Defective acute inflammation in Crohn’s disease: a clinical investigation

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Summary

Background The cause of Crohn’s disease has not been mechanistically proven. We tested the hypothesis that the disease is a form of immunodeficiency caused by impaired innate immunity.

Methods We investigated inflammatory responses in patients and controls by quantifying neutrophil recruitment and cytokine production after acute trauma, interleukin 8 secretion by cultured monocyte-derived macrophages after exposure to inflammatory mediators, and local inflammatory and vascular changes in response to subcutaneous injection of heat-killed *Escherichia coli*.

Findings In patients with Crohn’s disease, trauma to rectum, ileum, or skin led to abnormally low neutrophil accumulation (differences from healthy individuals of 79%, n=8, p=0.0003; 57%, n=3, p=0.05; 50%, n=13, p<0.0001, respectively) and lower production of proinflammatory interleukin 8 (63%, n=7, p=0.003; 63%, n=3, p=0.05; 45%, n=8, p<0.0001) and interleukin 1β (50%, n=8, p=0.0005). Interleukin 8 secretion by cultured macrophages was reduced after exposure to acute wound fluid (38%, n=50, p=0.05; 45%, n=8, p<0.0001), C5a (48%, n=41, p=0.0005), or tumour necrosis factor α (52%, n=27, p<0.0001). Local inflammatory reaction to inoculation with *E coli* was attenuated, as quantified by changes in bloodflow (ileal disease 50%, n=6, p=0.01; colonic disease 77%, n=6, p<0.0003). This response was mediated by nitric oxide in controls, was increased by sildenafil in patients, and was not related to *CARD15* genotype.

Interpretation In Crohn’s disease, a constitutionally weak immune response predisposes to accumulation of intestinal contents that breach the mucosal barrier of the bowel wall, resulting in granuloma formation and chronic inflammation. Polymorphisms in *CARD15* do not underlie this phenotype, but incapacitate the NOD2 pathway that can compensate for impairment of innate inflammation. Current treatment of secondary chronic inflammation might exaggerate the underlying lesion and promote chronic disease.

Introduction

Crohn’s disease is a chronic inflammatory disorder, the incidence of which rose greatly in the latter part of the 20th century.1 It primarily affects the bowel, but can also involve the musculoskeletal system, skin, and eyes. The origin remains an enigma, although many diverse causes have been proposed.2 The diagnosis is currently established on the basis of clinical, endoscopic, radiological, and histological findings. Characteristic pathological appearances include the formation of skip lesions (discrete regions of inflamed bowel separated by uninvolved mucosa), aphthous ulceration, and fistulation; these signs relate to the presence of an underlying granulomatous transmural inflammation. The search for a microbial cause has been extensive because of the similarity between features of Crohn’s disease and those produced by infection with organisms such as mycobacteria.3 The other main theory is that inflammation arises as a primary autoimmune process, for which both humoral and cellular mechanisms have been implicated.4

Detailed genome-wide linkage analyses in affected families have identified several susceptibility loci (*IBD1–IBD8*) for Crohn’s disease.5 The strongest association is attributable to the *CARD15* gene located within the *IBD1* locus.6–8 The risk of Crohn’s disease is increased two to four times in the presence of a single disease-associated polymorphism; it is increased 20–40 times if two variant alleles are present.

The mechanism of this predisposition remains unclear. *CARD15* encodes a cytoplasmic protein, NOD2, which is expressed predominantly in mononuclear phagocytes. The NOD2 protein is implicated in recognition of muramyl dipeptide (MDP), a component of peptidoglycan that is present in cell walls of gram-positive and gram-negative bacteria9–10 and is found in high concentrations in the bowel lumen.11 Binding of MDP activates the transcription factor nuclear factor (NF) κB,12 leading to induction of genes for proinflammatory cytokines.13 Consequently, the polymorphisms associated with Crohn’s disease that abolish the response to MDP14 should attenuate inflammation. This prediction contrasts both with the pathology of Crohn’s lesions, which are obviously inflamed and contain proinflammatory cytokines,15 and with the therapeutic efficacy of immunosuppressive drugs.16 The situation is further confused by observations from studies in transgenic mice with *Nod2* mutations that produce a truncated protein.17 Although this mutation was believed to be identical to one of the human polymorphisms, the effect seemed to be
opposite: MDP induced greater activation of NFκB and more efficient processing and secretion of interleukin 1β. This finding supported a proinflammatory mechanism.

