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Intracellular Cytokine Patterns of Peripheral Blood T Cells as a Useful Indicator of Activeness of Crohn's Disease

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ABSTRACT

Recently, the alteration of peripheral T cells has become a focus of attention in research on Crohn's disease (CD). To examine the characteristics of peripheral T cells in CD patients, we analyzed the expression of a memory T cell marker ($CD45RO^{Bright}CD3+$) and the cytokine production by peripheral helper and cytotoxic T cells in patients with CD. With the use of monensin to prevent the secretion of cytokines under stimulation, we measured the count of intracellular cytokine-positive cells for production of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-4, IL-6, IL-10, and granulocyte-macrophage colony stimulating factor (GM-CSF) in the peripheral T cell population using flow-cytometry. The counts of lymphocytes, T cells, and helper T cells in patients with CD were significantly lower than in normal volunteers. Although no difference in the counts of lymphocytes, total T cells, helper and cytotoxic T cells was observed, the counts of intracellular cytokine producing helper T cells in IFN-γ, TNF-α or GM-CSF were significantly higher in active cases than in quiescent cases. These results suggest that stable CD patients are immunosuppressive, and activation of some kinds of T-cells, especially Th1-associated cytokine producing T-cells, correlate with disease progression. Th1-associated cytokine analysis of peripheral T cells may be one of the useful markers to evaluate the activeness of Crohn's disease.

Key words: *Crohn's disease, Cytokine, CD45RO, Helper T cells, Peripheral blood*

The cause of Crohn's disease (CD) is now understood to be dysregulation of mucosal immune responses against enteric microflora, to which some patients have genetic susceptibility[10,28)], and abnormality of barrier function (intestinal permeability, etc). The initiation and perpetuation of intestinal inflammation in CD might be due to the aberrant secretion of pro- and anti-inflammatory cytokines from various intestinal immune cells[4,9,29,31)] forming gut-associated lymphoid tissue (GALT). It has been demonstrated in the past decade that the development of chronic inflammation is associated with pro-inflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, which are produced in the intestinal mucosa and lamina propria[5,6,26,27,30)]. Interleukin 10 (IL-10), which has an anti-inflammatory effect on immune cells, is also associated with severity of intestinal inflammation[18)]. We recently found that IFN-γ, TNF-α, and IL-10 were overexpressed in the inflamed intestinal tissues of CD patients[1)], indicating that dysregulation of cytokines produced from local immune cells plays a role in the pathogenesis of CD. These findings suggest that any new therapeutic strategy for CD requires the monitoring of mucosal immune status.

As laboratory parameters to easily assess the immunological status of CD, we investigated peripheral T cells and their cytokine production. Peripheral T cells may be affected by persistent intestinal inflammation in CD since they are believed to re-circulate into the peripheral blood from GALT[8,13,19,21)]. In fact, a recent experimental study using a spontaneous CD mouse model revealed that T cells are an essential cell component in the development of intestinal inflamma-

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tion[17]. Conventional investigations of peripheral blood lymphocytes in CD have shown that the counts of PBL (peripheral blood lymphocytes) and helper T cell subsets were reduced in active CD cases[35]. Fuss et al. reported that high IFN-γ production and low IL-2 production was detected at protein levels in CD4+ T cells derived from peripheral blood in patients with CD, when stimulated via the CD2/CD28[9]. Their results suggest that alterations of peripheral blood T-cell counts and functions, especially cytokine production, exist in CD.

In this study, we selected 7 cytokines for examination: IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and granulocyte-macrophage colony stimulating factor (GM-CSF). IFN-γ and TNF-α production by immune cells plays a major role in the consecutive episodes of inflammation in CD[22]. IFN-γ in particular activates macrophage/dendritic cells to express MHC class-II molecules[33], to release TNF-α and IL-6, and to produce IL-12[14]. Such activation of IFN-γ is considered to stimulate the macrophage-dendritic cell system toward pro-inflammatory responses[23,24], which may exacerbate lesions of the gut in CD. IL-2 is a well-known cytokine related to cell proliferation and interactions between several kinds of immune cells. IL-4 and IL-10 are known as counterpart cytokines against IFN-γ[24,25]. Administration of IL-6 receptor antagonist in immunized colitis mice resulted in protection against mucosal inflammation[11,37]. GM-CSF, a haematopoietic factor enhancing the qualitative function of mature neutrophils, monocytes, and macrophages, and stimulating the expansion and differentiation of haematopoietic progenitors, induces not only the priming of neutrophils to produce superoxide anions, but also the activation of macrophages to release superoxide anions, TNF-α, and IL-6. Moreover, GM-CSF accelerates the differentiation of monocyte-derived immature dendritic cells in cooperation with IL4[34]. Recently human recombinant GM-CSF was used as an immuno-modulatory agent for Crohn's disease[7,16]. Thus, in this study, we analyzed the number of memory T cells, the amounts of IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and GM-CSF production by stimulated peripheral helper T cells via the recently developed flow-cytometric intracellular cytokine staining technique.

