Redox State and $O_2^{\bullet -}$ Production in Neutrophils of Crohn's Disease Patients

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The aim of this *in vitro* study was to evaluate the intracellular redox state and respiratory burst (RB) in neutrophils of patients with Crohn's disease (CD). The intracellular redox state and RB in neutrophils was assessed by the superoxide anion ($O_2^{\bullet -}$) production induced in these cells after stimulation by various factors related to the molecular mechanisms that, if altered, may be responsible for an abnormal immune response. This can, in part, cause the onset of inflammation and tissue damage seen in CD. This study demonstrated a decreased glutathione/glutathione disulfide (GSH/GSSG) ratio index of an increased oxidative state in CD patient neutrophils. Moreover, our findings showed a decrease in tumor necrosis factor (TNF-α)– or phorbol 12-myristate 13-acetate (PMA)–induced $O_2^{\bullet -}$ production in CD patient neutrophils adherent to fibronectin as compared with controls. A decreased adhesion was also demonstrated. For this reason, the involvement of altered mechanisms of protein kinase C (PKC) and β-integrin activation in CD patient neutrophils is suggested. These data also showed that the harmful effects of TNF-α cannot be caused by excessive reactive oxygen species (ROS) production induced by neutrophils. Decreased cell viability after a prolonged time of adhesion (20 hrs) was also measured in CD patient neutrophils. The findings of this study demonstrate, for the first time, that granulocyte-macrophage colony–stimulating factor (GM-CSF), a compound recently used in CD therapy, is able to activate the RB for a prolonged time both in control and CD patient neutrophils. Increased viability of CD patient neutrophils caused by GM-CSF stimulation was also observed. In conclusion, our results indicate that decreased $O_2^{\bullet -}$ production and adhesion, caused, in part, by an anomalous response to TNF-α, together with low GSH level and low cell viability, may be responsible for the defective neutrophil function found in CD patients. This can contribute to the chronic inflammation and relapses that characterize this pathology. A possible role of GM-CSF in inducing $O_2^{\bullet -}$ production and in restoring the defensive role of neutrophils in CD patients is suggested. Exp Biol Med 231:186–195, 2006

Key words: Crohn's disease; GM-CSF; GSH; redox state; neutrophils $O_2^{\bullet -}$ production; TNF-α

Introduction

Crohn's disease (CD) is a chronic disorder characterized by transmural inflammation that can affect any part of the gastrointestinal tract. The cause of CD remains uncertain and several interacting elements are involved in its complex pathogenesis, that is, genetic factors, priming by the enteric microflora, and an abnormality in the immune-mediated response (1). In fact, it is thought that CD is related to a defect in mucosal immunity. Intestinal lesions occurring in CD are caused by prolonged and amplified inflammatory and immune responses and are characterized by a dense inflammatory cell infiltrate, mainly comprising macrophages, neutrophils, and lymphocytes (2). These cells secrete proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, involved in the initial steps of the inflammatory response and reactive oxygen species (ROS), which are the final effectors of cascade inflammation (3, 4). Excessive production of cytokines and ROS cause severe tissue damage of the affected organs, and both can represent interesting targets for CD treatment. In healthy individuals, the intestinal mucosa is in a state of physiologic inflammation that is controlled by an intrinsic balance between proinflammatory and contrainflammatory mediators (5). Disorders in the regulation of immune responsiveness are responsible for an altered mucosal homeostasis and predispose the individual to uncontrolled and pathologic inflammation. Human peripheral neutrophils are the first cells recruited at the site of infection *in vivo* and are important in the host's defense against bacterial infection *via* invading microorganisms through the release of ROS

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Table 1. Patient Characteristics

	Crohn's disease	Controls
Sex (M/F)	18/14	19/16
Age, range (years)	23–54	26–57
Ileal disease	All	None
Bowel resections	All	None
Primary/recurrence	12/20	None
CDAI score, range	220–400	None
5-ASA	7	None
Steroids	8	None
5-ASA + steroids	13	None
Anti–TNF-α	None	None
Untreated patients[a]	4	—

[a] Pharmacological therapy (5-ASA and/or steroids) was suspended for at least 1 month before the samples were taken.

and protease. When polymorphonuclear neutrophils (PMN) are exposed to various stimuli, extracellular O_2 is reduced to superoxide anion ($O_2^{\bullet-}$) by NADPH oxidase activity, and the respiratory burst (RB) is induced (6). Involvement of the reactive nitrogen intermediates, particularly nitric oxide (NO), has also been implicated in PMN-mediated tissue injury (7). Indeed, NO synthesized by a family of NO synthases (NOS) can interact with ROS, as $O_2^{\bullet-}$, to produce potent oxidants. In phagocyte cells, studies on ROS production have focused mainly on their function in the killing of bacteria, but a variety of recent evidence suggested that ROS in PMN, as well as in nonphagocyte cells (8), have an important role in activating and/or modulating the signaling pathways (9). Redox signaling can play a significant role in regulating the inflammatory response. It is evident that the mechanisms controlling PMN activation and $O_2^{\bullet-}$ production (SAP) are crucial for immune homeostasis and for the PMN inflammatory response. The levels of reducing agents, such as glutathione (GSH), are also important in regulating the oxidative intracellular state. Indeed, the activation of different enzyme systems that produce ROS is generally transient, thus, the antioxidant systems contribute to restoring the prestimulation steady-state level of ROS. Our previous reports demonstrated a decrease in GSH levels both in the normal and affected ileum of CD patients (10). These data show an altered metabolism of GSH and an increased intracellular oxidative state that may be a possible cause for relapse in this intestinal pathology.

For this reason, the aim of this *in vitro* study is to evaluate the intracellular redox state in CD patient PMN and the release of ROS induced in these cells after stimulation by TNF-α and adhesion factors related to molecular mechanisms that, if altered, may be responsible for an abnormal immune response. ROS production occurs through the activation of signaling pathways, but their precise regulation occurring *in vivo* is not yet completely understood. In fact, despite strong indications for a pathogenic role of TNF-α in intestinal bowel disease, the specific molecular mechanisms driving TNF-α–dependent disease remain poorly defined (11). For this purpose, the SAP from CD patient and control PMN has been investigated in PMN in suspension or adhesion after stimulation by TNF-α or phorbol 12-myristate 13-acetate (PMA), an analog of diacylglycerol, which is a specific activator of protein kinase C (PKC), an enzyme involved in the RB and in some signals, including TNF-α–signaling pathways (12, 13).

