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What Is Wrong with Granulocytes in Inflammatory Bowel Diseases?

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Abstract

The neutrophil plays a central role in the acute inflammatory response, a crucial mechanism required for the efficient clearance of invading microorganisms and antigenic material. Patients with primary immunodeficiencies of neutrophil function, particularly chronic granulomatous disease, are predisposed to develop bowel inflammation that is indistinguishable from Crohn's disease (CD) on the basis of clinical, endoscopic and histopathological features. The intrinsic function of the neutrophil is normal in the vast majority of patients with CD; however, there is clear evidence of an impairment of neutrophil recruitment to sites of trauma and bacterial infection. This is associated with an inability to adequately clear bacteria that have penetrated the tissues, resulting in the formation of granulomata, the histological hallmark of the disease, and the subsequent initiation of a chronic adaptive immune response. The reduced secretion of proinflammatory cytokines by macrophages, most notably TNF-α, may account for the attenuated neutrophil recruitment observed in CD. Stimulation of the innate immune system in CD, particularly in patients in remission, may be an alternative therapeutic strategy that could reduce the risk of future disease relapses.

Keywords

Inflammatory bowel disease; Crohn's disease; Acute inflammatory response; Neutrophil; Chronic granulomatous disease

Introduction

The inflammatory bowel diseases (IBD) Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing and remitting diseases of complex aetiology. Histopathologically, IBD lesions are characterised by the presence of a chronic inflammatory immune response [1]. Delineation of the immunological defect(s) that give rise to this chronic immune response has been a key area of research in IBD over the last half century. In 1976, Segal and Loewi [2] proposed the hypothesis that in CD the underlying aetiology may be one of a poor acute inflammatory response resulting in a delayed clearance of intestinal commensal microflora that have penetrated the bowel wall, their persistence in the tissue triggering the chronic

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inflammation observed. The basis of this hypothesis was the histological hallmark of CD, the granuloma, which is indicative of the failure to clear antigenic material, and the common occurrence of CD-like pathology in patients with inherited primary immunodeficiencies (PIDs) of neutrophil function, most notably chronic granulomatous disease (CGD) [3]. The timely and adequate recruitment of functional neutrophilic polymorphonuclear leukocytes is fundamental to the acute inflammatory response; accumulating evidence, predominantly from in vivo studies in patients, supports the notion that this is defective in CD [2, 4, 5]. This review will examine the role of the neutrophil and the acute inflammatory response in the aetiopathogenesis of CD.

Neutrophils in the Acute Inflammatory Response

Granulocytes are leukocytes that differentiate from myeloblasts in the bone marrow and are defined by a characteristic lobulated nucleus and granular cytoplasm [6]. Neutrophils (or polymorphonuclear leukocytes), the most common of the granulocytes and of all leukocytes found in the circulation, are highly motile cells capable of phagocytosing, killing and digesting invading microorganisms [7]. Identified by Elie Metchnikoff in the late 19th century, these cells constitute the first-line defence of the innate immune system and are rapidly recruited to sites of acute inflammation [7]. This is accomplished by increases in microvascular flow and permeability, coincidental with the secretion of cytokines and chemokines by macrophages and the upregulation of selectins and integrins on the vascular endothelium permitting the margination and diapedesis of the neutrophils into the tissues [6]. Once at their target site, neutrophils recognise foreign particles via pattern recognition receptors aided by the presence of serum antibody and complement opsonins. The particles are subsequently phagocytosed into a membrane-bound vacuole, the phagosome, which fuses with acidic enzyme-rich granules forming the phagolysosome. Phagocytosis is rapidly followed by a significant consumption of molecular oxygen in a process termed the respiratory burst. This is associated with increased activity of the hexose monophosphate shunt which generates the electron carrier, NADPH, the other principal substrate required for the respiratory burst. The rate at which neutrophils accumulate has a critical effect on the outcome of a local bacterial infection [8].

The enzyme NADPH oxidase transports electrons from NADPH in the cytosol to oxygen in the phagocytic vacuole to produce superoxide [7]. This electron transport is accomplished through a flavocytochrome b, gp91phox (encoded by the gene *CYBB*), which is associated with another membranous subunit, p22phox *(CYBA)*, that together form a conduit for electron flux. The enzyme is activated by a number of cytosolic proteins including p47phox *(NCF1)*, p67phox *(NCF2)*, p40phox *(NCF4)* and a small GTP-binding protein, Rac2. These proteins assemble at the cytosolic surface of the flavocytochrome when the cell is stimulated. The electron and ion fluxes associated with the respiratory burst are required to generate the optimal intravacuolar conditions for the killing and digestion of phagocytosed microbes and organic material by granule-derived digestive proteases, in particular cathepsin G and elastase [9, 10]. The failure of neutrophils to adequately kill and digest phagocytosed organisms may be compensated for by a second chronic phase of inflammation which aims to limit the spread of the potentially hazardous material by surrounding and containing it

within macrophages. The activated macrophages secrete proinflammatory cytokines which, in turn, activate T lymphocytes and initiate the adaptive immune response.