METHODS

Patients and Specimens

Twenty-nine patients with CD (24 males and 5 females, age at surgery 22–46 years old; average, 31.1 years old) and 11 healthy volunteers (10 males and 1 female, age 25–43 years old; average, 32.6 years old) were enrolled in this study. All patients had been medicated by physicians and admitted for surgical treatment. In this study, we divided these cases into two groups: 13 quiescent cases with Crohn's disease activity index[3,15] (CDAI) $\leq$ 150 and 16 active cases with CDAI > 150. Informed consent was obtained from all patients, and this study was approved by the Human Ethics Committee of Hiroshima University. Four patients were administered steroid treatment and only one patient received mercaptopurine (6-MP) treatment. The remaining patients received no immuno-suppressive agents (such as steroid or 6-MP). The majority of our patients had nutritional support with total parenteral nutrition and/or elemental diet and was medicated with 5-ASA (5-aminosalicylic acid).

Antibodies

We used fluorochrome-conjugated monoclonal antibodies specific for each cell surface antigen: anti-CD3 (Immunotec A Coulter Company, Marseille, France), and anti-CD8 and anti-CD45RO antibodies (Immunotec A Coulter Company, Marseille, France). For the staining of intracellular cytokines, the directly conjugated anti-cytokine monoclonal antibodies were as follows: anti-IFN-γ, anti-TNF-α, anti-IL2, anti-IL4, anti-IL10, anti-GM-CSF (BD Biosciences PharMingen, Franklin Lakes NJ, USA), and anti-IL6 (Bio-source International, London, UK).

Cell culture

Blood samples were drawn into preservative-free heparin tubes (heparin 50 I.U./ml), and used within two hours after venipuncture. These samples were diluted 1:1 with Hank's balanced solution (HBSS) (Invitrogen, Carlsbad, California, USA) and mononuclear cells were isolated by gradient centrifugation with Ficoll-Paque solution (Amersham Pharmacia Biotec, Uppsala, Sweden). The isolated cells were then prepared as 2.0×10^6 cells/ml in RPMI medium with 10% FCS (Invitrogen), and added to flat-bottom culture plates (Sumitomo Bakelite, Tokyo, Japan). The cells were then stimulated with 5 ng/ml phorbol 12-myristate 13-acetate (Sigma, St Louis, Missouri, USA) and 1 μM ionomycin (Sigma), with 2 μM monensin (Sigma) at 37°C in a humid 6% CO_2 atmosphere for six hours.

Cell staining

After the cells were harvested and washed once with HBSS, the pellet was re-suspended with 50

Abbreviations used:
GALT: Gut-associated lymphoid tissue, HBSS: Hank's balanced salt solution, FCS: fetal calf serum, PMA: phorbol 12-myristate 13-acetate, PBS: phosphate-buffered saline, IFN-γ: interferon gamma, TNF: tumor necrosis factor, Th2: Type 2 helper T cell, DC: dendritic cell, CD: Crohn's disease.

μl HBSS. Before cell fixation, monoclonal antibodies against surface antigen were added to the cells for 15 min in the dark at room temperature. The cells were fixed using a commercial fixation kit (IntraPrep reagent I, Immunotech A Coulter Company, Marseille, France) for 15 min, and then washed twice with PBS containing 0.1% NaN_3. For permeabilization of cell membranes, a commercial permeabilization kit reagent (IntraPrep reagent II) was added to each tube, and then the cells were incubated for 5 min at room temperature. Without washing, an aliquot of the sample was added to a FACS tube (Falcon Becton Dickinson, Oxford, UK), and was then mixed with anti-cytokine antibodies for 30 min in the dark for staining of intracellular cytokines. Finally, the cells were washed with PBS and fixed again with 0.5% paraformaldehyde.

Flow cytometric analysis for cytokine production of T cells

Prepared samples were analyzed with a multicolor flow cytometer (Coulter EPICS XL, Beckman Coulter, Fullerton, CA, USA) and System II Software (Beckman Coulter). Initially, we gated lymphocytes with forward and side scatter, and discriminated the gated lymphocytes as CD3+CD8- T cell subset and CD3+CD8+ T cell subset. Although the surface antigen CD3+CD4+ is commonly used as a marker for helper T cells, the level of expression of surface antigen CD4 is known to be remarkably down-regulated after increasing culture time (from 2 hours) during stimulation with phorbol 12-myristate 13-acetate /ionomycin[12]. Thus, we defined CD3+CD8- T cells as helper T cells in this study. We then measured the proportion of each cytokine-producing cell in this subset. For the reason given above, we measured CD3+CD8-/CD3+CD8+ as CD4/CD8 ratio in this study. The cytokine-positive cells were identified on the dot plot graph by delineating regions based on stimulated cells treated with isotype-matched control-antibodies. In addition, in each subset, we measured the positive proportion of surface antigen $CD45RO^{Bright}$, which is known to be a memory cell marker.