Despite the robust association, CARD15 is not strongly mechanistically related to the causation of Crohn’s disease. Polymorphisms show very limited penetrance and occur in only 40% of patients (predominantly those with ileal disease) as well as in 15% of healthy individuals. The possibility exists that polymorphisms in CARD15 are not in themselves causal, but modify the immune response in lesions that are elicited by some other mechanism.

An alternative theory is that the acute inflammatory response fails in Crohn’s disease, leading to delayed or incomplete removal of bacteria and other bowel contents that breach the mucosal barrier. Subsequent persistence of this foreign material within the tissues could provoke a granulomatous reaction and produce a secondary chronic inflammation. We undertook to examine acute inflammatory responses in Crohn’s disease and to relate them to the CARD15 genotype.

**Methods**

**Patients**

Patients who met inclusion criteria were identified in the gastroenterology outpatient clinics at University College London Hospitals (UCLH) and invited to participate in the study. Controls were identified through the gastroenterology and rheumatology outpatient clinics at UCLH or the Department of Medicine, University College London (UCL). Patients were recruited by DJBM, MWNH, SB, or AWS. Details of patients and controls included in each set of experiments are provided in the webtable and webfigure. Patients were not receiving immunosuppressive medication (and had not during the preceding 2 months) and all had quiescent disease (Harvey-Bradshaw score $<3$, serum inflammatory markers within normal limits), unless otherwise stated (webtable, webfigure). Although none of the patients had clinically overt disease activity, not all patients were specifically endoscoped to exclude occult gastrointestinal lesions. Patients with Crohn’s disease and controls were approximately matched for age, sex, and smoking history. No patients showed any clinical or biochemical evidence of an impaired nutritional state; serum concentrations of albumin were within the normal range for all patients (mean 45–41 g/L, SE 2–86). Where clinically indicated, concentrations of vitamin B12 and red cell folate in serum were measured, and were not abnormal in any individual. No patient had active fistulating disease at the time of study. These studies were approved by the Joint UCL/UCLH Committee on the Ethics of Human Research, project numbers 00/0004, 02/0324, and 04/Q0502/29. Written informed consent was obtained from all volunteers. No patient was studied more than once in each of the different sets of experiments.

**Procedures**

Genomic DNA was isolated from blood samples with the GCT Genomic DNA Purification Kit (DNA Research Innovations, Kent, UK). All participants were genotyped for three single nucleotide polymorphisms (SNPs) within the CARD15 gene: R702W, G908R, and L3020fsinsC (SNP reference numbers rs2066844, rs2066845, rs2066847, respectively; webappendix). Wildtype genotypes are referred to as w/w, the presence of any one SNP in the genotype (simple heterozygosity) as w/m, and any two (compound heterozygosity or homozygosity) as m/m.

Biopsy samples were taken from the posterior wall of the rectum 10 cm from the anus in nine controls with non-inflammatory bowel disease, six patients with Crohn’s disease, and three with ulcerative colitis. In all these patients, the endoscopic appearance of the mucosa was entirely normal, and histology on these initial samples showed no evidence of microscopic Crohn’s lesions. A second biopsy was done at the site of the initial one 6 h later (webmovie). Forceps were positioned directly above the previously sampled lesion so that the jaws encompassed the mucosal defect. Paired serial samples were also taken from both the rectum and neo-terminal ileum in a further two patients with familial adenomatous polyposis and two patients with Crohn’s disease, all of whom had undergone colectomy and ileorectal anastomosis. In another patient with Crohn’s disease, biopsy samples were taken only from an end-ileostomy since she had had a pan-proctocolectomy. Only one patient with Crohn’s disease and all three patients with ulcerative colitis were taking mesalazine, the remainder were taking no medication. Samples were fixed in formalin, embedded in paraffin wax, sectioned, and immunostained for myeloperoxidase (Dako, Glostrup, Denmark; 1:1000) and interleukin 8 (R&D systems, Minneapolis, MN, USA; 1:100) with the streptavidin–biotin immunoperoxidase method (webappendix). For negative controls, we used duplicate sections in which primary antibodies were omitted. Neutrophils and interleukin 8-producing cells were counted in a blinded fashion and averaged over five randomly selected high-power fields per section.