The effect of granulocyte-macrophage colony–stimulating factor (GM-CSF) on RB induction has also been investigated. GM-CSF is an important hematopoietic growth factor able to activate ROS production in PMN (14) and recently used in CD therapy (15). This study can be useful to identify specific targets involved in the onset of the CD inflammatory processes and to clarify the action of PMN in this pathology.

Materials and Methods

Drugs and Analytical Reagents. Dextran T500 and Ficoll-Paque were obtained from Amersham Biosciences (Little Chalfont, UK). Coomassie Brilliant Blue was purchased from Bio-Rad (Hercules, CA). Fetal bovine serum (FBS), PMA, GM-CSF, TNF-α, fibronectin-coated 96-well plates, and all other chemicals were obtained from Sigma Aldrich (St. Louis, MO).

Patients. A total of 32 patients with CD and 35 controls (healthy individuals or patients without intestinal or immune pathologies) participated in this study, and blood was drawn after receiving their informed consent. The figures show the exact number of patients that were studied in the different experiments conducted. Patient characteristics are reported in Table 1.

All patients were admitted into the Surgical Unit of the Department of Clinical Physiopathology because of increased clinical activity. This increased activity was documented through both a clinical assessment and the disease activity index (Table 1). We chose a group of CD patients similar to the controls with regard to sex and age range and, for homogeneity, all chosen CD patients suffered from ileal disease, had received bowel resections, and were in the active phase of the disease (Table 1). At the time the blood samples were taken, 7 patients were receiving treatment with salicylates (5-ASA), 8 with steroids, 13 with 5-ASA plus steroids, and 4 had not received these drugs for at least 1 month before the sample was taken; none of the subjects was being treated with anti-TNF (Table 1). Patients included in this study were treated with drugs prevalently used in CD pathology and we excluded patients submitted to anti-TNF therapy to eliminate possible interference in the studies performed with TNF-α.

The CD Activity Index (CDAI) provides a calculated composite score that incorporates various factors: number of stools, abdominal pain, abdominal mass, hematocrit, body weight, extraintestinal manifestations, and use of drugs for

the control of diarrhea. Patients with a score of less than 150 are considered to be in clinical remission and scores greater than 450 reflect severe active CD (16).

Preparation of Human and Neutrophil Cultures. Neutrophils were isolated from peripheral venous blood in the presence of EDTA; the separation procedure was performed in sterile conditions, as previously described (17).

Briefly, blood was mixed with an equal volume of 2.5% (w/v) dextran in 0.9% NaCl solution. After incubation at room temperature for 45 mins, cells in the leukocyte-rich plasma were collected and layered on Ficoll-Paque and centrifuged at 300 *g* for 30 mins. Residual erythrocytes in the PMN pellet were lysed by hypotonic lysis. The cells were pelleted at 300 *g* for 15 mins, washed in PBS, and resuspended in PBS containing 4.5 g/liter glucose for SAP determination in PMN in suspension. For experiments performed in adhesion, PMN were resuspended in RPMI 1640 with 2 m*M* L-glutamine and 10% heat-inactivated FBS (complete medium) and cultured for 1 hr or 20 hrs at 37°C in a 5% CO_2 atmosphere in fibronectin-coated 96-well plates at a concentration of 0.3×10^6 cell/150 μl. The cells were stained with 1% methyl-violet in 0.1 *N* isocitric acid and counted. After PMN isolation, the cell viability, assessed by exclusion of the vital dye, Trypan Blue, was greater than 99%, and the purity of the neutrophil preparation was greater than 95%. Protein concentrations were determined using the Bradford method (18). Bovine serum albumin was used as a standard.

PMN Viability. The colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (19) was used to determine PMN viability in all experimental conditions performed in adhesion. This method is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to reduce the yellow MTT and form dark blue formazan crystals, largely impermeable to cell membranes, thus, resulting in its accumulation within healthy cells. After incubation at 37°C for 3 hrs in a 5% CO_2 atmosphere, the cells in each well were solubilized with 50 μl of a detergent solution (20% sodium dodecyl sulfate in 0.01 *M* HCl) and incubated overnight. The absorbance, directly proportional to the number of surviving cells, was read at 570 nm, in an automatic 96-well plate reader (Model 550; Bio-Rad).

Determination of Cellular GSH. PMN (approximately 10^6) were centrifuged at 300 *g* for 10 mins, collected, and directly dissolved in 0.2 ml of 5% (v/v) aqueous $HClO_4$ plus 10 μl of 1 m*M* γ-glutamyl-glutamate (internal standard), and sonicated twice for 5 secs to measure GSH and glutathione disulfide (GSSG) using the high-performance liquid chromatography (HPLC) method (20).

Measurement of $O_2^{\bullet-}$ Production. The initial rate of SAP in PMN in suspension was assayed by the reduction of cytochrome-*c* by continuously monitoring optical density and calculated from the linear part of absorbance increase at 550 nm, using the extinction coefficient of 21.1 mM^{-1} (21). The specificity of the cytochrome-*c* reduction was verified by the addition of 160 U/ml superoxide dismutase (SOD) to control samples. PMA was added to 1×10^6 PMN/ml at final concentrations ranging from 5 to 100 n*M*, and TNF-α or GM-CSF ranging from 5 to 100 ng/ml after a preincubation period of 5 mins. No spontaneous metabolic activity was observed during the preincubation period. In inhibition experiments, PMN were preincubated for 30 mins at 37°C with inhibitors of various $O_2^{\bullet-}$ generating systems. Subsequently, the cells were stimulated with PMA, and the initial rate of SAP was determined.