The kinetics of IFN-γ, IL-2, and IL-4 production by helper T cells have been well-documented[12], but those of GM-CSF by peripheral helper T cells have not been investigated by flow cytometric analysis. We investigated the kinetics of GM-CSF production in our assay using T cells from 5 healthy adult volunteers. In the helper T cells (CD3+CD8-) from the 5 volunteers, GM-CSF positive cells were detected at 2 hours after stimulation, and gradually increased in number until 4 hrs, sustaining that level until 10 hours (2 hr: 2.0 ± 0.94%, 4 hr: 4.9 ± 1.35%, 6 hr: 5.3 ± 1.55%, 10 hr; 5.8 ± 1.51%, as mean values (n = 5, each case)). In a subsequent study, we set the optimal culture time at 6 hours after stimulation.

Statistical analysis

All values are expressed as mean ± SD. Mann-Whitney's U test was used for comparison of cytokine-producing cells, $CD45RO^{Bright}$ cells, and CD4/CD8 ratio among active and quiescent CD patients, and healthy controls. Two-tailed p values < 0.05 were considered significant.

RESULTS

Clinical parameters

The laboratory parameters of the patients were as follows: mean WBC was 5,866/mm^3 (2,700–10,730) (neutrophils 66.4%, lymphocytes 21.5%, monocytes 7.6%), mean level of serum C-reactive protein (CRP) was 0.88 mg/dl (serum CRP levels of 14 patients were < 0.3 mg/dl, when we calculated CRP < 0.3 mg /dl as CRP = 0.3 mg/dl). Although there were no differences in white blood cell counts between the patients (5865.5 ± 1813.1) and healthy volunteers (5232.3 ± 843.8), the lymphocyte count in patients was significantly lower than that in healthy volunteers (CD: 1192.5 ± 410.7 /mm^3, HV: 2152.9 ± 779.2 /mm^3, $p < 0.0001$). The counts of T cells, helper T cells and cytotoxic T cells in the patients were also significantly lower than those in healthy volunteers (CD: 860.7 ± 301.2 /mm^3, HV: 1653.5 ± 644.2 /mm^3, $p = 0.0002$, CD: 546.5 ± 241.5 /mm^3, HV: 1000.3 ± 441.6 /mm^3, $p = 0.0002$, CD: 314.2 ± 121.6 /mm^3, HV: 579.0 ± 274.2 /mm^3, $p = 0.0013$, each group). Among the CD patients, both active and quiescent cases had low counts of lymphocytes (quiescent CD: 1267.7 ± 424.8 /mm^3,

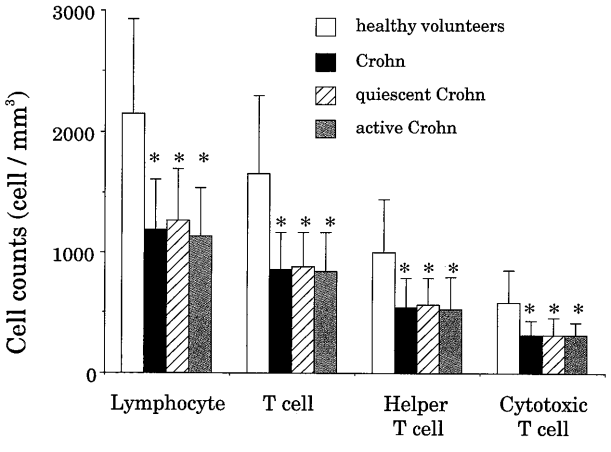

Fig. 1. The counts of lymphocytes, T cells (CD3+), helper T cells (CD3+CD8-) in healthy volunteers, patients with CD, patients with quiescent CD and patients with active CD. Lymphocytes, T cells, helper T cells and cytotoxic T cells were significantly lower in patients with CD, in patients with quiescent CD and in patients with active CD than in healthy volunteers. Each graph denotes cell numbers, mean ± standard deviation. *$p < 0.05$ compared with healthy volunteers.

