation

There is a neutrophil deficit in CD cantharidin blisters

CD disease tissue macrophages have an enhanced respiratory burst to opsonised *S.aureus*

Serum G-CSF is mildly raised in CD disease

G-CSF induced venous neutrophils have reduced respiratory burst and tissue penetration

During G-CSF, venous neutrophil respiratory burst and neutrophil and macrophage tissue penetration are normal in CD disease

G-CSF may ameliorate active fistulous CD disease *in vivo*

There is population variability in the ratio of p47^{phox} gene and pseudogene, which may be a susceptibility gene for CD disease

Haploinsufficiency for p47^{phox} can not be assessed by a genescan method

ACKNOWLEDGMENTS

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PUBLICATIONS

ABSTRACTS

R. Day, M. Harbord, A. Segal, A. Forbes.

Differences in micro-vascular blood flow at sites of skin blistering may account for reduced

leukocyte trafficking in Crohn's disease

Gut 2002; 50 (Suppl. 11): A77

Gastroenterology 2002; 122(Suppl. 4): A265

M. Harbord, M.R. Novelli, S.L. Bloom & A.W.Segal

A transgenic mouse model of chronic granulomatous disease

Experimental Models of Immune Dysregulation and Mucosal Inflammation

Keystone Symposia 1999, Santa Fe: 209P

ORAL PRESENTATIONS

M. Harbord, R.Day, A.Hankin, S.Bloom, A.Forbes, A.W.Segal.

Neutrophil respiratory burst and tissue penetration are normal in Crohns disease during G-CSF

administration

European Society for Clinical Investigation, 2002

Also presented in abstract form at the British Society of Gastroenterology (Gut 2002; 50 (Suppl.

11): A67) and at the American Gastroenterological Assosciation (Gastroenterology 2002; 122

(Suppl. 4): A433

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Neutrophil deficiency during acute inflammation in inflammatory bowel disease

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YM1 crystals in chronic granulomatous syndrome mice

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Ym1 is a neutrophil granule protein that crystallizes in p47phox deficient mice

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Cantharidin Blisters : A technique for investigating leukocyte trafficking and cytokine

production at sites of inflammation in humans

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PAPERS IN PREPARATION

Cells and mediators in skin blisters in inflammatory bowel disease

M.Harbord, R.Day, S.Bloom, A. Hankin, A.Forbes, A.W.Segal

Chronic granulomatous disease mice are susceptible to induced colitis

M Harbord, A Hankin, J Roes, M Novelli, AW Segal.

INVITED TALKS

Royal London Hospital GUT Club, 2002

Royal Free Hospital gastroenterology seminars 2000

Chronic Granulomatous Disease Research Trust family day 2000

Chronic Granulomatous Disease Research Trust consultant's conference 1999

Pearce Gould Visiting Professor G.I. symposium, UCL 1999

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Ym1 Is a Neutrophil Granule Protein That Crystallizes in p47^{phox}-deficient Mice*

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Crystals were discovered within the aged lung and at sites of chronic inflammation in a mouse model of chronic granulomatous disease. Following re-crystallization at neutral pH, the crystals were identified as the chitinase-like protein Ym1, expressed in organs of the lymphoreticular system, the lung, and distal stomach. Ym1 was shown to be a neutrophil granule protein and to have weak β-N-acetylglucosaminidase activity, indicating that it might contribute to the digestion of glycosaminoglycans. Crystal formation is likely to be a function of excess neutrophil turnover at sites of inflammation in the chronic granulomatous disease mouse. Failure to remove subcutaneous Ym1 crystals injected into knockout mice indicates that a failure of digestion may also contribute to crystallization.

Chronic granulomatous disease (CGD)[1] is a rare genetic disease that presents with recurrent life-threatening bacterial and fungal infections. The commonest autosomal recessive cause of CGD, affecting 25% of patients (1), is caused by defects in the p47^{phox} subunit of the NADPH oxidase. We have created a mouse model of CGD by disrupting this subunit in order to increase the understanding of the biochemistry and cell biology of this disease. During histological review of our p47^{phox} knockout (p47^{phox} −/−) mouse, we discovered crystals in the lung of aged mice, which was not described in the report of the original p47^{phox} −/− mouse in 1995 (2).

Recently Ym1, a member of a family of mammalian proteins (Table I) that shares sequences with lower organism chitinases, has been described as crystallizing in the lungs of three immunodeficient mice as follows: the moth-eaten mouse, the CD40L-deficient mouse, and a transgenic mouse with lung-specific human tumor necrosis factor receptor expression (3). Increased expression of Ym2, a Ym1 splice variant (4), has been found in a murine model of asthma, believed to be macrophage-derived

and dependent on CD4$^+$ T cells (5). Gastric hyalinosis in CYP1A2-null B6, 129 mice has recently been identified as Ym2 (6), and in the same study, anti-Ym1 immunoreactivity demonstrated to pulmonary macrophage crystals. Unidentified pulmonary crystalline deposits have been described in other transgenic, immune-deficient mice (7) and in certain strains of immune-competent mice, particularly on a 129Sv or C57BL/6 background (8), especially during experimental chronic pulmonary infection (9). These were previously thought to be Charcot-Leyden-like crystals, but it now seems likely that they are composed of Ym1 protein.

Ym1 mRNA is expressed in the spleen and lung and a highly homologous gene, ym2, in the thymus, stomach, and kidney (4). The distribution of Ym1 protein within the cells of these and other tissues has not been determined, although it has been assumed to be a macrophage protein because it was first discovered in a peritoneal macrophage cDNA library (10). Recently, enhanced macrophage ym1 gene expression, mediated by a homologue of macrophage migration inhibitory factor, has been found associated with Th2-cytokine inducing helminth infection (11). The function in vivo is unknown, although recently the same researchers (12) have both deduced the protein structure, which contains a saccharide-binding site, and shown that Ym1 functions as a lectin, binding multivalent hexosamines and heparin (10). Ym1 may have weak chitinase activity, thus cleaving several moieties of β-1,4-linked N-acetyl glucosamine (GlcNAc) (3), and therefore may have a defensive role. Finally, the protein has been shown to be chemotactic for eosinophils (13), and high concentrations are associated with eosinophil recruitment in vivo (11).

In this study, we demonstrate that p47^{phox} −/− murine pulmonary crystals are indeed Ym1 and that the protein is stored in neutrophil granules. In addition, we show that pure Ym1 can function as a β-N-acetylhexosaminidase and not a chitinase. We show that Ym1 crystals easily form in vitro and speculate that excessive Ym1 release during acute inflammation causes crystal formation in vivo.