In experiments performed during PMN adhesion, the incubation medium containing 80 μ*M* cytochrome-*c*, or a mixture of cytochrome-*c* and 25 ng/ml TNF-α or 25 ng/ml GM-CSF or 10 n*M* PMA was added to each well before adding the cells (0.3×10^6 cells). Cytochrome-*c* reduction caused by SAP was measured in a final volume of 200 μl at 550 nm using a microplate reader (Model 550; Bio-Rad). Experiments were carried out in the presence and absence of 160 U/ml SOD. Initial readings were performed immediately after the addition of cells; subsequent readings were made at 10-min intervals during 1 hr of incubation. The amount of $O_2^{\bullet-}$ produced was determined from the absorbance at 550 nm of samples without SOD minus the absorbance of matched samples with SOD, and expressed as nanomoles $O_2^{\bullet-}$ per milligram protein.

To perform experiments after 1 hr or 20 hrs of adhesion, PMN were plated in the presence or absence of 25 ng/ml of TNF-α or GM-CSF. Under these conditions, PMN were stimulated with 10 n*M* PMA; initial readings were performed immediately after stimulation, subsequent readings were made at 10-min intervals during 2 hrs of incubation. Experiments were carried out in the presence and absence of SOD. In inhibition experiments, PMN were preincubated for 30 mins at 37°C with 300 μ*M* N^G-nitro-L-arginine methyl ester (NAME) or 250 μ*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), and were subsequently plated and stimulated with various factors.

Adherence Assay. PMN (0.3×10^6 cells/150 μl of complete medium) for the adherence assay were plated on fibronectin-coated 96-well plates in the presence or absence of 25 ng/ml TNF-α or GM-CSF, or 10 n*M* PMA. After the incubation times, the monolayers were filled twice with PBS and centrifuged upside-down for 5 mins at 50 *g* to remove nonadherent cells. The wells were flicked empty, and the PMN remaining in adhesion were quantified by measuring myeloperoxidase activity through guaiacol oxidation monitored by change in absorbance at 470 nm, as previously described (22). Briefly, 0.1% (wt/v) cetrimethylammonium bromide in PBS, as a peroxidase solubilizing agent, was added to the wells and, after 2 mins, this solution was transferred into a mixture containing PBS, the substrate (13 m*M* guaiacol), and 1 m*M* 3-amino-1,2,4-triazol. This compound, a selective eosinophil peroxidase and catalase inhibitor, was added to the mixture to abolish the

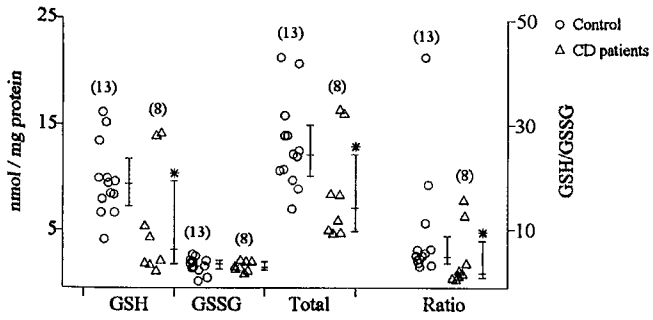

Figure 1. Levels of GSH system components in CD patient and control PMN. GSH and GSSG levels were measured in 10^6 PMN in suspension using the HPLC method. Total = [GSH] + 2[GSSG]; ratio = [GSH] / [GSSG]. Values are expressed as nanomoles per milligram protein. Median values and interquartile ranges are reported. Values in parentheses indicate the number of cases. $^*P \leq 0.05$ compared with control PMN.

interference by eosinophils (23) and to prevent H_2O_2 consumption by catalase (22). The reaction was started by the addition of 1 m*M* H_2O_2.

Statistical Analysis. All experiments were carried out three or more times. Data are expressed as the mean ± SEM, if normally distributed, and statistical significance of the differences was determined using Student's *t* test. Statistical significance of the differences for data that was not normally distributed was assessed using the Wilcoxon matched-pairs test; the results are given as the median with lower and upper quartiles. $P \leq 0.05$ was considered statistically significant.

Results

GSH and GSSG Levels in PMN in Suspension. Initially, we evaluated the intracellular redox state because it regulates several PMN functions and is affected by the balance between oxidant and reductant species. Thus, we measured GSH and GSSG levels in PMN in suspension, given that their ratio (GSH/GSSG) is an index of the intracellular oxidative status. GSH plays an important role in cellular functions, modulating the activities of various proteins by thiol-disulfide interchange reactions (24). Recently, it was demonstrated that GSH is involved in the signal transduction pathways regulating tyrosine kinase receptors and phosphatases, and it is also related to NADPH oxidase activation caused by platelet-derived growth factor receptor stimulation (25, 26). Moreover, intracellular GSH levels are involved in TNF-α–induced activation of the transcription factor, nuclear factor kB (NF-kB; Ref. 27), and in PMN adhesion processes mediated by integrins (28). Figure 1 shows that GSH and GSH/GSSG ratio levels are significantly lower in CD patient PMN with respect to control PMN, whereas GSSG values are similar. These results indicate that CD patient PMN are characterized by an increased oxidative state, consistent with our previous results obtained in CD patient intestinal mucosa (10).

Initial Rate of $O_2^{\bullet-}$ Production in PMN in Suspension. To determine PMN-induced RB, we measured SAP after PMN stimulation with three factors, PMA, or TNF-α, or GM-CSF, which can activate PMN and their SAP through different signaling pathways. In particular, we used PMA, because it activates PKC, a key participant in PMN signaling pathways (12), and because, at low PMA concentrations, it induces degranulation and the RB in PMN (29). In fact, PKC is involved in NADPH oxidase assembling and activation by inducing the phosphorylation of the cytosolic subunit, $p47^{phox}$ (30).

TNF-α stimulation was performed because TNF-α is largely released from CD patient cells (31), constitutes a pivotal proinflammatory cytokine, and it is involved in the activation of ROS production and in CD development (11). Moreover, TNF-α plays a crucial role in the signal transduction between immune and other cells and in the events that initiate and regulate the production of other proinflammatory cytokines. For these reasons, the anti–TNF-α antibody in CD therapy has been developed for patients who do not respond to standard treatment and for patients in whom pathology remission has been observed (31).