active CD: 1131.4 ± 402.0 /mm^3), T cells (quiescent CD: 884.6 ± 227.0 /mm^3, active CD: 841.4 ± 327.3 /mm^3), helper T cells (quiescent CD: 569.2 ± 218.2 /mm^3, active CD: 528.0 ± 264.6 /mm^3) and cytotoxic T cells (quiescent CD: 315.4 ± 144.3 /mm^3, active CD: 313.3 ± 104.6 /mm^3). There were no significant differences in WBC, lymphocytes, T cells, helper T cells or cytotoxic T cells between active and quiescent cases (p = 0.2451, p = 0.4560, p = 0.6295, p = 0.4299, p = 0.8608, respectively) (Fig. 1). There were no significant differences in CD4/CD8 ratio between HV and quiescent cases (HV: 2.003, quiescent CD: 2.079, p = 0.9769), between HV and active cases (HV: 2.003, active CD: 1.724, p = 0.2363), and between quiescent and active cases (quiescent CD: 2.079, active CD: 1.724, p = 0.2729), respectively.

Peripheral memory T cells, memory helper T cells and memory cytotoxic T cells

In the population of T cells, memory T cells (CD45RO^{Bright}CD3+) were lower in the patients (n = 28, 238.9 ± 115.0 /mm^3) than in the healthy volunteers (n = 11, 365.8 ± 152.3 /mm^3, p = 0.0209). Among the patients, memory T cells were higher in active cases (n = 15, 278.1 ± 110.0) than in quiescent cases (n = 13, 193.6 ± 107.3), but not significantly (p = 0.0841).

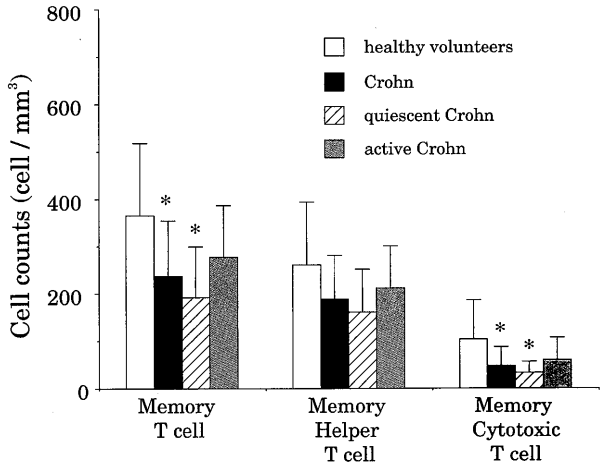

Fig. 2. The counts of memory T cells (CD3+CD45RO^{Bright}), memory helper T cells (CD3+CD8-CD45RO^{Bright}), memory cytotoxic T cells (CD3+CD8+CD45RO^{Bright}) in healthy volunteers, patients with CD, patients with quiescent CD and patients with active CD. Memory T cells, memory helper T cells, memory cytotoxic T cells are relatively lower in patients with CD, quiescent CD and active CD than in healthy volunteers. In a comparison between active and quiescent cases, the counts of memory T cells, memory helper T cells and memory cytotoxic T cells are relatively higher in patients with active CD than with quiescent CD, but not significantly (p = 0.0841, p = 0.1479, p = 01468). Each graph denotes cell numbers, mean ± standard deviation. *p < 0.05 compared with healthy volunteers.

Memory helper T cells (CD3+CD8-CD45RO^{Bright}) were also lower in the patients (n = 28, 189.5 ± 92.5 /mm^3) than in the healthy volunteers (n = 11, 262.6 ± 131.6 /mm^3), but not significantly (p = 0.1051). Memory cytotoxic T cells (CD3+CD8+ CD45RO^{Bright}) were significantly lower in patients (n = 28, 47.1 ± 39.8 /mm^3) than in healthy volunteers (n = 11, 103.2 ± 84.3 /mm^3, p = 0.0192). Among the patients, memory helper and cytotoxic T cells were higher in active cases than in quiescent cases, but again not to a significant extent (Fig. 2).

Cytokine-producing cells in the peripheral helper T cell (CD3+CD8-) subset in patients with Crohn's disease and healthy volunteers

In the peripheral helper T subset, IFN-γ-producing helper T cells in patients with CD were significantly lower than in healthy volunteers (p = 0.0260). TNF-α-producing cells and IL-2-producing cells in the peripheral helper T cell subset were significantly lower in patients with CD than in healthy volunteers (TNF-α; p = 0.0002, IL-2; p < 0.0001). Moreover, counts of each cytokine-producing cell, for IFN-γ, TNF-α and IL-2, among the peripheral helper T cells of quiescent cases were significantly lower than in healthy volunteers (IFN-γ; p = 0.0034, TNF-α; p = 0.0002, IL-2; p = 0.0001), while TNF-α producing cells and IL-2-producing cells in the peripheral helper T cell subset