EXPERIMENTAL PROCEDURES

Generation of CGD Mouse—Compounds were purchased from Sigma unless stated otherwise. The p47^{phox} gene was isolated from a P1 mouse ES cell library (Strain 129Sv; Genome Systems, St Louis, MO) and partially characterized. A neomycin resistance gene was inserted into the third exon of the p47^{phox} gene as an XhoI/SalI fragment after conversion of a BspMI site in exon 3 into a SalI site by linker ligation. A PstI site 5′ of exon 3 was converted into XhoI by linker ligation to provide a unique restriction site for linearization of the targeting vector before transfection. The HSV-Tk gene was attached to the 3′ end of the region of homology to permit negative selection of clones with random integration of the targeting vector. Gene targeting in mouse embryonic stem cells was done as described previously (14). p47^{phox} −/− mice were established on the 129 background by mating transmitting chimeras with 129Sv females. Mice were maintained in conventional housing

[1] The abbreviations used are: CGD, chronic granulomatous disease; p47^{phox} −/−, p47^{phox} knockout; GlcNAc, N-acetyl glucosamine; DSS, dextran sodium sulfate; PBS, phosphate-buffered saline; MPO, myeloperoxidase; MU, 4-methylumbelliferone; MU-(GlcNAc)$_n$, (n = 1, 2, and 3) β1→4-linked mono/di/trisaccharides of GlcNAc linked to MU; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline.
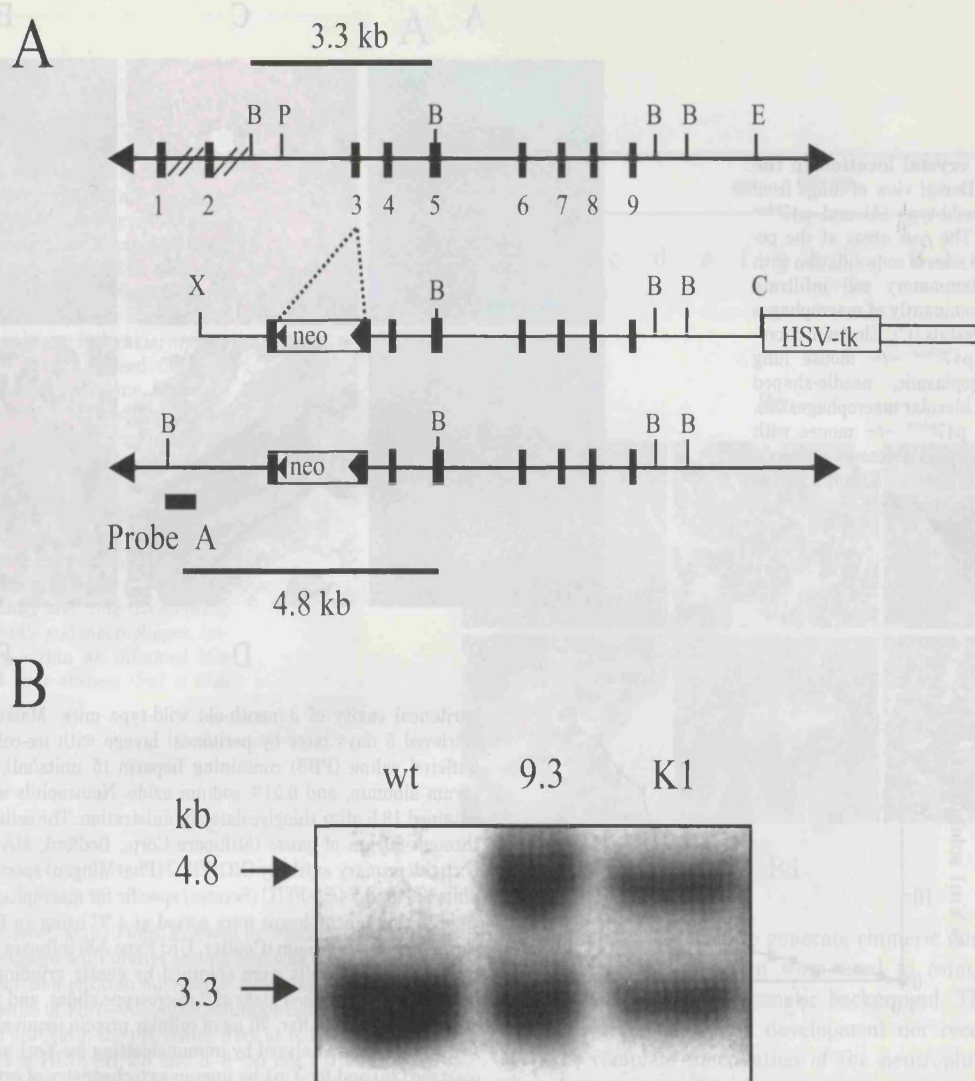
FIG. 1. **Targeted mutagenesis of the p47^phox locus in mouse embryonic stem cells.** *A*, the structure of the genomic locus (*top*), the targeting construct (*middle*), and the targeted locus after homologous recombination (*bottom*). *Solid boxes* represent exons, and the *open boxes* indicate either the neomycin resistance gene, the *HSV-Tk* gene as indicated, or remaining plasmid vector sequences. The *triangles* represent loxP sites flanking this version of the neomycin (*neo*) gene. *B*, *Bam*HI; *C*, *Cla*I; *E*, *Eco*RI; *P*, *Pst*I. *B*, Southern blot analysis of genomic DNA samples digested with *Bam*HI and probed with a genomic fragment (probe A) derived from the p47^phox locus. Targeted insertion of the neomycin resistance gene leads to an increase of the *Bam*HI restriction fragment of wild-type (*wt*) clones by 1.5 kb, evident in clones 9.3 and K1.

facilities. All mice received water and standard mouse chow (SDS-3 expanded, Lillico, UK) *ad libitum*. Acute colitis with portal pyemia, cholangitis, and liver abscess formation was induced in a cohort of mice using oral administration of dextran sodium sulfate (DSS) (15).