Unlike other organs, the gastrointestinal tract (in particular the terminal ileum and colon) contains a massive bacterial load [11] which can gain instant access to the lamina propria if the mucosal barrier is breached, and thus initiate an acute inflammatory response. Evidently, this occurs in CD, as evidenced by the accumulation of autologous indium-111-labelled neutrophils at sites of bowel inflammation [12], the abundance of neutrophils in histological sections of active disease tissue, and the presence of neutrophil-derived proteins, e.g. calprotectin, in the faeces which correlate with clinical disease activity [13]. The crucial role of the luminal contents in the development of CD lesions has been demonstrated by the reduced post-surgical recurrence of disease with faecal diversion [14, 15]. Direct evidence for an important role of neutrophils in the initiation of CD (or CD-like pathology) is provided by the PIDs of neutrophil function [16]. These disorders primarily affect the number of circulating neutrophils, the ability of the neutrophils to successfully extravasate into the tissues at sites of acute inflammation, and the normal biological functions of the neutrophils themselves. While the underlying aetiology of these disorders is clearly distinct from CD (the PIDs being caused by single gene defects), they can offer instructive insight into the pathogenesis of CD and are thus worthy of detailed consideration.

Chronic Granulomatous Disease and Other Primary Immunodeficiencies of Neutrophil Function

CGD [17], the prototype and most common of the neutrophil PIDs, affects approximately 1 in 250,000 of the population and results from dysfunction of NADPH oxidase. In approximately 65% of cases, CGD is caused by mutations in *CYBB*, and, as this gene is on the X chromosome, it almost exclusively affects males (although females who exhibit extreme lyonisation can also be affected). Mutations in *NCF1*, *NCF2*, and *CYBA* account for the remaining 25, 5 and 5% of cases, respectively, and segregate in an autosomal recessive manner. In CGD the complete or partial loss (as occurs in ‘variant CGD’) of NADPH oxidase activity has profound effects. It results in the failure to efficiently kill some bacteria and fungi resulting in recurrent or chronic pyogenic infections, predominantly in the skin, lymphatics, liver, bowel, bones and respiratory tract, which can be fatal; patients thus require prophylactic antibiotics and antifungal therapy [17]. However, given the importance of the oxidase in optimising the conditions within the vacuole for the activity of the digestive proteases, CGD neutrophils are unable to adequately digest the phagocytosed microorganisms [18]. The undigested antigenic material is subsequently contained (in the neutrophils undergoing apoptosis) within granulomata by macrophages, the histological hallmark that gives the disease its name. Lymphocytes are subsequently recruited and secrete cytokines giving rise to local (obstruction of hollow muscular organs and fibrosis) and systemic consequences.

A considerable proportion of patients with CGD (approx. 50%) develop a non-infectious chronic IBD that bears a striking resemblance to CD by clinical, endoscopic, and histopathological assessment [3] (reviewed by Marks et al. [19]). In our own case series of 25 adult CGD patients, 11 fulfilled the Lennard-Jones diagnostic criteria for CD [19].

Disease was most commonly observed in the mouth and distal bowel. The intestinal inflammation was discontinuous in nature creating skip lesions, transmural (thus predisposing to stenosis and fistulation) and associated with identifiable granulomata in over half the patients on routine clinical biopsies. All patients had perianal disease. Although CGD is typically a disease of young children, the median age of onset of gastrointestinal symptoms was in early adolescence (14 years, range 4–29). This suggests that the development of mature bowel lesions may require an extended exposure to antigenic material, possibly thus explaining the failure of many of short-lived animal models to accurately reproduce the features of CD [20]. Of particular interest, in some patients with CGD, bowel disease may be the predominant or exclusive feature of their disease [21].