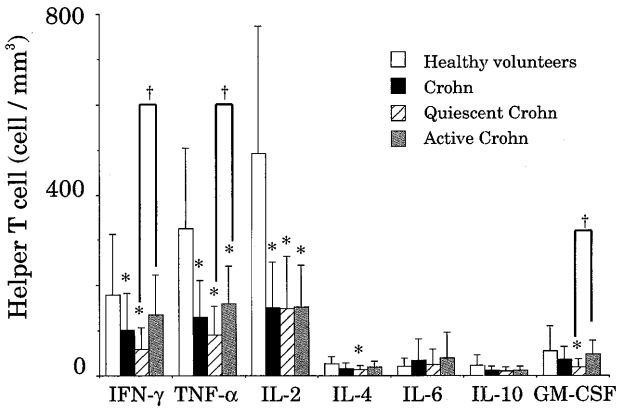

Fig. 3. The counts of cytokine producing helper T cells in healthy volunteers, patients with CD, patients with quiescent CD, and patients with active CD. Evaluated cytokine producing cells, except for IL-6, were lower in patients with CD, patients with quiescent CD and patients with active CD than in healthy volunteers. IFN-γ producing cells, TNF-α producing cells and GM-CSF producing cells were significantly higher in active cases than t in quiescent cases in the peripheral helper T cell subset (p = 0.0097, p = 0.0203, p = 0.0118, respectively). Each graph denotes cytokine producing helper T cells, mean ± standard deviation. *p < 0.05 compared with healthy volunteers. †p < 0.05 compared between quiescent CD and active CD.

of active cases were also significantly lower than in healthy volunteers (TNF-α; $p = 0.0026$, IL-2; $p < 0.0001$). However, the counts of IFN-γ-producing cells did not differ significantly from those in healthy volunteers (IFN-γ; $p = 0.2564$). The counts of IL-4-producing cells in quiescent cases were significantly lower than in healthy volunteers ($p = 0.0456$), but those in active cases were not ($p = 0.2363$). IL-10-producing cells in CD patients, active and quiescent cases tended to be lower than in healthy volunteers, but not to a significant extent ($p = 0.0662$, $p = 0.1077$, $p = 0.1124$, respectively). Concerning IL-6, few cases had large numbers of IL-6-producing cells, so data varied widely ($p = 0.9853$). GM-CSF-producing cells in quiescent cases were significantly lower than in healthy volunteers ($p = 0.0328$), while in active cases were not ($p = 0.8559$) (Fig. 3).

Cytokine-producing cells in the peripheral helper T cell (CD3+CD8-) subset in active and quiescent Crohn's disease

IFN-γ-, TNF-α- and GM-CSF-producing cells in the peripheral helper T subset were significantly higher in active cases than in quiescent cases ($p = 0.0097$, $p = 0.0203$, and $p = 0.0118$). In the production of other cytokines such as IL-2, IL-4, IL-6 and IL-10, there were no significant differences between active and quiescent cases (Fig. 3).

Cytokine-producing cells in the peripheral cytotoxic T cell (CD3+CD8+) subset in patients with Crohn's disease and healthy volunteers

IFN-γ-producing cells, TNF-α-producing cells and IL-2-producing cells in the peripheral cytotoxic T subset of patients were significantly lower than in healthy volunteers ($p = 0.0222$, $p = 0.0020$, and $p = 0.0004$, respectively). IFN-γ-producing cells, TNF-α-producing cells and IL-2-producing cells in the peripheral cytotoxic T subset of quiescent cases were also significantly lower than in healthy volunteers ($p = 0.0013$, $p = 0.0017$, and $p = 0.0009$, respectively). On the other hand, TNF-α- and IL-2-producing T cells in the peripheral cytotoxic T subset of active cases were significantly lower than in healthy volunteers ($p = 0.0179$, $p = 0.0031$), but counts of IFN-γ-producing cells were not ($p = 0.3237$). IL-4- and IL-10-producing cells tended to be lower in all CD patients, active cases and quiescent cases than in healthy volunteers (IL-4: $p = 0.0764$, $p = 0.0680$, $p = 0.1827$. IL-10: $p = 0.4397$, $p = 0.3088$, $p = 0.7016$, respectively), but not to a significant extent. The counts of IL-6-producing cells in quiescent cases were almost the same as in healthy volunteers. GM-CSF-producing cells in the group of all CD patients and quiescent cases were lower than in healthy volunteers ($p = 0.0283$, $p = 0.0078$) but those in active cases were not significantly lower ($p = 0.1857$) (Fig. 4).