GM-CSF was studied because it augments mature phagocyte functions (32), and, as previously reported, is able to activate ROS production in PMN (14). Moreover, GM-CSF constitutes a new therapeutic approach in the treatment of this pathology (15). However, the action mechanisms of this compound used as a drug for CD patients are not yet known. Therefore, it is interesting to evaluate the effect of GM-CSF on PMN-induced SAP and to compare it with the effect of TNF-α.

We measured SAP in PMN in suspension isolated from CD patient and control blood samples. This condition simulates *in vitro* PMN in circulation. PMN stimulation in suspension was performed with various concentrations of PMA (from 5 n*M* to 100 n*M*) able to activate SAP. Different concentrations of TNF-α or GM-CSF (from 5 to 100 ng/ml) were also used. In Figure 2, we report only the results obtained using 10 n*M* PMA, because this concentration caused the highest SAP, whereas low and similar SAP in PMN were obtained after treatment with the different concentrations of TNF-α or GM-CSF (data not shown). To identify the source of this production, we measured the initial rate of SAP in PMA-stimulated CD patient and control PMN after cell pretreatment with specific inhibitors of various $O_2^{\bullet-}$-generating systems. Significant inhibition in SAP occurs in both the controls and CD patients only after preincubation of PMN with NAME, a specific inhibitor of NOS, or with AEBSF, an inhibitor of NADPH oxidase (Fig. 2; Refs. 7, 33). AEBSF inhibits also serine protease activity, but this effect does not interfere with its inhibition action on NADPH oxidase activity (33). On the contrary, in PMN treated with rotenone, oxypurinol, or indomethacin, specific inhibitors of NADH dehydrogenase, xantine oxidase, and cyclooxygenase, respectively (21), no change in $O_2^{\bullet-}$ production was observed (data not shown). These data indicate that the initial rate of SAP is mainly caused by

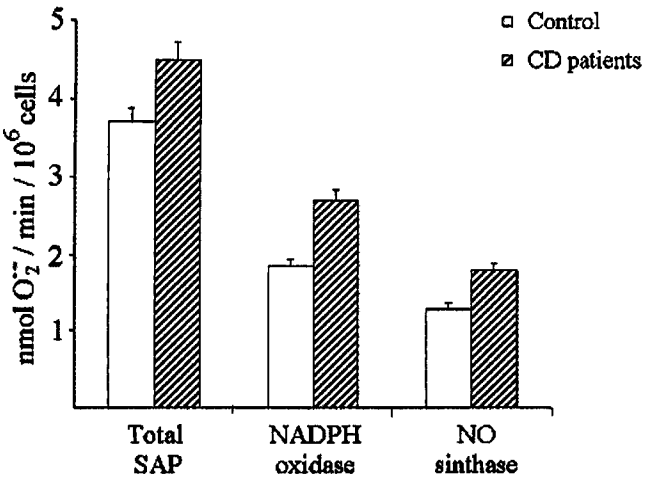

Figure 2. Effects of PMA on the $O_2^{\bullet-}$ production rate in CD patient and control PMN in suspension. The initial rate of $O_2^{\bullet-}$ production was measured in 10^6 PMN in suspension after the addition of 10 n*M* PMA. NADPH oxidase and NOS activities were obtained subtracting the values obtained in the presence of 250 μ*M* AEBSF or 300 μ*M* NAME to the total $O_2^{\bullet-}$ production, respectively. Data are expressed as nanomoles $O_2^{\bullet-}$ per minute per 10^6 cells and are presented as mean ± SEM of six experiments in triplicate.

NADPH oxidase and NOS activity, which are similar in both CD patients and control PMN (Fig. 2).

$O_2^{\bullet-}$ Production in PMN During and After 1 Hr of Adhesion on Fibronectin. PMN act in the inflammatory locus and react differently to physiologic stimuli after adhesion to other cells or proteins of the extracellular matrix (ECM), with respect to PMN in suspension (34). The ECM regulates cellular processes during tissue formation and organization, particularly modulating PMN functions and RB in the acute inflammatory response (35). In fact, previous studies show that TNF-α or other chemokines increase ROS production of PMN only after their adhesion on surfaces coated with a permissive substrate, such as

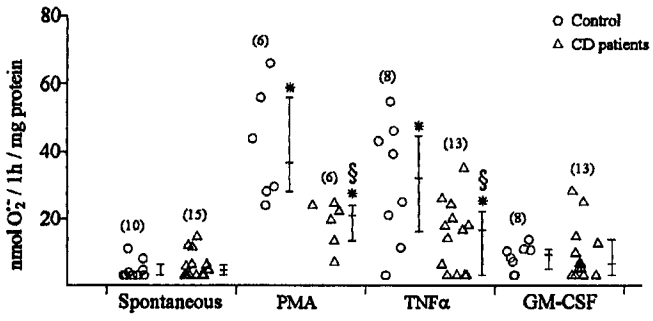

Figure 3. Effects of PMA, TNF-α, and GM-CSF on $O_2^{\bullet-}$ production during PMN adhesion. Control and CD patient PMN (0.3×10^6 cells) were plated on fibronectin-coated 96-well plates in the presence of cytochrome-*c* alone (spontaneous) or with 25 ng/ml TNF-α, or 25 ng/ml GM-CSF, or 10 n*M* PMA. Median values and interquartile ranges are reported. $O_2^{\bullet-}$ production values are expressed as nanomoles per hour per milligram protein. Values in parentheses indicate the number of cases. $^*P \leq 0.05$ compared with the respective spontaneous SAP; $^{\S}P \leq 0.05$ compared with the respective PMA- and TNF-α–induced SAP in control PMN.

fibronectin and fibrinogen (36). Therefore, considering that, during the active phase of the disease, PMN and monocytes migrate in response to bacterial products from peripheral blood to the intestinal mucosa, where cytokines are locally produced, we measured the RB and hence, the SAP, in CD patient and control PMN placed on fibronectin in the presence and absence of TNF-α or GM-CSF. This condition mimics the adhesion of these cells on the tissue, and the fibronectin represents an ECM protein on which TNF-α and GM-CSF induce RB in PMN (14, 35, 36).