Skin windows were made by dermal abrasion of a 3-cm by 1-cm area on the volar surface of the forearm with medium-grade sandpaper until capillaries were seen but before bleeding started. Abrasions were overlaid with filter paper saturated with either normal saline alone or saline containing 100 ng/mL MDP (Sigma, St Louis, MO, USA) or 10 μg/mL recombinant human interleukin 8 (PeproTech, Rocky Hill, NJ, USA). The filter paper was covered with a layer of Nescofilm sealing film, and then an adhesive dressing. Dressings and filter papers were removed after either 30 min (for measurements of early mediators) or 24 h (for measurements of myeloperoxidase and cytokines). Only one of the 13 patients with Crohn’s disease who underwent dermal abrasion was on any
medication (aspirin, hyoscine, and loperamide) and one patient with ulcerative colitis was on mesalazine.

Filter papers were incubated in 400 μL normal saline on a rotating wheel for 30 min at 4°C to elute proteins, and then centrifuged (15 000 g, 5 min, 4°C) to pellet filter papers and cells. Cytokine profiles were determined in the supernatant with commercially available protein arrays containing antibodies against 42 different cytokines (RayBiotech, Norcross, GA, USA). Commercially available ELISA kits were used to quantify absolute concentrations of cytokines and other secreted protein products, including interleukin 8, interleukin 1β (R&D Systems), albumin (Alpha Diagnostic International, San Antonio, TX, USA), histamine (IBL Hamburg, Hamburg, Germany), C3a-desArg (Progen, Heidelberg, Germany), prostaglandin E2, and leukotriene B4 (R&D Systems).

Cellular contents were extracted by incubation of the centrifuged filter papers in a solution of 0·5 mol/L NaCl and 1·5% Triton X-100 containing Complete Mini protease inhibitor cocktail tablets (Roche, Basel, Switzerland). The samples were sonicated (ten 1-s bursts), centrifuged (15 000 g, 5 min, 4°C), and the supernatants assayed for myeloperoxidase by oxidation of 4-aminoantipyrine (Sigma), with horseradish peroxidase (Sigma) as a standard.

To create macrophage cultures, peripheral venous blood was collected into 5 U/mL heparin and mixed with an equal volume of balanced salt solution (0·14 mol/L NaCl, 0·01% anhydrous D-glucose, 5 μmol/L CaCl₂, 98 μmol/L MgCl₂, 0·54 mmol/L KCl, 14·5 mmol/L Tris-HCl, pH 7·6). Mononuclear cells were isolated by centrifugation (15 min, 800 g, 20°C) over Ficoll-Paque PLUS (Amersham Biosciences, Chalfont St Giles, UK), and washed repeatedly with ice-cold phosphate-buffered saline (Oxoid, Nepean, ON, Canada) to remove platelets. Cells were resuspended in RPMI-1640 medium (Invitrogen, Paisley, UK) with 10% normal human serum. Cells were then lysed in TRIzol (Invitrogen) containing 1 mg protein or 100 ng/mL MDP for 15 h in RPMI-1640 with 10% normal human serum. Cells were then centrifuged (15 000 g, 5 min, 4°C), and the supernatants were thawed and resuspended at a protein concentration of 10 mg/mL in injection-grade normal saline. A sample of this suspension (100 μL containing 1 mg protein or 10' organisms) was then injected subcutaneously into the volar aspect of each forearm. Blood was collected before injection, and at 24 h and 48 h after injection, for full blood counts and measurements of C-reactive protein, serum amyloid A, and cytokines in serum. Bloodflow at the injection sites was assessed by laser doppler imaging (MoorLDI2; Moor Instruments, Axminster, UK). Five healthy controls and ten patients (five with ileal Crohn’s disease, five with colonic Crohn’s disease) were treated with 50-mg sildenafil (Pfizer, New York, NY, USA) at either 24 h or 48 h (two patients) after inoculation, and perfusion monitored every 30 min over the subsequent 90 min.

