

Phenotype and cytokine production of Lamina Propria Mononuclear Cells from Endoscopic Biopsy Specimens in Untreated Patients with Ulcerative Colitis

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The phenotype of, and the production of interleukin-1 β (IL-1 β) and interleukin 6 (IL-6) by lamina propria mononuclear cells (LPMNC) were studied in 25 patients with ulcerative colitis (UC) (11 with untreated active disease and 14 inactive disease), and 18 control subjects.

The percentage of CD4 $^+$ cells, CD20 $^+$ cells, CD3 $^+$, CD25 $^+$ cells and CD3 $^+$, HLA-DR $^+$ cells was significantly higher in patients with active UC than in control patients, however, the percentage of CD8 $^+$ cells was significantly lower in patients with active UC than in patients with inactive UC and controls. IL-1 β was detected in nine of the 11 patients (82%) with active UC but in only two of the 14 patients (14%) with inactive UC and three of the 18 control subjects (17%). The amount of IL-6 in patients with active UC was significantly higher than in patients with inactive UC and in controls.

These results suggest that IL-1 β and IL-6 mediate immune responses, and may be associated with inflammation and the etiopathogenesis of UC.

Key Words : Interleukin 1, Interleukin 6, Lamina Propria Mononuclear Cells, Ulcerative Colitis

INTRODUCTION

Immunological abnormalities of UC, which is a diffuse inflammatory bowel disease of the colonic mucosa, have been elucidated mainly from the aspects of activation of lymphocytes and associated cytokine secretion^{1,2)} and immunoglobulin production³⁾. These studies were initiated to seek abnormalities in peripheral blood^{4,5)}. However, it is more important to examine immunological abnormalities in the colonic mucosa, since UC is organ-specific, invading only the large bowel.

IL-1 and IL-6 play important roles in immune inflammatory responses^{6,7)}. IL-1 has been implicated as a major mediator released by monocytes/macrophages in inflammatory and immunological responses⁸⁾. The role of IL-1 as a key mediator in the cytokine cascade may be of importance⁹⁾. In addition, IL-1 facilitates the initiation of a T cell response¹⁰⁾, and orchestrates a wide spectrum of

immunoinflammatory activities pertinent to inflammatory bowel disease¹¹⁾. IL-6 is a pleiotropic cytokine produced by various cell types, including monocytes/macrophages, activated T cells, B cells, endothelial cells, fibroblasts, chondrocytes and mesangial cells^{7,12)}. IL-6 promotes terminal differentiation of B cells, induction of immunoglobulin secretion¹³⁾, growth and differentiation of T cells¹⁴⁾, and production of acute phase protein synthesis in hepatocytes¹⁵⁾.

Most past studies of UC were based on the analysis with resected tissue from patients with UC receiving corticosteroid or immunosuppressive drugs. These drugs may have an influence on the analysis, and thus it is important to investigate the patients with untreated UC.

The aim of this study was to investigate the production of IL-1 β and IL-6 production by lamina propria mononuclear cells (LPMNC) isolated from inflamed mucosa in untreated patients with a first attack of UC. We also searched for the phenotype of LPMNC.

MATERIALS AND METHODS

1. PATIENT POPULATION

Eleven patients with active ulcerative colitis (six women, five men; mean age 40.4 years (range 26-53)), 14 with inactive UC (six women, eight men; mean age 38.2 (range 15-45)), and 18 control patients (seven women, 11 men; mean age 61.4 (range 43-68)) were studied. Mucosal biopsy specimens were obtained, with informed consent, while these patients were undergoing endoscopy for clinical reasons. In the case of active disease, the biopsy specimens were obtained from the site where the most intense inflammation was observed macroscopically. In the case of inactive disease, the specimens were obtained from the site where inflammation had been evident during the active phase. The control group with normal colonic mucosa (confirmed on routine histology) were patients who were undergoing polypectomy for colonic polyps.

The diagnosis of UC was based on the clinical picture and laboratory findings, including colonoscopy and histological features of the biopsy. Active and inactive UC were defined by clinical features, colonoscopic findings, and histological features of the biopsy when available. All patients with active UC were in their first attack, and not being treated with any drugs. Nine patients with inactive UC were receiving sulfasalazine alone (median dose 3 g/day, range 1.5-4), and five were untreated.