Purification and Identification of Lung Crystals—Lungs from 12-month-old p47^phox −/− mice were gently Dounce-homogenized and incubated at 37 °C for 90 min with collagenase (1 mg/ml in water; 5 mg/lung), DNase (30 μg/ml in water), and protease inhibitors (1 μg/ml leupeptin; 1 μg/ml pepstatin A; 1 μg/ml tosyl-lysine chloromethyl ketone; 0.5 mM phenylmethylsulfonyl fluoride). The digest was layered on top of Lymphoprep (Nycomed, Oslo, Norway) and centrifuged (1000 × *g*, 4 °C, 20 min). The pellet containing the crystals was washed in water and centrifuged (100,000 × *g*, 4 °C, 30 min) 3 times. Enriched crystals were dissolved in 10 mM CHES, pH 9.5 (250 μl/lung), for 1 h and then 100 mM MES, pH 6, added to the supernatant for 18 h (1:3 v/v; 4 °C, resultant, pH 6.06) to re-crystallize the protein. This was washed 3 times in water and further purified by re-crystallization. Protein size and purity was assessed by staining with Coomassie Brilliant Blue (BDH Laboratory Supplies, Poole, UK) after SDS-PAGE (16), and the yield was determined by the Bio-Rad protein assay (Bio-Rad) (17). To determine the effect of pH on Ym1 solubilization, 20 mg/ml crystalline Ym1 in water was incubated with 50 mM Tris/HCl, pH 7.0–8.5, for 2 h at 25 °C, and the supernatant protein was quantified. Each protein band was identified by matrix-assisted laser desorption ionization mass spectroscopy of trypsin (Promega Corp.) digests using a Bruker Biflex III mass spectrometer (Bruker, UK) (18). The protein was identified using Mascot (www.matrixscience.com), PeptIdent (www.expasy.ch), and MS-FIT (prospector.ucsf.edu/ucsfhtml3.2/msfit.htm). Protein iden-

tity was confirmed by automated Edman degradation using an ABI Procise protein sequencer (Applied Biosystems, Foster City, CA) (19).

Ym1 Tissue Distribution—Formalin-fixed tissues for light microscopy were paraffin-embedded and stained with hematoxylin/eosin or Wright/Giemsa. Lung samples for electron microscopy were retrieved from formalin-fixed paraffin-embedded p47^phox −/− tissue using a combined toluene/osmium retrieval method. Silver to gold ultrathin sections were cut on a Reichart-Jung Ultracut II (Leica Microsystems, UK) and double-stained with Reynold's lead citrate and uranyl acetate. Sections were examined and photographed on a Jeol 1200 EX transmission electron microscope (JEOL Ltd.). For immunohistochemistry, rabbit polyclonal sera reactive to Ym1 (20) was affinity-purified on a HiTrap affinity column (Amersham Biosciences) (21). 3-μm sections from paraffin-embedded tissue were dried overnight at 60 °C, taken to absolute alcohol, blocked for 10 min with 0.5% hydrogen peroxide in methanol, and rinsed thoroughly in water. They were digested for 10 min in a 0.1% solution of chymotrypsin containing 0.1% calcium chloride (pH 7.8, 37 °C) and rinsed in water and then in 0.05% Tween 20 in Tris-buffered saline (pH 7.4). Endogenous biotin activity was blocked (Avidin/Biotin Blocking Kit, Vector Laboratories); the sections were incubated for 10 min in 1/10 normal swine serum (Dako Ltd., Ely, UK) and then incubated for 60 min with the primary antibody (1/3000 anti-Ym1 or rabbit polyclonal anti-thyroglobulin as a nonspecific control). They were developed using a biotinylated 1/200 swine anti-rabbit secondary antibody (Dako) and a streptavidin-biotin-peroxidase complex (Dako) and visualized with DAB (Kem-en-Tec, Copenhagen, Denmark). For immunoblotting, mouse organs from two p47^phox −/− and two wild-type mice aged 3 weeks, 6 weeks, 5 months, and 10 months

FIG. 2. **Ym1 crystal location in the CGD mouse.** Dorsal view of lungs from 10-month-old wild-type (*A*) and p47^{phox} −/− (*B*) mice. The *pale areas* at the periphery of *B* are sites of consolidation with a chronic inflammatory cell infiltrate composed predominantly of macrophages surrounding crystals (*C*). Electron micrograph of the p47^{phox} −/− mouse lung show intracytoplasmic, needle-shaped crystals within alveolar macrophages (*D*). A 6-month-old p47^{phox} −/− mouse with chronic liver and skin abscesses had crystals within the bile ducts (*E*) and adjacent to bacteria in the skin (*F*).
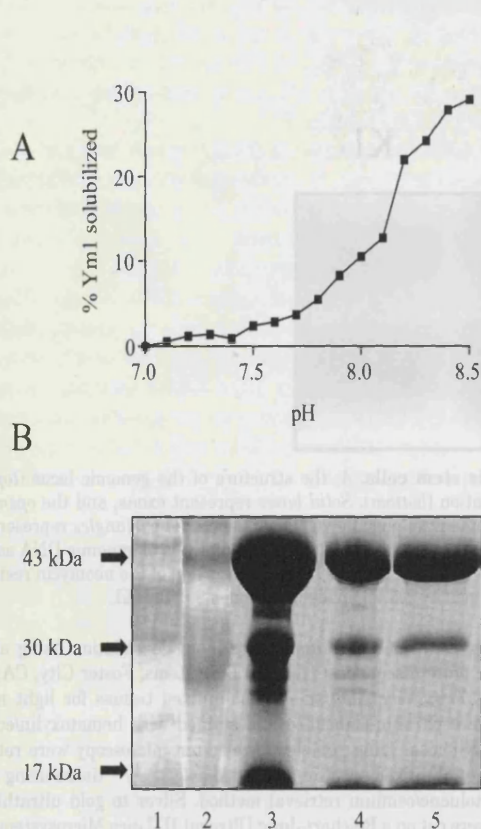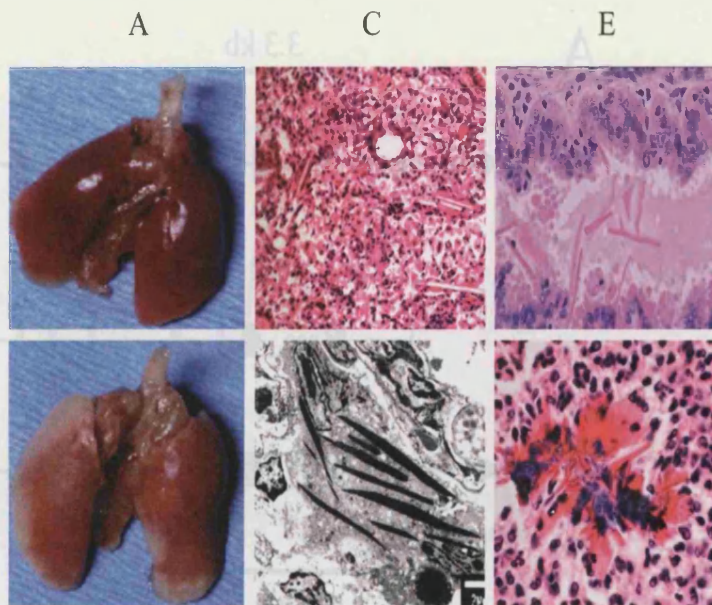