As mentioned above, a number of other disorders of neutrophil function also present with gastrointestinal inflammation resembling that of CD. These have been extensively reviewed by Rahman et al. [16] where a diagnostic algorithm is also outlined. Congenital neutropenia, cyclic neutropenia and autoimmune neutropenia are all associated with a reduction in the total number of circulating neutrophils in the peripheral blood which may result in the delivery of insufficient numbers of neutrophils to sites of acute inflammation. Leukocyte adhesion deficiency-1 is caused by mutations in β_2 -integrin CD18 which result in the absence or markedly reduced expression of functional CD18. This has a number of effects on leukocyte function, in particular defective chemotaxis, margination and adherence of neutrophils such that their ability to extravasate into the tissues at sites of acute inflammation is impaired. Hermansky-Pudlak syndrome and Chediak-Higashi syndrome are both caused by abnormalities of the formation and trafficking of vesicles of lysosomal lineage resulting in impaired phagocyte chemotaxis and bactericidal activity, neutropenia and abnormal natural killer (NK) cell function. Finally, glycogen storage disease-1b (GSD-1b), as with CGD, is associated with a reduction in the neutrophil respiratory burst, in this case, as a consequence of reduced availability of NADPH [16].

Neutrophil Function in CD

The fact that a considerable proportion of patients with CGD and other disorders of neutrophil function exhibit bowel disease indistinguishable from CD indicates the potential importance of the neutrophil in the aetiopathogenesis of CD. Various aspects of the function of neutrophils from CD patients have been investigated in vitro including chemotaxis, phagocytosis, respiratory burst, bacterial killing and digestion [22–24]. Some of these data have been conflicting; however, the general conclusion is that the intrinsic function of the neutrophil in CD is intact. In our own study of 100 patients with CD and 50 healthy controls, while there was a small reduction in the neutrophil respiratory burst in CD patients, bacterial digestion was normal [24]. Of considerable interest, 3 CD patients in the cohort of 100 had a respiratory burst below 30% of the mean of the controls, a level expected from patients with variant CGD. One patient was found to have congenital neutropenia due to a mutation in *G6PC3* [25] and another had GSD-1b. Screening for a defective respiratory burst in patients with CD, particularly those with extensive colonic disease, perianal involvement and oral manifestations, may be warranted [21, 24]. It is important to measure the kinetics of oxygen consumption or superoxide production rather than using an endpoint assay, such as the

nitroblue-tetrazolium (NBT) test, as the latter would fail to identify cases in which the oxidase activity is reduced but not absent, as occurs in variant CGD [24, 26].

Defective Acute Inflammation in CD

To further interrogate the acute inflammatory response in patients with CD, inflammatory disease controls and healthy controls (HC), we have conducted a number of in vivo and in vitro experiments [2, 4, 5, 24, 27–29]. To distinguish from primary defects preceding the development of the disease and those arising as a consequence of ongoing chronic inflammation, such studies would ideally be conducted on patients immediately preceding the development of their disease. This is obviously not possible and, as a compromise, the vast majority of our studies have been performed with patients with quiescent disease (as defined by a Harvey-Bradshaw score [30] less than 3 and serum inflammatory markers within the normal range) who have been on limited, if any, immunosuppressive medication at the time of study or in the preceding 2 months. When patients with active disease or those on medication were included, subgroup analyses were performed.

The first study, in 1976, examined the recruitment of neutrophils to skin windows, areas of acute inflammation created by dermal abrasion on the forearm [2, 31]. It was observed that the number of neutrophils passing out of the skin window was considerably lower (by approx. 80%) in patients with CD as compared with HC or patients with rheumatoid arthritis (an inflammatory disease control). This was unrelated to the site or extent of disease, current treatment or peripheral blood neutrophil count. There was no difference observed in in vitro chemotaxis or in the activity of the respiratory burst as assessed by the nylon column dye test [32]. The findings of this study were replicated in a larger cohort in 1981 [27] where it was also demonstrated that the impaired neutrophil recruitment was not due to the presence of inhibitory factors within the serum of CD patients and that neutrophil recruitment could be enhanced, although not to normal levels, by using zymosan-activated serum. These results suggested that the reduced emigration of neutrophils from skin windows in CD may be due to a deficient local inflammatory response. The impairment of neutrophil recruitment to skin windows in CD was subsequently independently replicated [33].

In 2006, we extended our skin window studies by quantifying neutrophil recruitment and local inflammation after acute trauma in the skin, rectum and ileum [4]. To measure neutrophil recruitment to the intestinal mucosa in an acute inflammatory response, a serial biopsy technique was employed in which an endoscopic pinch biopsy was taken from an area of macroscopically and microscopically non-inflamed mucosa to induce trauma. A second biopsy was then taken at the same site (such that the jaws of the forceps encompassed the visible mucosal defect) 6 h later. Neutrophil accumulation to this site of intestinal trauma was quantified by histological assessment staining for the neutrophil marker myeloperoxidase. As in the skin windows, there was a significant reduction in neutrophil recruitment in patients with CD. This was not observed in patients with UC.