2. ISOLATION OF LPMNC

LPMNC were isolated from inflamed mucosa and normal mucosa by a modification of the enzymatic technique of Bull and Bookman¹⁶⁾, as previously described¹⁷⁾. Briefly, tissue was obtained from fresh endoscopic biopsy specimens. After washing, the tissue was incubated in 1 mM dithiothreitol solution (DTT; Sigma, Poole, Dorset, U. S. A.) for 15 minutes at room temperature. To remove the epithelial cells, it was shaken with 5 mM ethylenediamine tetraacetic acid (EDTA; Sigma, Poole, Dorset, U. S. A.) for half an hour at 37 °C. After washing, the tissue was digested with collagenase (from *Clostridium histolyticum*, Boehringer, Germany) at a concentration of 1 mg/ml in 10 % fetal calf serum/RPMI 1640 medium (Gibco, U. S. A.), for three hours at 37 °C. LPMNC were obtained by Ficoll-Paque (Pharmacia, Sweden) gradient centrifugation 400 g for half an hour at 4 °C.

3. FLOW CYTOMETRIC ANALYSIS

LPMNC were processed for direct single or double staining using fluorescein isothiocyanate (FITC) - or phycoerythrin (PE) - labeled monoclonal antibodies, namely, SimulSET control reagent (IgG₁ FITC + IgG₂ PE), CD3 (Leu 4), CD4 (Leu 3a), CD8 (Leu 2a), CD14 (Leu M3), CD20 (Leu 16), CD25 (IL-2 receptor) and HLA-DR (Becton Dickinson Inc.). For staining of LPMNC, the suspension of LPMNC was adjusted to 2×10^5 cells/ml: 10 ml of each monoclonal reagent was placed into a polystyrene tube; 50 ml of the cell suspension (1×10^4 cells) was added to the tube. The tubes were incubated for 30 minutes at 4 °C in the dark. The samples were washed once in phosphate buffered saline containing 0.1 % sodium azide. The cell sediment was resuspended in 0.5 % paraformaldehyde in buffer. Analysis by flow cytometry was performed with FACScan (Becton Dickinson Immunocytometry system).

4. CELL CULTURE

LPMNC were suspended in culture medium RPMI 1640 containing 10 % fetal calf serum, at a concentration of 1×10^5 cells/ml, and 1 ml of the cell suspension were put

into each well of a 24-well flat-bottom plate (Nunc, Denmark). For IL-1 β production, LPMNC were cultured in the presence of lipopolysaccharide (LPS; 10 μ g/ml; Sigma, U. S. A.) for two days. For IL-6 production, LPMNC were cultured in the presence or absence of pokeweed mitogen (PWM; 2.5 μ g/ml; Sigma, U. S. A.) for five days. Incubation of the culture was performed in 5 % CO₂, 95 % air at 37 °C, and then the supernatant was collected by centrifugation at 400 g for 5 minutes and stored at -70 °C before analysis.

Efficacy of the LPS and PWM used in these studies was confirmed in experiments on peripheral blood mononuclear cells from healthy individuals.

5. MEASUREMENT OF IL-1 β AND IL-6

The amount of IL-1 β present in the supernatants was assayed using ELISA (IL-1 β , Ohtsuka, Japan). Briefly, a monoclonal IL-1 β antibody was absorbed onto microtitre plates and supernatant samples were added in duplicate together with known IL- β standards. Anti IL-1 β polyclonal antibody was added to each well, and then the wells were rinsed. Peroxidase-labeled anti-rabbit IgG antibody was added to the test wells. After addition of a substrate solution, optical density was measured at 492 nm. The amount of IL-1 β in each sample was determined according to a standard curve prepared concurrently. Intra-assay and inter-assay replicates gave results with a coefficient of variation of less than 2.9 % and 6.8 %, respectively. Furthermore, the kit is specific for the measurement of IL-1 β . No cross reactivity was apparent with human recombinant IL-1 α , IL-2, Tumor necrosis factor- α , Interferon- α and Interferon- γ . Immunoglobulins (IgA, IgG and IgM) were also measured by ELISA (IgA, IgG and IgM, Ohtsuka Assay Inc., Japan) as described above for the amount of IL-1 β . Assays were performed in duplicate according to the manufacturer's instructions.