FIG. 3. **Purification of Ym1.** *A*, purified crystalline Ym1 solubilization after 2 h of incubation at pH 7.0–8.5. *B*, SDS-PAGE separation of Ym1 protein at different stages of purification. *Lane 1*, wild-type lung homogenate; *lane 2*, p47^{phox} −/− lung homogenate; *lane 3*, after alkaline solubilization; *lane 4*, re-crystallization; and *lane 5*, re-alkalization. The bands in *lane 5* were Ym1 or Ym1 breakdown products.

were homogenized in a 10-fold volume (v/w) of ice-cold buffer (50 mM Tris, pH 6.8, containing protease inhibitors). Gastrointestinal tract contents, removed without any mucosal disturbance, were centrifuged (15,000 × *g*, 4 °C, 10 min), and the supernatant was then collected. Immunoblots of 2 μg of protein and Ym1 standards (50, 10, and 1 ng) were probed with anti-Ym1 (1 μg/ml)(22). Anti-Ym1 was detected using anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody) (Amersham Biosciences) and ECL Western blotting detection reagents (Amersham Biosciences) for qualitative evaluation, or ^{125}I-anti-rabbit immunoglobulin (Amersham Biosciences), detected using the FLA3000 PhosphorImager (FujiFilm, Japan), for quantitative evaluation.

For cellular origin, 0.8 ml of 3% thioglycolate was injected into the peritoneal cavity of 3-month-old wild-type mice. Macrophages were retrieved 5 days later by peritoneal lavage with ice-cold phosphate-buffered saline (PBS) containing heparin (5 units/ml), 0.5% bovine serum albumin, and 0.01% sodium azide. Neutrophils were similarly obtained 18 h after thioglycolate administration. The cells were filtered through 60 μm of gauze (Millipore Corp., Bedford, MA) and stained with the primary antibody GR1-FITC (PharMingen) specific for neutrophils (23) and F4/80-FITC (Serotec) specific for macrophages (24). Neutrophils and macrophages were sorted at 4 °C using an EpicsElite cell sorter, orifice size 75 μm (Coulter, UK; Expo 300 software, Applied Cyto Systems, UK). T cells were obtained by gently grinding the thymus between the roughened glass of 2 microscope slides, and the cells were washed free with buffer. 20 μg of cellular protein (equivalent to 25,000 neutrophils) was analyzed by immunoblotting for Ym1 and neutrophil elastase (25) and for Ym1 by immunocytochemistry of cytospin smears (Cytospin 2, Shadon, UK).

Subcellular Fractionation of Wild-type Murine Neutrophils—Thioglycolate-induced peritoneal neutrophils were obtained from eight wild-type mice. 2 μl of di-isopropyl fluorophosphate (1 M in ethanol) was added to the cell pellet, followed by 2.5 ml of break buffer (10 mM PIPES, pH 7.1; 100 mM KCl; 3 mM NaCl; 3.5 mM MgCl$_2$; protease inhibitors). The cells were sonicated (Soniprep 150, Sanyo, Japan) on ice for 3 cycles of 5 s, centrifuged (1000 × *g*, 4 °C, 10 min), the postnuclear supernatant layered onto a 10 ml of continuous sucrose gradient (15–55% w/w in break buffer containing 1 mM EDTA, pH 7.4; 5 units/ml heparin; and protease inhibitors), and centrifuged in a Beckman TLS55 head (150,000 × *g*; 4 °C, 1 h). The gradient was collected in 40 fractions by aspiration from the superior surface. Sucrose concentration was determined by refractometry. Fractions were analyzed for Ym1 and lactoferrin by immunoblotting and for myeloperoxidase (MPO) activity (26).

Immunogold Electron Microscopy of Peritoneal Neutrophils—Wild-type thioglycolate-induced peritoneal neutrophils, fixed in 2% paraformaldehyde containing 0.5% glutaraldehyde in PBS, were dehydrated through a graded series of alcohol, followed by two changes of propylene oxide, and embedded with araldite CY212 resin. Silver to gold ultrathin sections were cut with a diamond knife, collected onto 200-mesh carbon-coated nickel grids, incubated with 1% periodic acid for 10 min, washed with distilled water, incubated with 50 mM glycine for 5 min, washed in PBS, and incubated for 5 min in PBS containing 0.5% bovine serum albumin, 0.1% Triton X-100, 0.1% lysine, and 0.1% sodium azide. After overnight incubation in a 1:2000 dilution of anti-Ym1, the cells were washed with PBS and incubated for 1 h with a 1/100 dilution of nanogold goat anti rabbit Fab′ conjugate (Nanoprobes, particle size 1.5 nm) in PBS containing 2% normal goat serum. After washing for 30 min in PBS, the sections were post-fixed with 1% glutaraldehyde for 10 min and washed for a further 15 min. The detection of gold particles was enhanced with HQsilver (Nanoprobes). Equal parts of initiator and moderator were mixed before adding activator and mixing thoroughly. The mixture was put on ice under safe lights, and the sections were