Initially, the SAP was measured in PMN during adhesion to fibronectin in the presence or absence (spontaneous SAP) of TNF-α, GM-CSF, or PMA. SAP values were determined in PMN immediately after placing the cells on fibronectin and at 10-min intervals thereafter, up to 1 hr. Figure 3 reports the values measured after 1 hr and shows that, in CD patient and control PMN, the spontaneous SAP is similar. Moreover, the presence of TNF-α or PMA significantly increases the SAP with respect to spontaneous SAP in both CD patient and control PMN. However, it is evident that subjects with CD exhibit a significantly lower SAP than that obtained in control PMN. On the contrary, during the time of adhesion, GM-CSF does not affect the SAP, either in CD patient or control PMN (Fig. 3). It should be noted that a similar picture was also obtained in PMN placed on fibronectin for only 10 mins (data not shown).

Subsequently, we studied SAP in PMN after longer times of adhesion on fibronectin. PMN were plated in the absence or presence of TNF-α or GM-CSF and, after 1 hr, were stimulated or not stimulated with PMA, and the $O_2^{\bullet-}$ content was measured after different times (30, 60, and 120 mins). This experimental condition was performed taking into account the fact that TNF-α and GM-CSF may cause an enhancement of neutrophil function, called "priming," by inducing an increase in SAP, adherence, and migration in response to various stimuli (37, 38). Figure 4A and B report the SAP measured in PMN stimulated or not with PMA for 60 mins; at all studied times, the effects in SAP values were similar.

A significant increase in SAP was observed in control PMN plated in the presence of TNF-α or stimulated with PMA alone compared with spontaneous SAP (Fig. 4A), similar to that observed during adhesion (Fig. 3). On the contrary, PMA or TNF-α does not significantly increase SAP in CD patient PMN (Fig. 4B). GM-CSF increases SAP compared with spontaneous SAP in both control and CD patient PMN (Fig. 4A and B), in contrast to that observed during adhesion (Fig. 3). Moreover, SAP production was not augmented in TNF-α–treated control PMN after PMA stimulation compared with SAP measured in PMN treated with PMA or TNF-α alone (Fig. 4A), also in contrast to the values obtained from CD patient PMN (Fig. 4B). However, PMA stimulation significantly increased SAP values in GM-CSF-treated control and CD patient PMN (Fig. 4A and B).

These results, overall, demonstrate that TNF-α and PMA differently affected the RB observed in control and

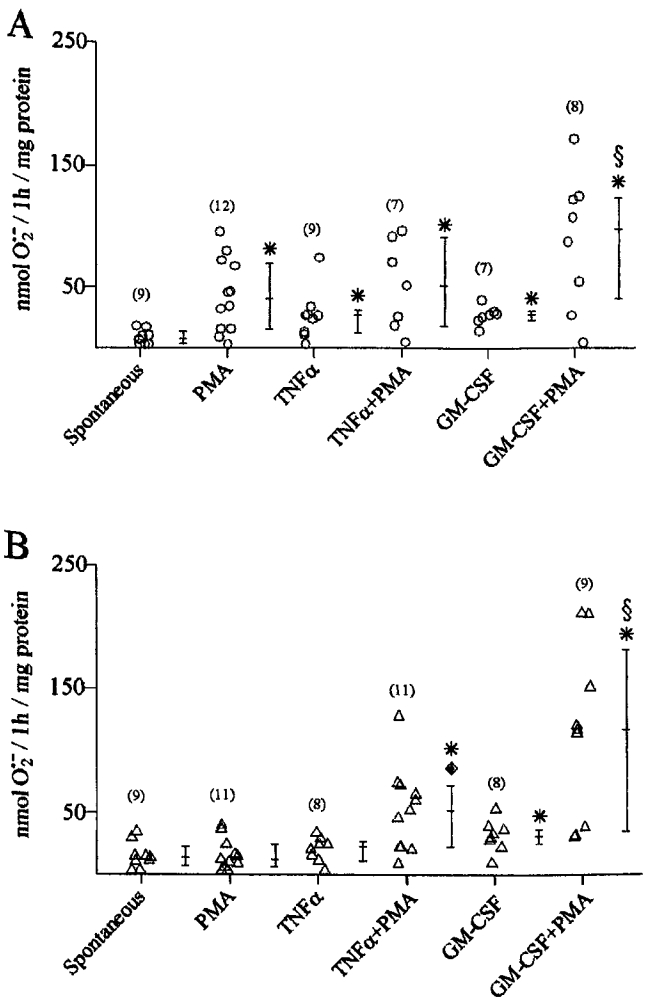

Figure 4. Effects of PMA, TNF-α, and GM-CSF on $O_2^{\bullet-}$ production after 1 hr of PMN adhesion. Control PMN (A) and CD patient PMN (B) (0.3×10^6 cells) were cultured for 1 hr in fibronectin-coated 96-well plates in the presence or absence of 25 ng/ml TNF-α or GM-CSF. Subsequently, SAP was measured in PMN with or without stimulation for 1 hr with 10 n*M* PMA. Median values and interquartile ranges are reported. $O_2^{\bullet-}$ production values are expressed as nanomoles per hour per milligram protein. Values in parentheses indicate the number of cases. $^*P \leq 0.05$ compared with the respective spontaneous SAP; $^{\circ}P \leq 0.05$ compared with PMA- and TNF-α–induced SAP; $^{\S}P \leq 0.05$ compared with the respective PMA- and GM-CSF–induced SAP.

CD patient PMN during and after 1 hr of adhesion. Reduced SAP with these factors occurs in CD patient PMN, indicating possible alterations in the mechanisms generating ROS through PKC- and TNF-α–dependent signaling pathways. On the contrary, the GM-CSF effect is similar in the PMN from the two groups and, in agreement with the literature, the RB increase is obtained later than the increase induced by TNF-α (14).