We studied a further three healthy individuals, 24 h after inoculation, to assess the effects on bloodflow of intra-arterial norepinephrine (Clinalfa AG, Laeufelfingen, Switzerland; 240 pmol/min) followed by NG-monomethyl-L-arginine acetate (L-NMMA; Clinalfa AG; 4 μmol/min), with a washout of normal saline between the two drugs. The participants were supine in a quiet, temperature-controlled laboratory. The brachial artery of the nondominant arm was cannulated with a 27-gauge needle inserted under local anaesthesia (2 mL 1% lidocaine). Baseline bloodflow was allowed to return to normal after needle insertion before the infusion of vasoactive agents. Drugs or saline were infused continuously at 0·5 mL/min for 15 min each.

Of the 12 patients with Crohn’s disease who took part in the bloodflow experiments, five (with colonic and two with ileal disease) were receiving mesalazine; of these patients, one (ileal disease) was also receiving azathioprine and one (colonic disease) methotrexate and metronidazole. One patient with ulcerative colitis was on olsalazine.
Statistical analysis
Statistical tests were done with Graphpad Prism version 4.01. The two-tailed Student’s t test was used for single comparisons, and Kruskal-Wallis ANOVA with Dunn post-tests or two-way ANOVA with Bonferroni post-tests used for multiple comparisons. Significance values refer to comparison with healthy controls under the same conditions unless otherwise stated.

Role of the funding source
The funding sources of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results
Figure 1 shows the effects of trauma on the intestinal mucosa. After rectal biopsies in nine controls, an acute inflammatory response ensued, with a large increase in numbers of neutrophils and interleukin 8-positive cells (figure 1). Accumulation of both cell types was substantially lower in traumatised rectal mucosa from eight patients with Crohn’s disease (79% reduction [184·2 cells/hpf], n=8, p=0·0003 for neutrophils; 63% reduction [74·9 cells/hpf], n=8, p=0·003 for interleukin 8-positive cells). Of these patients, four were homozygous/compound heterozygous for CARD15 polymorphisms (m/m) and four were wildtype (w/w). No significant differences in any variable were noted between these two sets of patients, especially between the numbers of myeloperoxidase or interleukin 8-positive cells in traumatised tissue. In experiments to establish whether the impairment also applied to the small bowel; similar defects were observed (57% reduction [115·3 cells/hpf], n=3, p=0·05 for neutrophils; 63% reduction [183·25 cells/hpf], n=3, p=0·05 for interleukin 8-positive cells). By contrast, samples taken from patients with ulcerative colitis showed slightly raised numbers of neutrophils in the bowel in the resting state, with elevation after trauma to numbers similar to those in controls.

To confirm whether this abnormal response in Crohn’s disease was localised to the bowel, we investigated the consequences of trauma to the skin (figure 2). Neutrophil efflux into skin windows is low in patients with Crohn’s disease after 5 h.20 We noted that it was still reduced after efflux into skin windows is low in patients with Crohn’s disease was localised to the bowel, we investigated the consequences of trauma to the skin (figure 2). Neutrophil efflux into skin windows is low in patients with Crohn’s disease. To confirm the cellular basis underlying this abnormality, we assayed numbers of myeloperoxidase and interleukin 8-positive cells in traumatised tissue. In experiments to establish whether the impairment also applied to the small bowel; similar defects were observed (57% reduction [115·3 cells/hpf], n=3, p=0·05 for neutrophils; 63% reduction [183·25 cells/hpf], n=3, p=0·05 for interleukin 8-positive cells). By contrast, samples taken from patients with ulcerative colitis showed slightly raised numbers of neutrophils in the bowel in the resting state, with elevation after trauma to numbers similar to those in controls.