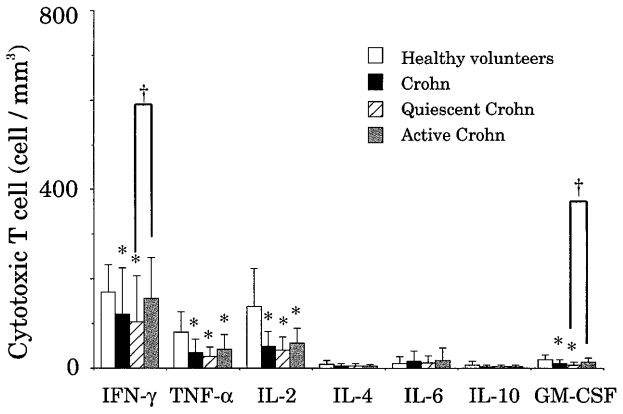

Fig. 4. The counts of cytokine producing cytotoxic T cells in healthy volunteers, patients with CD, patients with quiescent CD, and patients with active CD. Evaluated cytokine producing cells, except for IL-6, were lower in patients with CD, patients with quiescent CD and patients with active CD than in healthy volunteers. IFN-γ producing cells and GM-CSF producing cells were significantly higher in active cases than in quiescent cases in the peripheral helper T cell subset ($p = 0.0033$, $p = 0.0240$, respectively). Each graph denotes cytokine producing cytotoxic T cells, mean ± standard deviation. *$p < 0.05$ compared with healthy volunteers. †$p < 0.05$ compared between quiescent CD and active CD.

Cytokine-producing cells in the peripheral cytotoxic T cell (CD3+CD8+) subset in active and quiescent Crohn's disease

The counts of IFN-γ-producing cells and GM-CSF-producing cells in the peripheral cytotoxic T cell subset were significantly higher in active than in quiescent cases ($p = 0.0033$, $p = 0.0240$). In the production of other cytokines such as TNF-α, IL-2, IL-4, IL-6 and IL-10, there were no significant differences between active and quiescent cases (TNF-α; $p = 0.1374$, IL-2; $p = 0.2173$, IL-4; $p = 0.5393$, IL-6; $p = 0.8501$, IL-10; $p = 0.3520$, Fig. 4).

DISCUSSION

In our analysis of peripheral blood cells obtained from CD patients, the counts of lymphocytes, T cells, helper T cells, and cytotoxic T cells in the patients were significantly lower than those in healthy volunteers. These parameters exhibited no significant differences between quiescent and active cases. Since the CD patients we analyzed were stable after medication, stable status in CD patients might be comparative to the immunosuppressive state in lymphocytes in peripheral blood.

An analysis using memory cell marker CD45RO, one of the cell activating markers, was also reduced in the PBL (peripheral blood lymphocyte) of the patients. The suppression of memory T cell numbers in patients would also indicate a stable status. Neither the counts nor the subsets (T cells, helper T cells, cytotoxic T cells) of PBL could dis-

tinguish between active and quiescent cases. The memory cell marker CD45RO (Fig. 2), however, increased in active cases, indicating the presence of some immune reaction, and that memory T cells might be reactivated by some antigens in active cases. Thus, the increase of the memory T cell population in CD patients might indicate an exacerbation of the diseased status of Crohn's disease concomitant with the activation of local inflammation. To elucidate this hypothesis, we tried to discriminate between active cases and quiescent cases by measuring the cytokine production of helper T and cytotoxic T cells.

Helper T cells are believed to play a central role in amplifying immune reactions together with antigen-presenting cells[20]. Cytokine-producing T cells existed mainly in the helper T cell subset. In the present study, the counts of cytokine-producing cells among stimulated helper T cells were two- to three-fold of those among cytotoxic T cells, except for IFN-γ. As shown in Fig. 3, the counts of all cytokines except IL-6 were lower in CD patients than in healthy volunteers, also suggesting the immunosuppressive status of helper and cytotoxic T cells. Interestingly, comparing quiescent and active cases, Th1 cytokine (pro-inflammatory cytokine) -producing helper T cells, including IFN-γ-producing and TNF-α-producing cells, were significantly increased in active CD cases. GM-CSF-producing cells were also increased in active cases. On the other hand, concerning Th2 cytokines (anti-inflammatory cytokines), IL-4-producing and IL-10-producing cells did not significantly differ in number between active and quiescent cases. These findings suggest that patients with an exacerbation of Crohn's disease exhibit an increase of Th1-associated cytokines in the peripheral T cells, which might reflect a cytokine imbalance in the intestine. Neither IL-2- nor IL-10-producing peripheral helper T cells were related to exacerbation, indicating that these helper T cells do not reflect a disease state in CD.