Because, in neutrophils stimulated by N-formyl-L-methionyl-L-leucyl-L-phenylalanine, it has been demonstrated an activation of NOS with an NO content increase, that reacting with $O_2^{\bullet-}$ can produce peroxynitrite and decrease $O_2^{\bullet-}$ levels (7), we evaluated SAP in CD patient PMN preincubated with NAME and subsequently stimulated by PMA or TNF-α. Under these experimental conditions, an increase of SAP should be detected, as reported by other researches (7); however, we did not observe changes in $O_2^{\bullet-}$ levels (data not shown). This indicates that an enhancement of NO and peroxynitrite production cannot be the cause of the lower $O_2^{\bullet-}$ level detection in CD patient PMN (Figs. 3 and 4B).

Cell viability, evaluated in all of the experiments, was 95%, and was similar in both PMN groups.

Effect of PMA, TNF-α, and GM-CSF on PMN Adherence to Fibronectin. It is well known that PMN adhesion to fibronectin, as well as to other ECM proteins, involves β-integrins, a family of cell adhesion receptors present on the PMN surface (39). These integrins, through the binding of specific cell adhesion molecules (CAM), are able to transduce extracellular signals into cells and *vice versa*, modulating numerous cellular functions (39). Moreover, a relationship between TNF-α–induced $O_2^{\bullet-}$ release and PMN adhesion mechanisms involving β_2-integrin activation has been found (36, 40). For this purpose, to relate the changes previously obtained on SAP to factors involved in PMN adhesion, we examined the adherence of PMN plated for different times on fibronectin in the presence or absence of PMA, TNF-α, or GM-CSF. As shown in Table 2, PMA and TNF-α significantly stimulate the adherence of control and CD patient PMN on fibronectin, but a lesser adherence occurs in CD patient PMN compared with control PMN. On the contrary, in the

Table 2. Effect of TNF-α, GM-CSF, or PMA on Adherence to Fibronectin of Control and CD Patient PMN[a]

	20 mins		60 mins		120 mins	
Incubation time	Control	CD	Control	CD	Control	CD
None	9 ± 2	10 ± 2	20 ± 2	18 ± 3	19 ± 4	17 ± 3
TNF-α (25 ng/ml)	21 ± 2*	15 ± 2	60 ± 8*	33 ± 4*, **	55 ± 6*	30 ± 4*, **
GM-CSF (25 ng/ml)	10 ± 2	11 ± 1	17 ± 3	19 ± 2	35 ± 3*	33 ± 4*
PMA (10 n*M*)	20 ± 3*	14 ± 2	53 ± 6*	30 ± 2*, ***	54 ± 8*	32 ± 5*, ***

[a] PMN (0.3×10^6) were plated in fibronectin-coated well plates in the presence or absence of TNFα, GM-CSF, or PMA. After different times at 37°C, cell adherence was quantified by measuring myeloperoxidase (MPO) activity. Data are expressed as percent with respect to the activity value of MPO present in 0.3×10^6 cells, and are presented as mean ± SEM of six experiments repeated in triplicate.
* $P \leq 0.05$ compared with respective adherence in the absence of factors; ** $P \leq 0.05$ compared with adherence of control PMN in the presence of TNF-α; *** $P \leq 0.05$ compared with adherence of control PMN in the presence of PMA.

presence of GM-CSF, the adherence of PMN from both groups is similar until 60 mins of incubation, compared with that measured in the absence of factors, and it was significantly increased after 120 mins.

These data seem to be in accordance with the changes obtained in SAP. It is possible that, in TNF-α– and PMA-treated CD patient PMN, the decreased adherence will be responsible, in part, for the $O_2^{\bullet-}$ defective production assayed in these conditions. In GM-CSF–stimulated PMN, the delayed increase of adhesion could be related to the delayed onset of SAP. However, both of these parameters in GM-CSF–treated control and CD patient PMN are similar.

Cell Viability, $O_2^{\bullet-}$ Production, and Adhesion in PMN After 20 Hrs on Fibronectin. Of all of the leukocytes, neutrophils have the shortest life span. They remain in circulation a short time (6–10 hrs) before entering tissues, where they survive for 1 to 2 days (41) and can be stimulated by various factors. PMN spontaneously undergo apoptosis and this represents an important mechanism for the removal of neutrophils from inflammation sites and, thus, the resolution of the inflammatory process (42). Moreover, in neutrophils, programmed cell death may be important to minimize nonspecific damage in surrounding tissue caused by the high amounts of ROS and proteases that are generated by PMN. Therefore, we have mimicked the prolonged permanency of neutrophils in tissues by experiments in which neutrophils were treated with TNF-α or GM-CSF for 20 hrs in adhesion on fibronectin.

Therefore, we measured $O_2^{\bullet-}$ production and cell viability after 20 hrs of adhesion on fibronectin in the presence or absence of TNF-α or GM-CSF to compare these parameters and to evaluate PMN activity during a longer time. The results showed a high percentage of living cells in the control PMN, whereas a significant decrease in cell viability was measured in CD patient PMN (Fig. 5). TNF-α and GM-CSF did not change cell viability in control PMN, nor did TNF-α stimulation in CD patient PMN. On the contrary, GM-CSF increased the percentage of living cells in CD patient PMN compared with untreated PMN, and the value of cell viability reached that measured in control PMN (Fig. 5).

With regard to SAP determined under the same experimental conditions, the data reported in Figure 6 show that SAP is similar in the PMN from both groups treated with TNF-α, and no significant increase was obtained compared with spontaneous SAP.

On the contrary, in PMN from both groups, GM-CSF treatment induced a similar RB that was significantly higher than that observed in untreated PMN, indicating that GM-CSF, unlike TNF-α, is able to induce a prolonged release of $O_2^{\bullet-}$ after adherence to fibronectin, and that RB activation mechanisms induced by GM-CSF are not altered in CD patient PMN compared with the controls.

Moreover, these data do not indicate a clear relationship between changes in cell viability and the ability to induce RB in both PMN groups. However, the increased viability induced by GM-CSF in CD patient PMN seems to be related to the increased SAP measured in GM-CSF–treated cells.