A

43 kDa →

a b c d e f g h i j k l m n o p

B

Sp   Bm   Ga

L   Bd   Sa

FIG. 4. **Ym1 expression in the CGD mouse.** *A*, anti-Ym1 (1 μg/ml) immunoblot of wild-type mouse organs demonstrating the major sites of expression; *a*, Ym1 (1 μg); *b*, venous blood; *c*, colon; *d*, ileum; *e*, duodenum; *f*, gastric antrum; *g*, proximal stomach; *h*, bone marrow; *i*, lung; *j*, brain; *k*, heart; *l*, thymus; *m*, kidney; *n*, testis; *o*, liver; *p*, spleen. 50 μg of protein was loaded in each lane. *B*, immunohistochemistry for Ym1 confirmed the major tissues of protein expression in the $p47^{phox}$ −/− mouse as spleen (*Sp*) showing scattered positive cells in the red pulp, bone marrow (*Bm*) showing staining of the granulocytic cell lineage, non-specialized type gastric mucosa in the gastric antrum (*Ga*) showing both parietal and chief cell staining, and lung (*L*) showing staining of crystals and macrophages. Immunoreactivity within an inflamed bile duct (*Bd*) and skin abscess (*Sa*) is also shown.
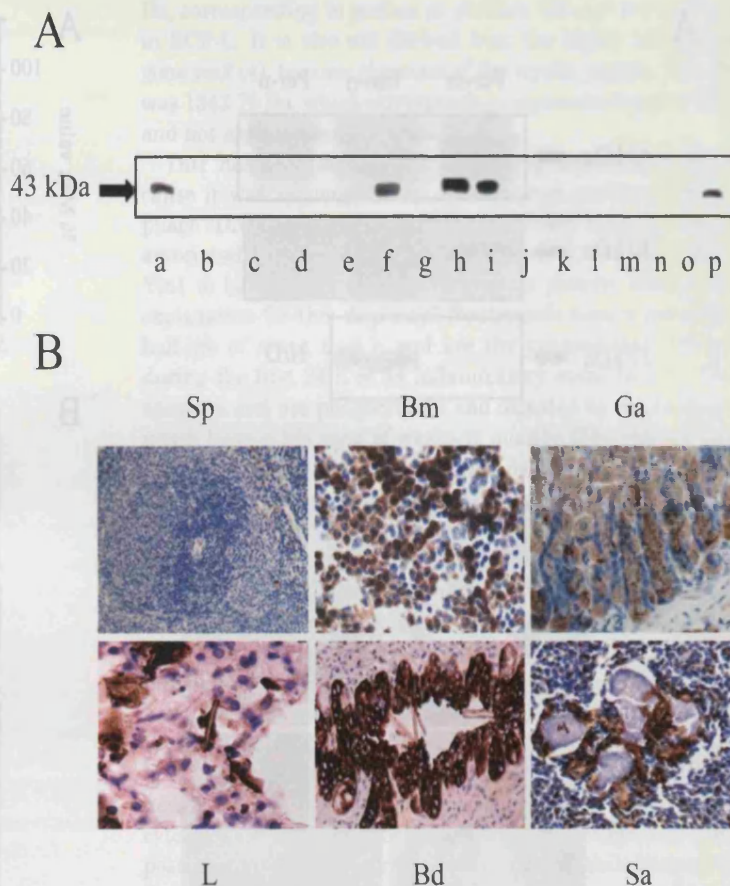
floated for between 2 and 10 min, followed by washing for 15 min. The sections were stained with uranyl acetate and lead citrate before viewing in a transmission electron microscope operating at 80 kV.

Enzyme Kinetics of Ym1—All buffer solutions contained 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM NaCl; the pH values were as follows: pH 1.25 and 2 (0.2 M KCl, 0.2 M HCl); pH 2.5 and 3–6 (50 mM citric acid, 100 mM Na_2HPO_4); pH 7, 7.5, and 8–10 (50 mM Tris/HCl). Purified Ym1 crystals (18 mg/ml in water) were solubilized by adding 11 volumes of 10 mM CHES, pH 9.5. Three volumes of acidic buffer, pH 2.5–5.5, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM NaCl, citric acid, and Na_2HPO_4, were added to the supernatant. Under these conditions Ym1 (<400 μg/ml) remained in solution. 50-μg aliquots of soluble Ym1 or buffer alone were incubated at 37 °C with 110 μM of 4-methylumbelliferyl-*N*-acetyl-BD-glucosaminide (MU-(GlcNAc)$_1$), 4-methylumbelliferyl-diacetylchitobioside (MU-(GlcNAc)$_2$), or 4-methylumbelliferyl triacetylchitotrioside (MU-(GlcNAc)$_3$) (dissolved respectively in Me$_2$SO, *N,N*-dimethylformamide, and Me$_2$SO) at a final pH 2.5–5.5, in 0.5 pH unit increments. Net fluorescence (emission 445 nm; excitation 365 nm) was measured over 80 min (Cytofluor multiwell plate reader, Perspective Biosystems). MU released by Ym1 was determined against standard curves for each pH of 4-methylumbelliferone (MU) (in *N,N*-dimethylformamide). In the original method (27) an alkaline stop buffer was added to enhance the measured fluorescence from MU. We obtained equivalent results for enzymatic activity with or without the addition of alkaline stop buffer because fluorescence from MU is stable at acidic pH (27). The assays were performed in triplicate using two preparations of Ym1. Concurrent experiments were performed using chitinase from *Serratia marcescens* as a positive control.

Subcutaneous Injection of Crystalline Ym1—Soluble Ym1 protein was sterilized (0.22-μm filter, Millipore, Bedford, MA), re-crystallized at pH 7.4, and 20 μg injected subcutaneously into three $p47^{phox}$ −/− and three wild-type mice. 20 μg of uric acid (pH 7.4) was injected as a control. Sterility of the injected material was confirmed pre/post-procedure by aerobic culture. The injection sites were excised after 10 days and assessed histologically.

RESULTS

Generation of $p47^{phox}$-deficient Mice—The $p47^{phox}$ gene was disrupted in mouse embryonic stem cells by insertion of a neomycin resistance gene into the third exon of the $p47^{phox}$ gene (Fig. 1). Two targeted embryonic stem cell clones (9.3, K1)

were obtained and used to generate chimeric mice. Chimeras transmitting the mutation were used to establish mutant mouse lines on the 129 genetic background. This mutation neither affects neutrophil development nor recruitment but leads to complete inactivation of the neutrophil respiratory burst in response to stimulation with phorbol myristate acetate or *Candida albicans* as shown previously (25).

Crystal Location and Identification—Spontaneous pulmonary crystal formation was noted in the majority of $p47^{phox}$ −/− mouse lungs but never in wild-type lungs ($n = 25/33$ $p47^{phox}$ −/−, 5.5 ± 2.6 months (mean ± S.D.); $n = 0/33$ wild-type, 5.6 ± 1.9 months). Crystals were seen in mice aged greater than 2 months, with density increasing with age ($R^2 = 0.39$). The distribution was patchy, greatest in basal and peripheral foci. Crystals were mainly extracellular, multifaceted, ~10–100 μm in length, and often associated with giant cells and macrophages. In addition, they were frequently noted in chronically inflamed $p47^{phox}$ −/− bile ducts following oral DSS and in spontaneous skin abscesses (Fig. 2). In two wild-type mice they were seen in DSS-induced, severely inflamed bile ducts. Ym1 crystals were resistant to degradation in $p47^{phox}$ −/− mice, because copious crystals persisted in the $p47^{phox}$ −/− mice 10 days after subcutaneous Ym1 injection, associated with a giant cell response, whereas none were seen in wild-type mice. No Ym1 crystals were seen at sites injected with uric acid, suggesting that the findings were not due to neo-crystal formation in response to the introduction of skin bacteria at the time of injection.