The amount of IL-6 in the culture supernatants was assayed using radioimmunoassay (RIA) (IL-6, Amersham, U. K.). Briefly, the assay is based on the competition between unlabeled IL-6 and a fixed quantity of ¹²⁵I-labeled IL-6 (human, recombinant) for a limited number of binding sites on an IL-6 specific antibody. The fixed amounts of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand. The antibody bound IL-6 is then reacted with the Ameriex-M second antibody reagent which contains a second antibody that is bound to magnetizable polymer particles. Separation of the antibody bound fraction is effected by either magnetic separation or centrifugation of the Ameriex-M suspension and decantation of the supernatant. Measurement of the radioactivity in the pellet enables the amount of labeled IL-6, the bound fraction to be calculated. The concentration of unlabeled IL-6 in the sample is then determined by

interpolation from a standard curve.

6. STATISTICAL ANALYSIS

Mann-Whitney U test was used for comparison of IL-6 concentrations and the percentage of LPMNC, and the chi-square test was for IL-1 β concentrations. All data were expressed as mean \pm standard deviation (mean \pm SD), and a probability less than 5 % was considered to be significant.

RESULTS

1. MUCOSAL SPECIMENS STUDIES

The biopsy specimens were obtained with ordinary biopsy forceps. The average mononuclear cell yield was $50.9 \pm 8.4 \times 10^5/g$ in active UC. In contrast, the average mononuclear cell yield was $36.2 \pm 4.8 \times 10^5/g$ in inactive UC and $36.3 \pm 7.4 \times 10^5$ in normal controls. Viability was $90.7 \pm 2.2 \%$ in active UC, $90.0 \pm 8.8 \%$ in inactive UC and $92.8 \pm 4.2 \%$ in normal controls, as determined by 0.1 % trypan blue exclusion.

2. FLOW CYTOMETRIC ANALYSIS OF LPMNC

As shown in Figure 1, the percentage of CD4⁺ cells (helper/inducer T cells) and CD20⁺ cells (B cells) of LPMNC from active UC was significantly higher com-

pared with normal controls ($p < 0.05$ for CD4⁺ cells ; $P < 0.01$ for CD20⁺ cells). In contrast the percentage of CD8⁻ cells (suppressor/cytotoxic T cells) was significantly lower in patients with active UC than in patients with inactive UC and controls ($p < 0.05$). The percentage of CD3⁺, CD25⁺ cells and CD3⁺, HLA-DR⁺ cells was higher in patients with active UC than in control patients ($P < 0.05$; Figure 2).

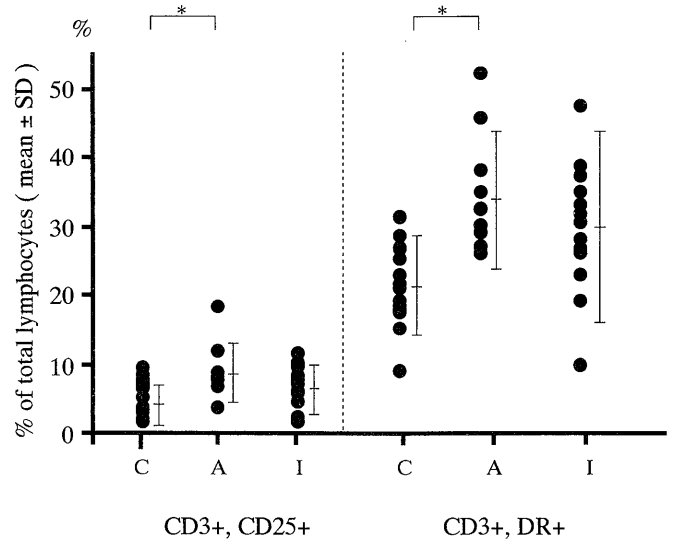


Figure 2. Percentage of activated T cells in lamina propria mononuclear cells in patients with active UC (A), patients with inactive UC (I) and normal controls (C) (* $p < 0.05$)