In this study, the reduced neutrophil recruitment to skin windows was replicated and was shown to be independent of *NOD2* genotype status. *NOD2* was the first gene to be associated with CD [34, 35]. CD-associated *NOD2* variants impair the secretion of

proinflammatory cytokines including interleukin-8, tumour necrosis factor-α (TNF-α) and interleukin-1β from peripheral blood mononuclear cells in response to the NOD2-ligand muramyl dipeptide (MDP) [36]. NOD2 variants are thus thought to predispose to CD by impairing the recruitment of neutrophils and the priming of the immune system to the presence of pathogens. This observation would be consistent with the original hypothesis of defective acute inflammation in CD [2]. In our 2006 study [4], CD patients that were wild type for *NOD2* variants demonstrated an augmentation of neutrophil recruitment with the application of MDP to the skin window, presumably due the stimulation of tissue macrophages to secrete proinflammatory cytokines and chemokines. This augmented neutrophil recruitment did not occur in CD patients that were homozygous or compound heterozygous for *NOD2* variants. On the basis of these results, we proposed that the underlying impairment of acute inflammation in CD might be able to be boosted by a second tier of immune enhancers such as MDP signalling via NOD2.

Given the proposition that the defective neutrophil recruitment may be a consequence of inadequate stimulation of the local inflammatory response and thus that handling of bacteria within the tissues may be abnormal in CD, we assessed the effect of subcutaneous injection of heat-killed *Escherichia coli* on local blood flow in patients with CD and controls [5]. As described previously, the acute inflammatory response is associated with capillary dilatation and increased microvascular flow and local blood flow may therefore be used as a surrogate for the robustness of acute inflammation. *E. coli* was chosen on the basis that it is a major aerobic microorganism of the bowel flora [37] which has been implicated in the pathogenesis of the disease [38]. The injection of 10^9 heat-killed *E. coli* into the volar aspects of the forearms elicited a vigorous inflammatory response with erythema, discomfort and swelling ensuing within the first 2–4 h and lasting for 48 h. The superficial appearance in patients with CD and controls was the same; however, local blood flow was markedly attenuated in CD [5].

To assess neutrophil recruitment in response to a bacterial stimulus and examine the effects of the failure of neutrophil recruitment on bacterial clearance in vivo, we conducted experiments using autologous radiolabelled neutrophils and radiolabelled heat-killed *E. coli* [5]. Autologous indium-111-labelled neutrophils were injected intravenously at the same time that unlabelled heat-killed *E. coli* were injected subcutaneously into the forearms. The rate of neutrophil accumulation was ascertained by measuring the radioactivity at the bacterial injection site. In HC and patients with UC, significant cellular recruitment could be measured at 4 h and this increased steadily over a 24-hour period. In this study it was possible to demonstrate by Gamma camera imaging that the impaired neutrophil recruitment at the forearm in CD was not as a consequence of increased accumulation within the bowel. To directly test the hypothesis that the failure of neutrophil recruitment and impaired local inflammatory response results in defective clearance of bacteria penetrating the tissues, phosphorous-32-labeled heat-killed *E. coli* were injected subcutaneously into the forearm and its clearance (measured by the decreasing radioactivity counts) was monitored over time. In CD, HC and UC it was observed that the clearance of radioactivity followed biphasic non-exponential kinetics with a rapid initial phase followed by a slower secondary phase. The bacterial clearance was similar in HC and UC but was profoundly delayed in CD

such that if the clearance curves were extrapolated to a point at which 99% of the inoculated material would have been cleared, the total clearance times in HC and UC were 10 and 7 days, respectively, as compared with 44 days in CD [5].

If there is a gross delay in the recruitment of neutrophils to sites of acute inflammation and subsequent clearance of bacteria in CD, it is relevant to consider why patients with CD do not demonstrate an unusual predisposition to pyogenic infections, as occurs in CGD [17]. It appears, however, as if the deficit of the acute inflammatory response in CD, in terms of local blood flow and bacterial clearance, is dependent upon the load of bacteria involved [5]. At injected doses of between 10^5 and 10^6 organisms there was no difference between HC and CD; however, gross differences became apparent at concentrations of 10^7 and 10^8. It is these higher bacterial loads that occur within the terminal ileum and colon, the sites in which CD most commonly occurs [11].