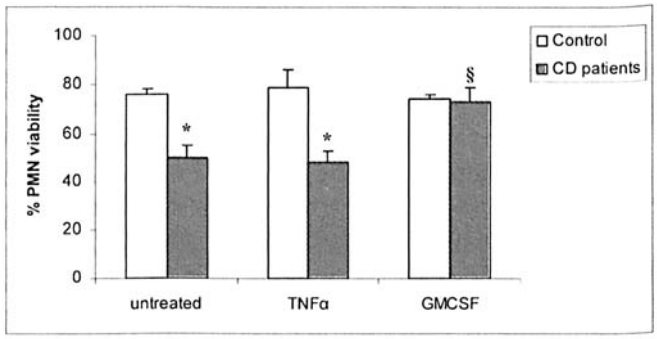

Figure 5. Effects of TNF-α and GM-CSF on cell viability after 20 hrs of PMN adhesion. Control and CD patients PMN (0.3×10^6 cells) were cultured for 20 hrs in fibronectin-coated 96- well plates in the presence or absence of 25 ng/ml TNF-α or GM-CSF. Data are expressed as the percent ± SEM of six experiments in triplicate with respect to the viability measured using MTT in 0.3×10^6 PMN before plating. $^*P \leq 0.05$ compared with the viability of the control PMN in the absence and presence of TNF-α; $^§P \leq 0.05$ compared with the viability of untreated CD patient PMN.

Other experiments were also performed in PMN treated for 20 hrs with TNF-α or GM-CSF and subsequently stimulated with PMA, but no significant change in SAP was obtained (data not shown).

After 20 hrs with cell adhesion, SAP was measured and the values obtained show that TNF-α–induced adhesion decreases in both groups of neutrophils, reaching the values obtained in untreated cells after 120 mins (Table 2), on the contrary, the adhesion values in GM-CSF–treated PMN remain higher and similar to those measured in both groups after 120 mins (data not shown).

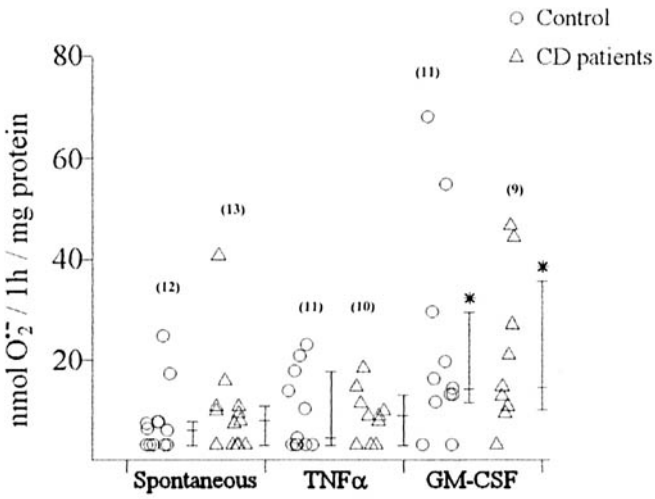

Figure 6. Effects of TNF-α and GM-CSF on $O_2^{\bullet-}$ production after 20 hrs of PMN adhesion. Control and CD patients PMN (0.3×10^6 cells) were cultured for 20 hrs in fibronectin-coated 96-well plates in the presence or absence of 25 ng/ml TNF-α or GM-CSF. Median values and interquartile ranges are reported. $O_2^{\bullet-}$ production values are expressed as nanomoles per hour per milligram protein. Values in parentheses indicate the number of cases. $^*P \leq 0.05$ compared with the respective spontaneous SAP.

Finally, we did not find relationships between SAP values obtained under the different experimental conditions, in CD patients treated with 5-ASA and/or steroids or not treated with these drugs for at least 1 month before the sample taking (data not shown).

Discussion

This study demonstrates, *in vitro*, that CD patient PMN adherent to ECM show a decreased SAP after TNF-α or PMA stimulation as compared with controls. In particular, altered mechanisms of PKC and β-integrin activation related to the TNF-α signaling pathway seem to be involved in impaired SAP and adhesion on fibronectin. Moreover, for the first time, the findings of this study support the involvement of GM-CSF in activating the RB directly and by the priming effect for a prolonged time, both in control and CD patient PMN, and in increasing the viability of the latter. An increased oxidative state in CD patient PMN caused by a decreased GSH/GSSG ratio has also been identified.

In particular, we demonstrate that the initial rate of PMA-induced SAP is similar in CD patient and control PMN in suspension, indicating the normal function of CD patient PMN in suspension with regard to PMA activation mechanisms of $O_2^{\bullet-}$ enzyme production.

However, a number of conflicting studies reported in the literature show impaired, normal, or enhanced SAP in PMA-stimulated PMN in suspension from intestinal bowel disease patients (43–45). These differences can be caused by the different experimental conditions used. Moreover, in contrast to other reports, we have demonstrated the involvement of NADPH oxidase and NOS activities in PMA-stimulated SAP, and no change of their activity in PMN in suspension from subjects affected by CD was found.

In contrast to results observed in suspension, TNF-α and GM-CSF increase SAP in PMN in adhesion, indicating that this condition favors SAP activation by these compounds. PMA and TNF-α significantly increase SAP in PMN in adhesion, but the results obtained in CD patient PMN in adhesion show lower levels of $O_2^{\bullet-}$ compared with the controls. These results indicate a possible defect in the activation mechanisms of PKC activity in CD patient PMN cause by adhesion on fibronectin, because PMA, a specific activator of this enzyme, induces similar SAP in both PMN groups in suspension. Moreover, the lack of stimulation of $O_2^{\bullet-}$ production by PMA, observed in TNF-α–treated control PMN, suggests that this factor also induces SAP, probably *via* PKC; therefore, PMA is not able to cause an additional increase. Indeed, PKC activation is also related to the TNF-α signaling pathway (13).

We have also excluded, through NAME experiments, the possibility that the decrease in SAP (detected in CD patient PMN stimulated by PMA or TNF-α) can be caused by the increase in NO, which, reacting with $O_2^{\bullet-}$, yields peroxinitrite, a potent oxidant agent (7).