Cytotoxic T cells usually attack target cells, and some portion of cytotoxic T cells possess helper functions. Although the counts of cytokine-producing cytotoxic T cells were lower, there was a similar tendency to helper T cells in cytokine production. Comparing quiescent and active cases, Th1-associated cytokine-producing cytotoxic T cells, such as those producing IFN-γ or GM-CSF, significantly increased with disease status. On the other hand, in the case of Th2 cytokines (anti-inflammatory cytokines), IL-4- and IL-10-producing cytotoxic T cells were unrelated to disease status in CD. Thus, a cytokine imbalance also occurred in peripheral cytotoxic T cells in patients with CD.

Pro-inflammatory cytokine-producing cells increased in number in the peripheral blood in active CD, but IL-4 or IL-10 production of helper and cytotoxic T cells did not. This suggests that pro-inflammatory cytokines work under the suppression of the anti-inflammatory cytokines in active CD patients and that production of pro-inflammatory cytokines might be one of the deteriorating factors in CD. Low counts of IL-2-producing cells among peripheral helper and cytotoxic T cells were unrelated to the disease state in CD. Since IL-2 promotes T cell proliferation, suppression of IL-2-producing T cells might contribute to maintaining immunosuppression in CD.

Analyses of peripheral blood, peripheral memory T cells and peripheral cytokine production by T cells revealed that patients with post-medicated CD were in an immunosuppressive state and maintained an immunological equilibrium with low antigen —reactivity and low cytokine production. When the quiescent phase turns into the active phase with a challenge by antigen stimulation, peripheral T cells begin to exhibit a cytokine imbalance, especially the promotion of pro-inflammatory cytokines.

Recently, cytokine targeting therapies using anti-TNF-α antibody[2,36], IL-6 receptor antagonist, recombinant human IL-10 and recombinant human GM-CSF have focused on their effectiveness for CD, but such therapies are not always effective for CD patients. For example, anti-TNF-α antibody targets cases that are intractable for steroid therapies, but was effective for only 60% of challenged cases[2,36]. Such therapies as anti-TNF-α antibody and IL-6 receptor antagonist administration may inactivate the overproduced cytokines in active CD patients. Meanwhile, Dieckgrafe et al. have reported the effectiveness of recombinant human GM-CSF (Sargramostim) as a new drug for CD[7,16] The effectiveness of this therapy seems to controvert GM-CSF overproduction of helper T-cells in active CD. This controversy may be explained as follows: intracellular GM-CSF activates the Th1-associated cytokines in helper T cells, so that the administration of recombinant human GM-CSF (rhGM-CSF) might suppress the GM-CSF intracellular production by a negative feedback reaction. On the other hand, rhGM-CSF might improve patients by mechanisms other than the activation of Th1 cells.

In this study, we have shown the relationship between disease status and peripheral cytokine production by T cells. If we could analyze cytokine production by peripheral helper T cells before treatment with cytokine-targeting drugs of patients with CD, we might be able to evaluate the effectiveness of each drug prior to medication. For example, anti-TNF-α antibody could be effective for patients with large numbers of TNF-α-producing peripheral helper T cells, or human recombinant GM-CSF for patients with high or low numbers of GM-CSF-producing peripheral helper T cells. Thus, estimation of the effective-

ness of each cytokine-targeting drug will contribute to the design of tailor-made therapies in the near future. Toward this end, evaluation of the relationship between cytokine production by peripheral T cells and local inflammation in the intestine is needed. In addition, to analyze the intracellular or intercellular cytokine network, we should examine all cytokine producing cells in memory helper T cells in Crohn's disease in future.