We analysed the cytokine profile of fluid from skin windows with an antibody array. Of the 42 cytokines assayed, only interleukin 8 and interleukin 1β were detected in any individual (figure 2). Concentrations of these cytokines were substantially diminished in skin windows from patients with Crohn’s disease (interleukin 8, 45% reduction [23·3 ng/window], n=8, p<0·0001; interleukin 1β, 50% reduction [2·6 ng/window], n=8, p<0·0005), irrespective of CARD15 genotype, but normal in rheumatoid arthritis and ulcerative colitis (figure 2). Concentrations of both cytokines were raised by topical application of MDP in all groups (interleukin 8, p=0·0004; interleukin 1β, p=0·01, for comparison with normal saline window in w/w patients) except patients with Crohn’s disease who were m/m, validating in patients the findings of previous studies in vitro21 and in animals.22 Trauma applied in creating the windows was similar in all individuals, as shown by equivalent concentrations of C3a (measured as its rapidly generated stable conversion product C3a-desArg), histamine, prostaglandin E2, and leukotriene B, determined at 30 min, and by albumin extravasation at 24 h (figure 2).

Interleukin 8 is a potent neutrophil chemoattractant,23 and we postulated that its reduced production might have a primary role in the failure of cellular migration. Consistent with this theory, addition of exogenous interleukin 8 to skin windows returned neutrophil efflux to normal (p=0·02, compared with saline alone) in all three patients with Crohn’s disease tested (two w/w, one m/m; figure 2) indicating that cells were able to respond in the presence of an appropriate stimulus. Augmentation of endogenous interleukin 8 secretion by topical MDP was similarly effective in w/w patients (p=0·006, compared with saline alone) but not, as expected, in m/m patients (figure 2).

These data help to resolve the controversy about the nature of the CARD15 defect: MDP was proinflammatory in vivo in healthy individuals and this action was ablated by the polymorphisms associated with Crohn’s disease. To confirm the cellular basis underlying this
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effect, cultured macrophages were exposed to MDP and the pattern of gene expression investigated (figure 3). In accord with results of a similar previous study, several inflammation-related genes, including those for interleukin 8 and interleukin 1\(\beta\), were strongly upregulated by healthy control macrophages (figure 3). No such induction was seen in cells from \(m/m\) patients with Crohn’s disease (figure 3), whereas normal patterns of expression were observed in macrophages from \(w/w\) patients with ileal or colonic disease (figure 3), whereas normal patterns of expression were observed in macrophages from \(w/w\) patients with ileal or colonic disease (figure 3).

Figure 2: Accumulation of neutrophils, cytokines, and early inflammatory mediators in skin windows

HC=healthy controls. CD=patients with Crohn’s disease. UC=patients with ulcerative colitis. RA=patients with rheumatoid arthritis. IL=interleukin. Data are means with SE bars and number of patients. (A) Neutrophil migration into skin windows was impaired in CD, irrespective of CARD15 genotype. Topical application of MDP corrected impairment only in CARD15 \(w/w\) patients. (B) Neutrophil emigration in CD corrected by topical recombinant IL8. (C) IL8 and IL1\(\beta\) were the principal cytokines detected in skin windows at 24 h. IL8 (D) and IL1\(\beta\) (E) low in CD compared with HC, UC, and RA. MDP amplified response, except in CD CARD15 \(m/m\). (F) Concentrations of C3a, histamine, prostaglandin E\(2\), and leukotriene B\(4\) similar in HC and CD. (G) Albumin concentrations similar in all groups.
Figure 3: Changes in gene expression in, and cytokine secretion by, peripheral blood monocyte-derived macrophages exposed to MDP

HC=healthy controls. CD=patients with Crohn’s disease. IL=interleukin. (A) Cells from six HC (i), three CD CARD15 m/m (ii), three CD CARD15 w/w ileal disease (iii), and three CD CARD15 w/w colonic disease (iv) assayed on Affymetrix U133A microarrays comparing gene expression by cells with and without MDP. Transcripts for IL8 (black arrows) and IL1/H9252 (white arrows) were identified except in CARD15 m/m cells, which did not respond (ii). Transcripts with the greatest increases in expression are listed in the table (B). None of the changes in CD m/m patients were significant. MIP=macrophage inflammatory protein. GRO=growth-related oncogene. IDO=indoleamine 2,3-dioxygenase. TSG=tumour necrosis factor-stimulated gene-6. PDE=phosphodiesterase. CD=cluster of differentiation. These transcripts are translated as shown by induction of secretion of IL8 (C), IL1/H9252 (D), TNF/H9251 (E), and IL10 (F) by macrophages in response to MDP, except in CD m/m cells.