Defective Cytokine Secretion from Macrophages

The migration of neutrophils to sites of inflammation requires the presence of a chemotactic gradient and the upregulation of adhesion molecules on the vascular endothelium [23]. Macrophages are recognised to play an important role in orchestrating this response through the secretion of proinflammatory cytokines such as TNF-α. The failure of macrophages to secrete such cytokines in response to immune activation could account for the reduction in neutrophil recruitment observed in CD. Monocyte-derived macrophages from controls and patients with CD and UC were therefore studied. A reduced secretion of a range of cytokines, most notably TNF-α, was observed in CD macrophages stimulated with a variety of stimulants including heat-killed *E. coli* and specific toll-like receptor ligands including lipopolysaccharide and Pam_3CSK_4 [5, 29]. This was not as a consequence of a failure of cell signalling in response to the stimuli, abnormal cytokine gene transcription or the processing and translation of the mRNA. Rather, it appeared to be as a result of defective vesicle trafficking resulting in the abnormal routing of the cytokine protein for degradation in the lysosomal compartment as opposed to being exocytosed and thus secreted [5]. The molecular basis of this defect is currently under investigation.

A Three-Stage Model for the Immunopathogenesis of CD

We have proposed a three-stage model to explain the development of bowel lesions in CD [20]. The first stage of this model involves the penetration of the bowel wall by luminal contents facilitated by environmental factors (e.g. infection) or defects of the mucosal barrier. In the second stage, macrophages fail to secrete proinflammatory cytokines, in particular TNF-α, in sufficient quantities to adequately trigger an acute inflammatory response such that there is a reduced influx of neutrophils. Finally, as a consequence of the impaired neutrophil recruitment, the bacteria persist within the tissue and are phagocytosed by macrophages which form a granuloma in an attempt to contain the bacteria. The resulting macrophage activation triggers a secondary phase of proinflammatory cytokine and chemokine secretion that drives the recruitment of T lymphocytes to the tissue resulting in a chronic adaptive immune response as seen on histopathological examination of CD lesions. This chronic immune response causes local tissue damage (including fibrosis, stricturing and

fistulisation) and systemic responses that give rise to the symptoms of CD. The secondary compensatory phase of immune activation is temporarily distinct from the initial acute inflammatory response. There is a growing appreciation of this concept of CD as an immune deficiency [39].

On the basis of this model, treating CD with immunosuppressive medication may reduce the symptoms of the disease by treating the secondary chronic adaptive immune response at the expense of innate immune function, the loss of which might predispose patients to relapse. An alternative strategy may be one in which low-level immunostimulation is provided to patients with quiescent disease to maintain them in remission. An attempt to employ this strategy was made in the 1970s in which an elemental diet was successfully used to induce remission [40] and the immunostimulator levamisole was then given [41]. More recently, trials of granulocyte macrophage colony-stimulating factor showed this drug to have some efficacy in the treatment of CD [42]; restricting its use to patients in remission or in the post-operative setting may be more effective.

Conclusion

The neutrophil plays a central role in the acute inflammatory response, a crucial mechanism required for the efficient clearance of microorganisms and antigenic material that penetrate into tissues. Patients with PIDs of neutrophil function, most notably CGD, are predisposed to develop bowel inflammation that is indistinguishable from CD on the basis of clinical, endoscopic and histopathological features. This provides strong evidence for the potential role of neutrophils in the aetiopathogenesis of CD. However, in the vast majority of patients with CD, the intrinsic function of the neutrophil in terms of chemotaxis, phagocytosis, respiratory burst, bacterial killing and digestion are normal, although there is clear evidence of an impairment of neutrophil recruitment to sites of trauma and bacterial infection. This impaired neutrophil recruitment in CD is associated with an inability to adequately clear bacteria that have penetrated the tissues. As a consequence, a secondary phase of proinflammatory cytokine and chemokine secretion is triggered resulting in a chronic adaptive immune response causing the signs and symptoms of the disease. The reduced secretion of proinflammatory cytokines by macrophages, most notably TNF-α, may account for the attenuated neutrophil recruitment observed in CD. The mechanism underlying the defective cytokine secretion is an abnormality in vesicle trafficking which aberrantly directs cytokines to the lysosome for degradation; the molecular basis of this is currently under investigation. Stimulation of the innate immune system in CD, particularly in patients in remission, may be an alternative therapeutic strategy that could reduce the risk of future disease relapses.

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