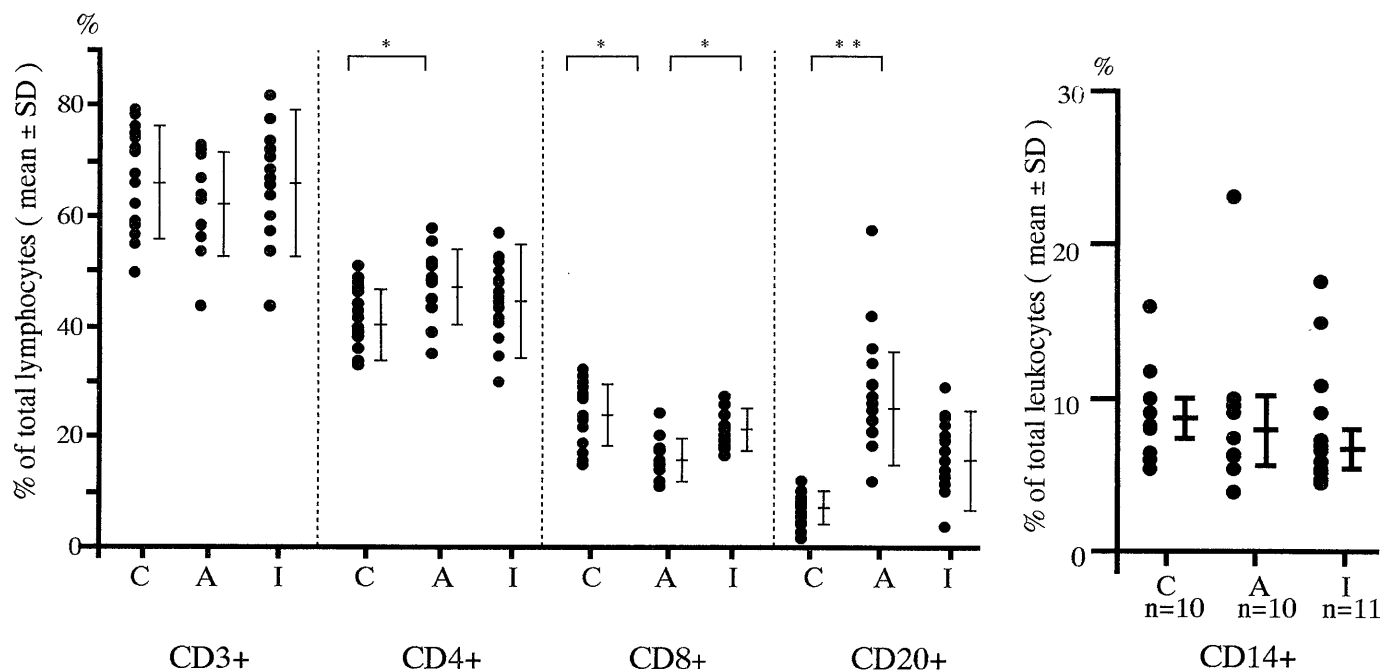


Figure 1. Percentage of T cells, B cells and Macrophages in lamina propria mononuclear cells in patients with active UC (A), patients with inactive UC (I) and normal controls (C) (* $p < 0.05$ and ** $p < 0.01$).

3. IL-1 β PRODUCTION

Samples containing IL-1 β at the level of 20 pg/ml or higher were considered IL-1 β positive, and those containing less than 20 pg/ml, negative; the level of 20 pg/ml being the detection limit of the ELISA. IL-1 β was detected in nine of the 11 patients (82%) with active UC but in only two of the 14 patients (14%) with inactive UC and three of the 18 normal control subjects (17%) (Figure 3). The IL-1 β levels in patients with active UC were significantly higher than those of patients with the inactive UC and normal control subjects ($p < 0.01$).

4. IL-6 PRODUCTION

Significantly more IL-6 was produced by LPMNC isolated from active UC mucosa, compared with LPMNC from inactive UC mucosa and normal control mucosa ($p < 0.01$), whether they were stimulated by PWM or not (Figure 4).