On the contrary, the effect of GM-CSF is similar in PMN from both groups, and PMA enhances the PMN response to GM-CSF, suggesting the involvement of PKC-independent mechanisms. The effect of GM-CSF on SAP after a longer stimulation time confirms findings in the literature that demonstrate a delayed GM-CSF–induced RB in adherent neutrophils (14). It is notable that, after 1 hr of adherence, PMA induces SAP similar to the controls in CD patient PMN plated in the presence of TNF-α and/or GM-CSF. This effect suggests that mechanisms triggered by TNF-α and PMA are able to activate the RB in CD patients when both are present, indicating that only the simultaneous presence of TNF-α and PMA induces an adequate signal. It is possible that PMA increases the p60TNF receptor serine phosphorylation induced by TNF-α (46), and that TNF-α or GM-CSF can activate proteins contributing to PKC activation.

Considering that, in the presence of TNF-α and PMA, a defective adherence on fibronectin occurs in CD patients PMN, we suggest that this may reflect an SAP adhesion decrease related to an anomalous expression or activation of integrins. In fact, TNF-α–induced SAP in PMN requires both binding of TNF-α to its specific receptor and binding of β-integrins to CAM (34). This can explain the lack of a RB in neutrophils in suspension after TNF-α treatment, as reported by others (37).

The lower adhesion induced by TNF-α and PMA observed in CD patient PMN seems to be in disagreement with the increased oxidative status measured in these cells because oxidative stress can activate the expression of redox-sensitive integrins and/or adhesion molecules in neutrophils (28). However, we suppose that the decreased adhesion and SAP observed in CD patient PMN can be related to the decreased GSH levels. In fact, GSH or GSSH are involved in the activation of specific adhesion molecules by reactions of *S*-thiolation and formation of protein-mixed disulfides (28). It should also be noted that the adhesion levels of untreated PMN in both groups are very low and that GM-CSF increases their adhesion similarly, but this is delayed as compared with values obtained in TNF-α– or PMA-treated cells, as also reported by other authors (47). These data can be related to delayed and similar SAP induced in CD patient and control PMN after GM-CSF stimulation. Therefore, a relationship between PMN adhesion and SAP stimulation induced by these factors is found. However, PKC activity seems to be involved in TNF-α–induced adhesion and SAP, whereas GM-CSF mediates its action by different signaling mechanisms that are not altered in CD patient neutrophils.

Taken together, these data indicate that an anomaly in the PKC and/or integrin activation related to various steps of the TNF-α signaling pathway can be responsible for altered adhesion and ROS production in adherent CD patient PMN. Indeed, several cytoskeletal proteins are PKC substrates (48), and this enzyme activity is also essential for phosphorylation of the $p47^{phox}$ subunit necessary for

NADPH oxidase assembly and activation (30). Therefore, defects in the enzyme activation mechanisms involved in ROS production, together with low GSH levels, can modulate the inflammatory response and contribute to chronic inflammation (49). A GSH deficiency in immune cells is also related to an increased NF-kB transcription (50) and increased levels of cytokines (51, 52). Considering that we previously determined a decrease in GSH content in both the normal and affected ileum of CD patients (10), also confirmed by other researchers in CD and ulcerative colitis patient intestinal cells (53), it is probable that the reduced GSH levels constitute a generalized effect regarding cells involved in the inflammatory intestinal processes.

Finally, data obtained in PMN plated for 20 hrs on fibronectin and treated with TNF-α show that no increase on SAP was observed in either PMN group, and this effect does not seem to be related to cell viability (which was approximately 80% in control PMN). Moreover, also after 1 hr of adhesion, although the percentage of living cells was approximately 95% in both PMN groups, increased TNF-α–induced SAP was measured only in control PMN and not in CD patient PMN, indicating that no clear relationship between SAP and viability was found. However, after 20 hrs, the adhesion values decreased in both PMN groups stimulated by TNF-α, confirming an involvement of adhesion mechanisms related to TNF-α signaling on SAP induced by TNF-α. It is also possible that, after long times of stimulation, these results obtained regarding the decrease of $O_2^{\bullet -}$ production are caused by processes of slow and downregulation. In contrast, GM-CSF continued to induce the RB both in control and CD patient PMN, similar to that observed after 1 hr of adherence. This effect, similar in both PMN groups, may be because of the ability of GM-CSF to maintain a high level of survival in CD patient PNM and high adhesion values in both PMN groups, confirming that SAP induced by GM-CSF occurs through signals that are different from TNF-α. Moreover, the decreased viability observed in CD patient PMN also suggests an increased apoptosis and/or necrosis that could be related to an increased oxidative state. However, further studies should be performed to evaluate apoptosis and to identify the factors involved in the decrease in viability or the increase in apoptosis, and in the possible relationship between these events and the effects on SAP induced by TNF-α or GM-CSF in CD patient PMN.

In conclusion, the decreased SAP and adhesion after TNF-α treatment, together with decreased cell viability and GSH levels, can be responsible for the functional defects and the altered immune response observed in CD patient neutrophils (15). All of these factors can contribute to the decreased defensive action of CD patient PMN against luminal microbes in the mucosa before activation and recruitment of other inflammatory cells. This can be responsible for a delayed and disorganized mucosal response dominated by the macrophage and lymphocyte activation that characterizes the CD pathology and induces the chronic immune response, secretion of inflammatory cytokines, and tissue injury. The results of this report also indicate that the harmful effects of TNF-α in CD patient PMN, where it is largely produced, cannot be caused by excessive ROS production, which, in some cases, has been indicated as a probable cause of the onset of the typical CD inflammatory state. On the contrary, CD patient PMN are normally stimulated by GM-CSF, which induces an SAP similar to that obtained in control PMN, indicating that this compound can contribute to the reestablishment of a correct PMN activity and immune response. Therefore, treatment with GM-CSF in some CD therapeutic strategies can favor the normal function of CD patient PMN.

These data, overall, may contribute, in part, to explaining TNF-α–mediated intestinal pathology and the CD pathogenesis; they also support the exploration of new targets and therapies in the treatment of CD. However, further studies are necessary to clarify the molecular processes responsible for the different roles that GM-CSF and TNF-α have in SAP in CD patient PMN, and the effective physiologic role of the alterations demonstrated in CD patients PMN, also in relation to the increased oxidative state and decreased cell viability.

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