### Table B

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We cultured these cells from peripheral-blood monocytes. The formation of the skin window involves acute trauma to the skin. Consequently, we exposed cultured macrophages to wound fluid recovered from acute surgical incisions in healthy individuals undergoing inguinal hernia repair (figure 4), and to two other mediators produced at acute inflammatory sites: C5a and TNFα (figure 4). In response to all these stimuli, macrophages from patients with Crohn’s disease secreted significantly less interleukin 8 than did those from healthy controls (for wound fluid 38% reduction [14.8 ng/mL], n=50, p<0.0001; C5a 48% reduction [3.4 ng/mL], n=41, p=0.0005; TNFα 52% reduction [5.7 ng/mL], n=27, p<0.0001) or patients with ulcerative colitis, although the response to lipopolysaccharide was normal (figure 4). Interleukin 8 secretion was unrelated to CARD15 genotype for any stimulus. These results show that macrophages from patients with Crohn’s disease are constitutionally less able than those from controls to produce interleukin 8 in response to proinflammatory agonists, even when removed from the body and placed in culture.

Bowel diversion experiments have shown the crucial role of luminal contents in development of Crohn’s disease lesions, which typically arise at the sites of highest bacterial concentrations. Our present findings suggest that reduced or delayed recruitment of neutrophils to sites at which bacteria penetrate the mucosa might lead to persistence of bacteria and other organic debris in the tissues, possibly within macrophages. Secondary secretion of proinflammatory cytokines, after the failure of initial clearance, could drive the development of chronic inflammation. To assess directly whether the response to the presence of bacteria in the tissues was abnormal in Crohn’s disease, we injected heat-killed E coli subcutaneously. This injection elicited a vigorous inflammatory response manifested by discomfort, erythema, and swelling within 2–4 h. By 24 h, the erythema (figure 5) and swelling were more extensive, although discomfort was reduced from 8 h. At 48 h the discomfort and swelling had disappeared, although the erythema was more diffuse. In healthy controls, bloodflow in the area of inflammation increased by about five times at 8 h and nine times at 24 h, and had almost returned to baseline by 48 h (figure 5).

The response in patients with Crohn’s disease was very different. Although superficial appearances at the injection site were similar, increases in bloodflow were abnormally low at 8 h and 24 h (figure 5), particularly in patients with colonic disease (77% reduction [6.7 relative units], n=6, p=0.0003) but also in those with ileal disease (50% reduction [4.1 relative units], n=6, p=0.01). Of the six patients with ileal lesions, three carried two polymorphisms in CARD15 and one was a simple heterozygote. The response of all four of these patients was indistinguishable from that of the wildtype patients with ileal disease. None of the patients with colonic

Figure 4: Secretion of interleukin 8 by macrophages in culture in response to (A) wound fluid, (B) C5a, (C) TNFα, or (D) lipopolysaccharide. HC=healthy controls. CD=patients with Crohn’s disease. UC=patients with ulcerative colitis. Response in CD independent of CARD15 genotype. Mean values shown.
disease carried the polymorphisms. Bloodflow response was unrelated to smoking status (four patients with Crohn’s disease and five healthy controls were smokers).

In two patients with ulcerative colitis, bloodflow response was greater than that in patients with Crohn’s disease and did not show the normal resolution after 48 h (**p**/0.0001), clearly distinguishing it from the hyporesponsiveness characteristic of Crohn’s disease. The inflammatory response was so florid in one patient with ulcerative colitis that we terminated these studies in patients with this condition.