DISCUSSION

Although the etiology of UC is still unknown, some abnormalities have been detected by studies from the immunological point of view¹⁸. However, in order to understand the immunological abnormalities of UC, it is essential to elucidate the immune response mechanism of the large bowel which is the major site of the disease, as well as the systemic immune response mechanism. Recently, a shift in focus from systemic to intestinal immunity has been established, and the literature reflects this trend^{19,20}. To our knowledge, however, there are no

reports which describe the study of immunological abnormalities of the large bowel using specimens from untreated patients in their first attack of UC. Several studies have reported that steroid hormone and sulfasalazine significantly inhibit IL-1 or IL-6 release in a dose-dependent fashion in vitro^{21,22}, suggesting that we may be able to investigate a true immune response of the large bowel in patients with UC by using specimens from

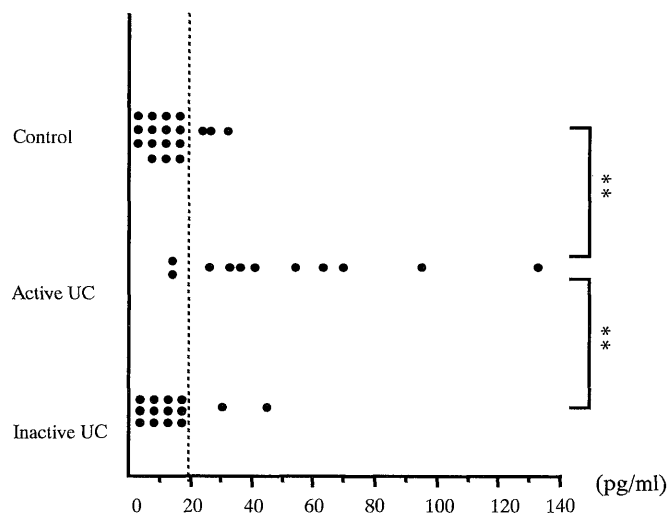


Figure 3. Interleukin 1- β (IL-1 β) production by lamina propria mononuclear cells (LPMNC) in the presence of LPS in untreated patients with active UC, patients with inactive UC and normal control subjects. Each point represents the level in a different individual. Dotted line indicates detection limit of the assay (20 pg/ml). The IL-1 β production by LPMNC in patients with active UC were significantly higher than those of patients with the inactive UC and normal control subjects (** $p < 0.01$).

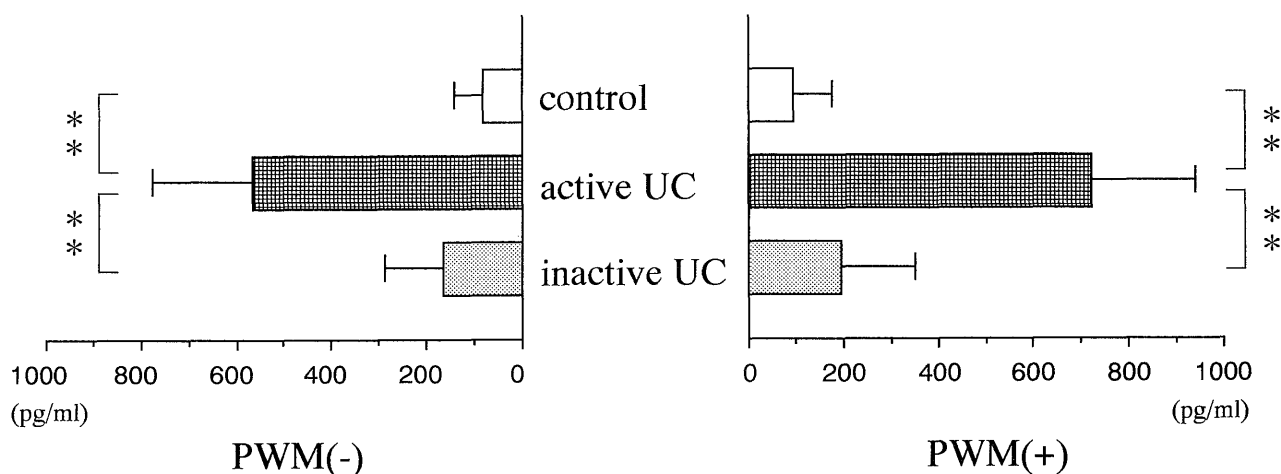


Figure 4. Interleukin 6 (IL-6) production by lamina propria mononuclear cells (LPMNC), either spontaneously or in the presence of PWM in patients with active UC (▨), patients with inactive UC (▤) and normal controls (□). There was significantly more IL-6 produced by LPMNC isolated from active UC mucosa, compared with LPMNC from inactive UC mucosa and control mucosa (** $p < 0.01$), whether they are stimulated by PWM or not.

untreated patients.