We discovered that Ym1 crystals entered solution at alkaline (and acid) pH but were insoluble at pH 7.0 (Fig. 3). This allowed the purification of ~2.5 mg of protein from each aged $p47^{phox}$ −/− mouse (Fig. 3). Mass fingerprinting identified the major band at 43 kDa as mouse secretary protein Ym1 and lower molecular weight bands as Ym1 cleavage products. This finding pre-dated and therefore was independent of the report by
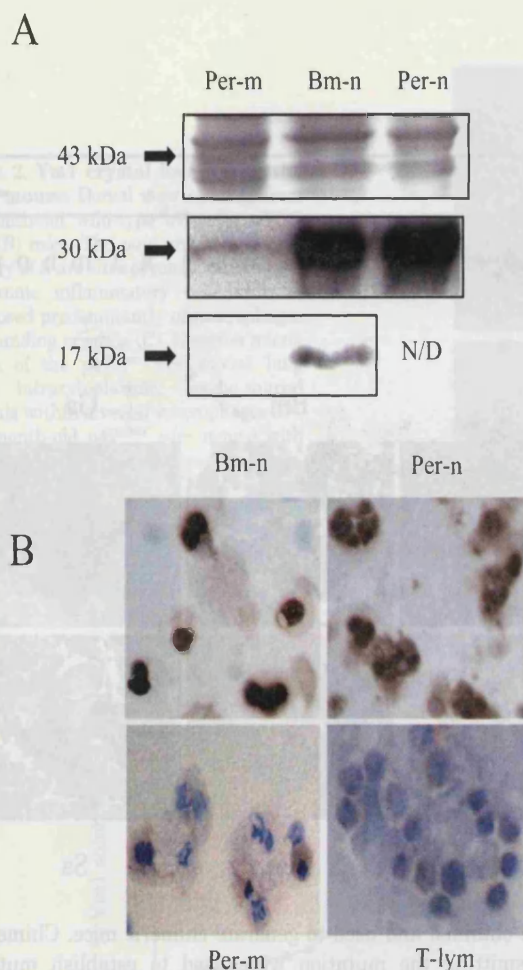
A

B

FIG. 5. **Ym1 is preferentially expressed in bone marrow (*Bm-n*) and peritoneal (*Per-n*) neutrophils compared with peritoneal macrophages (*Per-m*).** *A*, Coomassie Brilliant Blue (BDH)-stained gel (*top*) demonstrates equal protein loading of immunoblots with anti-Ym1 (*middle*); anti-elastase (*bottom*) confirms correct sample assignation; *N/D*, not done. *B*, cytospin preparations of bone marrow (*Bm-n*) and peritoneal (*Per-n*) neutrophils, peritoneal macrophages (*Per-m*), and T-lymphocytes (*T-lym*) stained with anti-Ym1. Occasional Ym1 staining in the thymus extract was within macrophages.
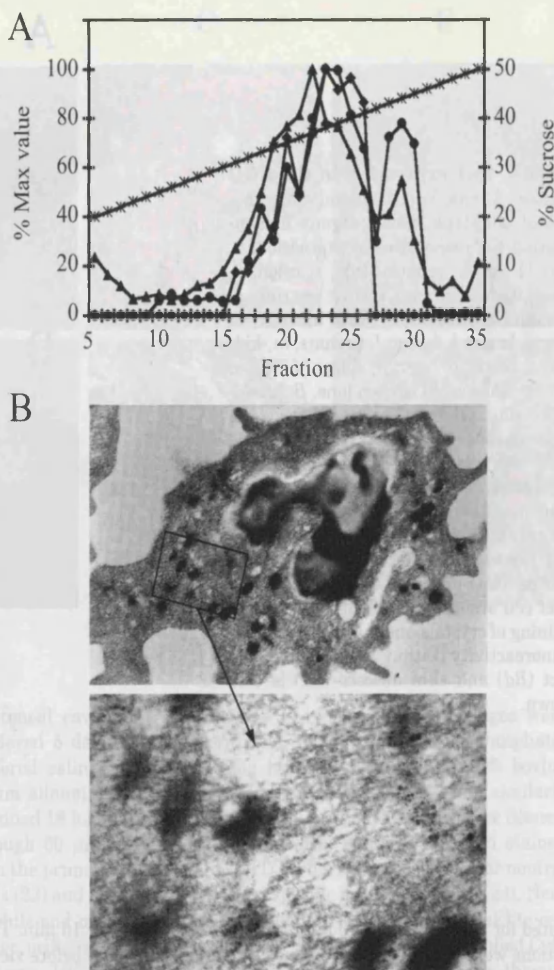
A

B

FIG. 6. **Ym1 is a neutrophil granule protein.** *A*, aliquots from a sucrose density gradient (*) separation of murine neutrophils probed with anti-Ym1 and anti-lactoferrin and assayed for MPO. Ym1 (●) and MPO (▲) are in similar proportions in the same subcellular fractions of the neutrophil. Lactoferrin (♦) is not seen in the denser fractions. *B*, electron micrograph of a neutrophil sectioned through its center, showing its nucleus (*top*, ×16,000; print magnification ×44,000). The cytoplasmic granules are electron dense and heavily labeled with silver-enhanced gold particles, adherent to anti-Ym1 antibody; there is no labeling elsewhere in the cytoplasm (*bottom*, ×40,000; print magnification ×110,000).

Guo *et al.* (3), identifying pulmonary crystals in other mice strains as Ym1. By using Mascot, the probability-based Mowse score with 10/13 peptide matches was 105 ($p < 0.05$). Edman degradation confirmed the identity.

Ym1 Tissue Distribution—Western blot analysis showed that protein expression was greatest in the lung, spleen, bone marrow, and gastric antrum of normal and $p47^{phox}$ $-/-$ mice. The results were confirmed by immunohistochemistry (Fig. 4). Smaller amounts of Ym1 were seen in the liver, kidney, brain, heart, proximal duodenum, and submandibular gland and in one mutant mouse in the thymus. There was similar Ym1 expression (with a gradual increase with mouse age) in $p47^{phox}$ $-/-$ and wild-type mice, except in the aged $p47^{phox}$ $-/-$ lung, where very high levels were observed consistent with the development of crystals (at 42 weeks $p47^{phox}$ $-/-$ 20.6 ± 1.0 ng of Ym1/μg of lung protein; wild-type 0.9 ± 0.2 ng/μg). Gastric antrum immunoreactivity occurred at a different molecular weight to the other tissues (45 and 42 kDa) and probably represents antibody cross-reactivity with Ym2, recently shown to cause gastric hyalinosis in CYP1A2-null B6, 129 mice (6). The finding of Ym1 immunoreactivity within the gastrointestinal fluid suggests the protein is secreted into the stomach lumen, where it may aid digestion (stomach 0.8 ± 0.9 ng of Ym1/μg of protein; ileum 3.8 ± 2.1; colon 5.9 ± 4.1).