Since lipopolysaccharide induces vasodilation through release of nitric oxide, we investigated the role of this mediator in the normal increase in bloodflow induced by...
the killed bacteria. Intra-arterial infusion of L-NMMA, a non-selective inhibitor of nitric oxide synthase,34 rapidly reduced bloodflow by about 50% (p=0.008, compared with pretreatment bloodflow, figure 4), whereas norepinephrine had little effect. Nitric oxide causes smooth muscle relaxation by increasing intracellular concentrations of cyclic GMP. Consequently, we examined the effect of sildenafil (Viagra, Pfizer, New York, NY, USA), a phosphodiesterase-5 inhibitor and vasodilator,35 to see if it would correct the deficient bloodflow in Crohn’s disease. Oral administration of 50-mg sildenafil to five healthy individuals and ten patients with Crohn’s disease at 24 h or 48 h (two patients) after bacterial injection resulted in marked increases in bloodflow (p=0.02, compared with pretreatment bloodflow) in most participants (figure 4).

To quantify the systemic inflammatory response, we measured interleukin 1β, interleukin 6, interleukin 8, interleukin 10, interleukin 12, TNFs, interferon γ, C-reactive protein, and serum amyloid A, in the serum 24 h after bacterial injection. Of these cytokines, we could only consistently detect interleukin 6, a potent mediator produced by macrophages that stimulates lymphocytes to proliferate and differentiate, and induces the secretion of acute-phase proteins by the liver.36 Despite showing little increase in forearm bloodflow, patients with colonic Crohn’s disease showed the highest concentrations of serum interleukin 6 and C-reactive protein, and the greatest increase in peripheral-blood neutrophil count (figure 5). This finding illustrated the principle that a weak local inflammatory reaction can engender a systemic proinflammatory state similar to that observed in active Crohn’s disease.

**Discussion**

Our investigations identified defective innate immunity in Crohn’s disease. The findings showed reduced neutrophil accumulation and interleukin 8 production, not only at sites of acute inflammation in the bowel, but also in the skin, indicative of a general constitutional abnormality. That a systemic defect existed was confirmed by the deficient responses of patients’ macrophages, cultured in vitro for 5 days, to acute inflammatory mediators.

What causes this abnormality, and what does it mean for the general health of the individual? We do not yet know enough about the dynamic interplay of the many influences on the immune system that differentiate a robust acute inflammatory response from one that is more lethargic. The regulation is almost certainly polycyclic, and the effects will follow a normal Gaussian distribution. We propose that individuals at the lower end of this response are predisposed to Crohn’s disease. Lesions manifest most frequently in the gastrointestinal tract because of its high commensal bacterial load, and within this organ at sites with the heaviest colonisation in the terminal ileum and colon.37 This theory is also consistent with the crucial role shown for the faecal stream contents in the development of intestinal inflammation.38

Patients with Crohn’s disease might also be more susceptible to other consequences of an impaired acute inflammatory response; in addition to developing arthritis, uveitis, and aphtous ulceration, pyogenic infection could be more frequent. Such infections would be difficult to identify in patients with varying degrees of nutrition, immunosuppression, and surgical intervention. Even patients with the most severe defect of neutrophil function, chronic granulomatous disease, can go for years or even decades without infection.39 At least 20% of these patients develop a granulomatous colitis that is indistinguishable from Crohn’s disease.40

The primary function of neutrophils is to kill and digest bacteria, accomplished by the potent digestive enzymes released into the phagocytic vacuole from the cytoplasmic granules.41 They eradicate infecting microorganisms and are discharged as pus, or apotose, resulting in resolution. Macrophages, by comparison, are also phagocytic but with less killing and digestive capacity. They have more of a containing role, forming granulomata to wall off foreign material from the remainder of the body and secreting cytokines that prime and amplify the immune system; the latter results in florid local and systemic reactions.42 Although the mucosa provides a very effective barrier in health, insults such as infection and trauma allow the luminal contents access to the tissues of the bowel wall. In the absence of adequate numbers of neutrophils for the effective clearance of bacteria43 they will be taken up by macrophages44 to form the granulomata45 and focci of chronic inflammation characteristic of Crohn’s disease.