FACScan analysis of LPMNC in UC showed that the percentage of CD4⁺ (helper/inducer T) cells and CD20⁺ (B) cells was significantly higher in patients with active UC than in control patients, and that the percentage of CD8⁺ (suppressor/cytotoxic T) cells was significantly lower. These results suggest an appropriate environment for the production of immunoglobulin in the lamina propria in patients with active UC. The proportion of activated T cells defined by HLA-DR or CD25 (IL-2 Receptor) was higher in patients with active UC than in control patients. Though there was no significant difference in the proportion of macrophages present in LPMNC between UC patients and normal controls, macrophages from patients with active UC are more activated²³⁾. Thus, activated macrophages and T cells contribute to produce a wide range of cytokines (IL-1, IL-2, IL-4, IL-5, IL-6, tumor necrosis factor- α , interferon- α , γ) and activate the cytokine cascade²⁴⁾.

It was found in this study that the concentrations of IL-1 β and IL-6 in lamina propria mononuclear cells were significantly increased only in patients with active UC. IL-1 is a polypeptide cytokine produced by various cells²⁵⁾ and has been shown to be a mediator of a number of inflammatory and immunological responses^{26, 27)}. Though there is no difference in the population of macrophages between the three groups (Figure 1), the amount of IL-1 in LPMNC in patients with active UC was significantly higher. These results suggest that macrophages from patients with active UC individually have a greater potential to produce IL-1 β than do macrophages from normal controls and patients with inactive UC. Enhanced production of IL-1 β is likely to be the result of enhanced production of the cytokine on a per cell basis. Mahida et al.⁶⁾ have described that enhanced production of IL-1 β is caused by the presence of activated macrophages. Thus, the increased IL-1 β level in patients with active UC facilitates the initiation of a T cell response and orchestrates a wide spectrum of inflammatory activities pertinent to UC.

As IL-6 has been shown to promote terminal differentiation B cells, induction of immunoglobulin secretion¹³⁾ and to have an accessory role in T cell activation and proliferation²⁾, IL-6 may be involved in a wide range of immunological abnormalities observed in UC^{3, 28)}. Our study shows that the amount of IL-6 produced by LPMNC isolated from active UC is higher than those of inactive UC and normal control subjects. There are several possible explanations for the enhanced production of IL-6 in patients with active UC.

Firstly IL-6 can be produced by a variety of cells, but when inflammation occurs macrophages, endothelial cells and fibroblasts are predominant sources²⁾. Though there was no significant difference in the proportion of macrophages between three groups, Choy et al.²³⁾ reported that activated macrophage had been found in patients with

active inflammatory bowel disease. Therefore, it is possible that enhanced production of IL-6 could be due to enhanced synthesis of this cytokine by activated macrophages.

Secondly it has been shown that IL-1 is a potent inducer of IL-6 synthesis²⁹⁾. LPMNC failed to respond to PWM with increased IL-6 production. One possible explanation is that the mucosal B cells are already activated and can not respond further to stimulation with mitogens. It is also possible that suppressor cells may contribute to the lack of response³⁰⁾. The high amount of IL-1 may contribute to the increased IL-6 production. The report of Stevens et al.³¹⁾, who described increased mRNA for IL-6 in mucosa from active UC compared with inactive UC or normal mucosa, support our findings. IL-6 has been implicated in the pathogenesis of autoimmune disease, for example rheumatoid arthritis³²⁾, proliferative glomerulonephritis³³⁾, and cardiac myxoma³⁴⁾. Thus, the abnormality of IL-6 production may contribute to the abnormality of immunoglobulin production system in patients with UC.

In conclusion, the synthesis of IL-1 β and IL-6 by LPMNC in UC is increased, but whether these findings are a primary or secondary phenomenon remains unclear. Nevertheless IL-1 β and IL-6 mediate immune responses, may be associated with the etiopathogenesis of UC.

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