Ym1 Is a Neutrophil Granule Protein—Before this study, the

main cell of origin of Ym1 was presumed to be the macrophage, and the subcellular location was not known. We demonstrated here that Ym1 was neutrophil-derived (2.5 ng/μg neutrophil protein) and present equally in both peritoneal and bone marrow-derived neutrophils. Ym1 was also present within purified peritoneal macrophages but at a much lower concentration (0.2 ng/μg) (Fig. 5). Ym1 was not detected in T or B cells (data not shown). Subcellular fractionation by sucrose density centrifugation showed that Ym1 colocalizes with neutrophil granule proteins. This was confirmed by immunogold electron microscopy of murine neutrophils, which demonstrated very clearly a granular distribution. The peaks of Ym1, lactoferrin, and MPO occurred at sucrose densities of 37–39%, but Ym1 and MPO were also present in denser fractions (43–45%) (Fig. 6). This suggests Ym1 may be present in the azurophilic granules as it coincided with the azurophilic granule protein MPO rather than with the specific granule protein, lactoferrin (28).

Ym1 Is a β-N-Acetylhexosaminidase—The production of pure Ym1 allowed for the first time the accurate characterization of its chitinase-like activity. It was discovered that at low concentrations, the protein could be maintained in solution when reacidified, which allowed the analysis of pure, soluble Ym1 through an acidic pH range. The low enzymatic activity was
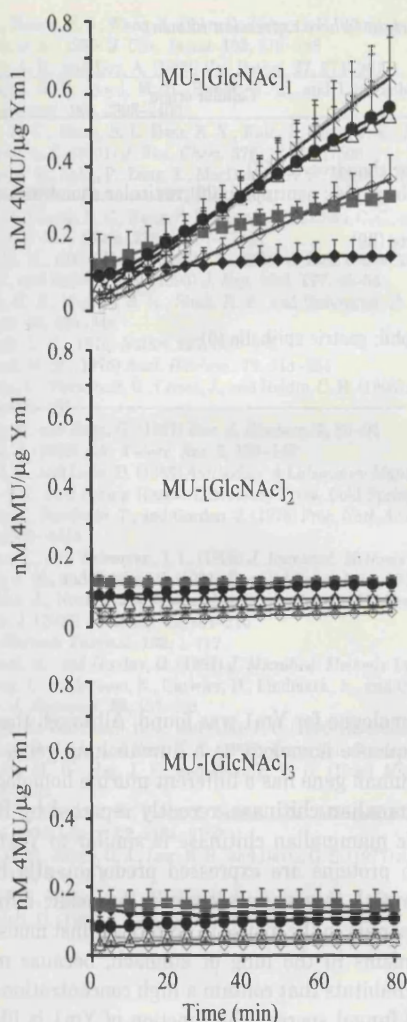
FIG. 7. **Ym1 is a weak β-N-acetylhexosaminidase.** Cleavage of the substrate MU-(GlcNAc)$_{1,2,3}$ at pH 2.5 ($\blacklozenge$), 3.0 ($\blacksquare$), 3.5 ($\triangle$), 4.0 ($\square$), 4.5 ($\times$), 5.0 ($\bullet$), and 5.5 ($\diamond$) by pure Ym1 in solution to release MU demonstrates only β-N-acetylhexosaminidase activity, maximal at pH 4 and 4.5.

assessed using sensitive assays based on the release of free MU. There was maximal cleavage of the GlcNAc$_2$ analogue, MU-(GlcNAc)$_1$, at pH 4 and 4.5, but no significant cleavage of MU-(GlcNAc)$_2$ or MU-(GlcNAc)$_3$ (Fig. 7). This defines Ym1 as having β-N-acetylhexosaminidase and not chitinase activity (29). We confirmed the finding (3) that cleavage was specific to the β-D-linkage of GlcNAc because Ym1 did not cleave MU-N-acetyl-α-D-glucosamine (data not shown). The V_{max} and K_m values were 0.023 μmol/min/mg Ym1 and 120.8 μM, respectively (30). The K_m is similar to known values for chitinases found in *S. marcescens* (31), but the V_{max} is several orders of magnitude less. Ym1 has a small amount of autofluorescence, equivalent to a maximum of 0.07 μmol/mg protein, which represents a part of the small amount of net fluorescence emitted with MU-(GlcNAc)$_2$ and MU-(GlcNAc)$_3$. Chitinase from *S. marcescens* cleaved MU-(GlcNAc)$_1$, MU-(GlcNAc)$_2$, and MU-(GlcNAc)$_3$ equally with an activity ~1000-fold more than Ym1 (data not shown).

DISCUSSION

Lungs from aged p47^{phox} −/− mice showed patchy consolidation with a heavy intra-alveolar chronic inflammatory cell infiltrate composed predominantly of macrophages together with numerous crystals, which we identified as the chitinase-like protein Ym1. We also demonstrated that similar crystals within the inflamed biliary tree were Ym1. The protein was confirmed as Ym1 and not the very similar protein ECF-L (13) because the mass of the tryptic peptide 102–117 was 1722.82

Da, corresponding to proline at position 106 and not serine as in ECF-L. It is also not derived from the highly homologous gene *ym2* (4), because the mass of the tryptic peptide 220–231 was 1343.70 Da, which corresponds to aspartate at position 220 and not asparagine as in Ym2.

Ym1 has been assumed to be macrophage-derived (5) because it was sequenced from an activated peritoneal macrophage cDNA library (10), and microscopically Ym1 crystals are associated with macrophages (3). However, we have shown Ym1 to be mainly a neutrophil granule protein. What is the explanation for this disparity? Neutrophils have a circulation half-life of about 6–8 h and are the predominant cell type during the first 24 h of an inflammatory stimulus (32). They apoptose and are phagocytosed and digested by macrophages, which have a life span of weeks to months (33), and can very rapidly degrade apoptotic neutrophils (up to 1 neutrophil/macrophage/30 min) (34). Extracellular non-degradable crystalline Ym1, or Ym1 contained within apoptotic neutrophils, will appear to be mainly associated with macrophages. This is compatible with the low concentration of Ym1 in thioglycolate-induced peritoneal macrophages, where the majority of cells will have been recently synthesized from bone marrow promonocytes and blood monocytes (35) and therefore will not have had the opportunity to phagocytose many neutrophils. It is noteworthy that another member of this family of proteins, YKL-40, has been found in the specific granules of human neutrophils (36) and that chitinase activity in human leukocytes has been shown to be mainly from granulocytes (37). It is possible that Ym1 is expressed also by eosinophils because the experimental induction of murine macrophage crystalline inclusions has been described after the intraperitoneal injection of human eosinophil-rich granulocytes (38), and both alveolar macrophage crystals in C57/BL6 mice (9) and Ym2 expression during experimental allergy (5) increase with peripheral eosinophilia.