Our data indicate that Crohn’s disease is associated with a failure of the translation of acute tissue damage into an effective neutrophil response. Whether this is a primary constitutive abnormality or a consequence of the disease or its treatment is difficult to prove. The fact that it is present in patients with apparently quiescent disease, is systemic involving the skin as well as the bowel, and is not seen with general systemic inflammation in rheumatoid arthritis or bowel inflammation in ulcerative colitis, argues strongly for the former. The basic lesion probably operates at the level of macrophages, since Crohn’s disease macrophages responded poorly to wound fluid and other inflammatory mediators. The fact that these cells were cultured for 5 days before stimulation also makes suppression by some humoral factor or therapeutic agent highly unlikely. Macrophages from patients with the disease produced reduced amounts of interleukin 8, a potent neutrophil chemoattractant,46 concentrations of which were also low in acutely traumatised bowel and skin in these patients. Neutrophil emigration became normal when interleukin 8 was applied to the skin window of patients with Crohn’s disease, confirming that there is nothing intrinsically wrong with the chemotaxis of these cells.47
We propose that the underlying impairment of acute inflammation that predisposes to Crohn’s disease can be boosted by a second tier of immune enhancers. One of these, MDP, stimulates the proinflammatory NOD2/CARD15 pathway to cytokine production in macrophages. This stimulation led to increased neutrophil migration into skin windows in patients with Crohn’s disease, except in individuals homozygous or compound heterozygous for polymorphisms shown to predispose to the disorder. These polymorphisms are associated only with disease of the ileum, a region with fluid contents where a small soluble bacterial product such as MDP (that is present at high concentration) is more likely to penetrate through a damaged mucosa than in the large bowel where contents are more solid. It is highly probable that similar compensatory stimuli and pathways operate in the large bowel, in which the triggering molecules and pathways remain to be identified. Toll-like receptor-4 and CD14 receptors on macrophages which recognise lipopolysaccharide, have been linked to Crohn’s disease, and are strong candidates to play such a part.

There is clearly a major problem in the handling of E. coli in the tissues, as shown by the very abnormal vascular responses in Crohn’s disease. We could not accurately define the differences in the handling of bacteria by the patients into whom they were injected, because serial skin biopsies would not be practicable. One possible scenario is that in healthy individuals E. coli evoked an efficient acute inflammatory response leading to rapid phagocytosis and digestion of the bacteria, and subsequent release of lipopolysaccharide, known to induce synthesis of inducible nitric oxide synthesen and vasodilatation. In Crohn’s disease, the intact bacteria might remain in the tissues as a consequence of suboptimal destruction by neutrophils, to be phagocytosed instead into macrophages. The very high concentrations of circulating interleukin 6, secreted by macrophages, indicate that the organisms were recognised and engulfed by these cells. This finding is also consistent with the demonstration of bacterial DNA within Crohn’s granulomata.

Causation of Crohn’s disease by failure of the acute inflammatory response would fit very well with the so-called hygiene hypothesis, in which the increased incidence of the disease has been attributed to improved standards of sanitation. The inflammatory response to bacterial ingress would be much more effective if it were primed from a state of subclinical inflammation induced by repeated mild infections or parasitic infestation, than if it has to start de novo in a relatively unstimulated bowel. Other epidemiological risk factors for Crohn’s disease are the adverse effects of smoking and possibly stress on the incidence of the disease; both reduce mucosal blood flow, which is closely related to rates of neutrophil emigration into the tissues. Smoking is also generally immunosuppressive as well as specifically reducing concentrations of mucosal interleukin 8.

These findings provide hope for the development of more effective therapies for Crohn’s disease. Current treatments are immunosuppressive, but although they reduce symptoms by dampening the proposed secondary inflammation, they might actually accentuate the underlying immunodeficiency. It might be feasible to introduce interleukin 8 or other proinflammatory stimuli directly into acute lesions, either by direct enteral administration or through synthesis by genetically modified gut organisms, since this cytokine would penetrate the bowel wall only through damaged mucosa. Agents that increase bloodflow, such as long-acting phosphodiesterase-5 inhibitors or other vasodilatory or proinflammatory drugs, might be useful in healing or preventing lesions in Crohn’s disease.