Ym1 was able to cleave the GlcNAc$_2$ analogue, MU-(GlcNAc)$_1$ but not MU-(GlcNAc)$_2$ or MU-(GlcNAc)$_3$. Therefore, it can be classified as a β-N-acetylglucosaminidase rather than a chitinase (40) which is compatible with the mutated chitinase active site of Ym1(41). The minimal activity with MU-(GlcNAc)$_2$ and MU-(GlcNAc)$_3$ shows that Ym1 can not cleave N-acetylhexosamine monomers sequentially from the same molecule of MU-(GlcNAc)$_2$ or MU-(GlcNAc)$_3$, as has been described for chitinase A and B of *S. marcescens* (31). Contrary to our findings, Guo *et al* (3) recently suggested that Ym1 also could cleave MU-(GlcNAc)$_{2,3}$. However, it is possible that their preparation contained cellular debris including chitinases of a similar molecular weight as Ym1 (see Table I) or enzymes capable of sequential β-N-acetylglucosaminidase or exo-chitinase activity, allowing Ym1 to release 4MU from MU-(GlcNAc)$_1$. Such problems in distinguishing between exo- and endo-chitinase activity have been described (42). We used purified Ym1, and therefore the enzyme kinetics are specific for Ym1. It is noteworthy that the enzyme activity had the same pH dependence as shown recently for Ym1 binding to certain (oligo)saccharides with a free amine group (10), although binding and cleavage sites of the protein may be distinct. After invasion by chitin containing microorganisms, chitin and other foreign antigens must be digested to prevent a granulomatous tissue response. Chitin can be degraded with a binary enzyme system using a combination of a chitinase and an β-N-acetylglucosaminidase (31). The weak activity demonstrated here may underestimate *in vivo* kinetics because functionality may require synergy with other proteins, shown previously for chitinase (43).

What can be deduced about protein crystallization from our findings? First, crystals may form due to a high local concen-

TABLE I

Mammalian "chitinases" molecular weight and cellular origin (where expression known)

NK, not known.

Name	Mass	Cellular origin
	kDa	
Human YKL-39[a]	43,039	Articular chondrocyte (53)
Human HCgp-39 (YKL-40)[b]	42,613	Macrophage (54); neutrophil (36); articular chondrocyte (55)
Human oviductal glycoprotein[c]	75,421	NK
Human chitotriosidase[d]	51,681	Monocyte (39)
Human TSA1902-L[e]	40,082	NK
Mouse AMCase[f]	50,000	NK
Mouse ECF-L[g]	44,448	NK
Mouse Ym1[h]	44,528	Neutrophil; gastric epithelia (6)
Mouse BRP39[i]	43,001	NK
Mouse oviductin[j]	78,807	NK

[a] NCBI protein database accession number AAC50597.
[b] NCBI protein database accession number AAA16074.
[c] NCBI protein database accession number AAA86946.1.
[d] NCBI protein database accession number AAC50246.1.
[e] NCBI protein database accession number BAA86980.1.
[f] NCBI protein database accession number AAG60018.
[g] NCBI protein database accession number BAA13458.2.
[h] NCBI protein database accession number AAB62394.2.
[i] NCBI protein database accession number CAA63603.1.
[j] NCBI protein database accession number Q62010.

tration of Ym1 secondary to neutrophil degranulation during repeated episodes of inflammation. This is analogous to the deposition of α_1-antitrypsin variants within the liver, where polymerization of the protein is enhanced during episodes of inflammation (44), and to Charcot-Leyden crystal formation, where lysophospholipase, a granule protein comprising 10% of an eosinophil's protein (45), crystallizes during asthmatic inflammatory reactions. In support of this, Ym1 crystals increased with age and were found within the lung, a major portal of antigen entry, in peripheral and basal sites, suggesting a response to inhaled matter; and increased Ym1 expression and Ym1 crystals occurred at extrapulmonary sites of inflammation. Second, a mildly acidic pH milieu within the CGD phagolysosome may contribute to crystallization. Inflammation causes a mildly acidic extracellular environment (46), and the phagolysosome of the human CGD neutrophil has been shown to be abnormally acidic (6 min post-phagocytosis, pH 6.1 *cf.* pH 7.4 in control subjects (47)). Assuming similar pH changes occur in the murine CGD phagolysosome, the concentration of Ym1 protein may exceed the crystallization threshold due to excessive degranulation. There is 1 ng of Ym1 in 10,000 murine neutrophils; the volume of granules in one human neutrophil is 52.5 fl (48); assuming human and murine neutrophils have a similar granule volume, the granule concentration of Ym1 is 1.9 μg/μl. At neutral pH, Ym1 crystallizes *in vitro* when the concentration exceeds 0.4 μg/μl. Third, crystalline Ym1 may be resistant to degradation within the CGD macrophage due to absence of functional NADPH oxidase. This is likely because skin injection experiments showed that only wild-type mice were able to clear Ym1 crystals from their subcutis. In contrast to the CYP1A2-null B6, 129 mouse, no protein crystals were seen within the stomach. This may be due to the extreme acidic environment and/or a lower Ym2 concentration in the p47^{phox} $-/-$ mouse.

It is interesting that a protein that may play a role in the digestion of bacteria and fungi is found as crystal deposits in the CGD mouse, which is susceptible to repeated bacterial and fungal infections. Could a similar phenomenon occur in humans? There are no reports of similar crystals in human patients with CGD. After comprehensive searches against protein, nucleotide, and expressed sequence tag data bases using the Basic Local Alignment Search Tool (National Center for Biotechnology Information (www.ncbi.nlm.nih.gov)), no known

human homologue for Ym1 was found. Although there is 61% protein sequence homology to a human lung gene, *TSA1902* (50), the human gene has a different murine homologue called acidic mammalian chitinase, recently reported by Boot *et al.* (51). Acidic mammalian chitinase is similar to Ym1/Ym2 because both proteins are expressed predominantly by gastric epithelium with maximum activity in an acidic milieu. Ym1/Ym2 may persist in the mouse to protect against mouse-specific microorganisms in the lung or stomach, because mice have evolved in habitats that contain a high concentration of chitin-containing fungal spores. The function of Ym1 is likely to be digestive rather than fungicidal, because neither Ym1 nor anti-Ym1 has anti-*C. albicans* activity *in vitro* at an acidic or normal pH (data not shown).

The function of Ym1 is still unclear but by defining the cellular location as the neutrophil granule and gastric epithelium, it is plausible to suggest that it has a major role in digestion during acute inflammation. Although knockout mice may help determine the function of Ym1, redundancy within this family of proteins may mean that a specific deficiency can be tolerated without a phenotypic change. This is known to be the case with human chitotriosidase deficiency (52). It will be important to study chitinase/β-N-acetylhexosaminidase activity in the human CGD macrophage and neutrophil because it is possible that similar proteins to Ym1 are sequestered, which could enhance the inflammatory reaction seen in these patients.

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