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REFERENCES

1. **Akagi, S., Hiyama, E., Imamura, Y., Takesue, Y., Matsuura, Y. and Yokoyama, T.** 2000. Interleukin-10 expression in intestine of Crohn disease. Int. J. Mol. Med. **5:** 389–395.
2. **Asakura, H., Yao, T., Matsui, T., Koganei, K., Fukushima, T., Takazoe, M., Hobara, R., Nakano, H., Okamura, S., Matsueda, K., Kashida, H., Makiyama, K., Hiwatashi, N., Kashiwagi, K. and Hibi, T.** 2001. Efficacy of treatment with chimeric monoclonal antibody (Infliximab) to tumor necrosis factor-alpha for Crohn's disease in Japan: evaluation by rapid turnover proteins, and radiologic and endoscopic findings. J. Gastroenterol. Hepatol. **16:** 763–769.
3. **Best, W.R., Becktel, J.M. and Singleton, J.W.** 1976. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. Gastroenterology **70:** 439–444.
4. **Breese, E. and MacDonald, T.T.** 1995. TNF alpha secreting cells in normal and diseased human intestine. Adv. Exp. Med. Biol. **4:** 371B821–824.
5. **Breese, E.J., Michie, C.A., Nicholls, S.W., Murch, S.H., Williams, C.B., Domizio, P., Walker-Smith, J.A. and MacDonald, T.T.** 1994. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. Gastroenterology **106:** 1455–1466.
6. **Camoglio, L., Te Velde, A.A., Tigges, A.J., Das, P.K. and Van Deventer, S.J.** 1998. Altered expression of interferon-gamma and interleukin-4 in inflammatory bowel disease. Inflamm. Bowel. Dis. **4:** 285–290.
7. **Dieckgraefe, B.K. and Korzenik, J.R.** 2002. Treatment of active Crohn's disease with recombinant human granulocyte-macrophage colony-stimulating factor. Lancet **360:** 1478–1480.
8. **Fiocci, C.** 1998. Inflammatory bowel disease: Etiology and pathogenesis. Gastroenterology **115:** 182–205.
9. **Fuss, I.J., Neurath, M., Boirivant, M., Klein, J.S., de la Motte, C., Strong, S.A., Fiocchi, C. and Strober, W.** 1996. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J. Immunol. **157:** 1261–1270.
10. **Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M. and Thomas, G.** 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature **411:** 599–603.
11. **Ito, H., Hirotani, T., Yamamoto, M., Ogawa, H. and Kishimoto, T.** 2002. Anti-IL-6 receptor monoclonal antibody inhibits leukocyte recruitment and promotes T-cell apoptosis in a murine model of Crohn's disease. J. Gastroenterol. **37:** 56–61.
12. **Jason, J. and Larned, J.** 1997. Single-cell cytokine profiles in normal humans: comparison of flow cytometric reagents and stimulation protocols. J. Immunol. Methods **207:** 13–23.
13. **Jo, Y., Matsumoto, T., Yada, S., Fujisawa, K., Esaki, M., Onai, N., Matsushima, K. and Iida, M.** 2003. CCR4 is an up-regulated chemokine receptor of peripheral blood memory CD4+ T cells in Crohn's disease. Clin. Exp. Immunol. **132:** 332–338.
14. **Kakazu, T., Hara, J., Matsumoto, T., Nakamura, S., Oshitani, N., Arakawa, T., Kitano, A., Nakatani, K., Kinjo, F. and Kuroki, T.** 1999. Type 1 T-helper cell predominance in granulomas of Crohn's disease. Am. J. Gastroenterol. **94:** 2149–2155.
15. **Kirsner, J.B. and Shorter, R.G.** 1995. Inflammatory bowel disease, 4th ed. Williams & Wilkins, Baltimore.
16. **Korzenik, J.R. and Dieckgraefe, B.K.** 2000. Is Crohn's disease an immunodeficiency? Dig. Dis. Sci. **45:** 1121–1129.
17. **Kosiewicz, M.M., Nast, C.C., Krishnan, A., Rivera-Nieves, J., Moskaluk, C.A., Matsumoto, S., Kozaiwa, K. and Cominelli, F.** 2001. Th1-type responses mediate spontaneous ileitis in novel murine model of Crohn's disease. J. Clin. Inv. **107:** 695–702.
18. **Kucharzik, T., Stoll, R., Lugering, N. and Domschke, W.** 1995. Circulating antiinflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). Clin. Exp. Immunol. **100:** 452–456.
19. **Mackay, C.R., Andrew, D.P., Briskin, M., Ringler, D.J. and Butcher, E.C.** 1996. Phenotype, and migration properties of three major subsets of tissue homing T cells in sheep. Eur. J. Immunol. **26:** 2433–2439.
20. **Mayer, L.** 2000. IBD: Inflammatory Bowel Disease. Mount Sinai Journal of Medicine **67:** 208–213.
21. **Meenan, J., Spaans, J., Grool, T.A., Pals, S.T., Tytgat, G.N. and van Deventer, S.J.** 1997. Altered expression of alpha 4 beta 7, a gut homing integrin, by circulating and mucosal T cells in colonic mucosal inflammation. Gut **40:** 241–246.
22. **Monteleone, G., Biancone, L., Marasco, R., Morrone, G., Marasco, O., Luzza, F. and**

Pallone, F. 1997. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. Gastroenterology **112:** 1169–1178.

23. **Moser, M. and Murphy, K.M.** 2000. Dendritic cell regulation of TH1-TH2 development. Nature Immunology **1:** 119–205.
24. **Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L.** 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. **136:** 2348–2357.
25. **Mosmann, T.R. and Moore, K.W.** 1991. The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol. Today **12:** A49–53.
26. **Niessner, M. and Volk, B.A.** 1995. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). Clin. Exp. Immunol. **101:** 428–435.
27. **Noguchi, M., Hiwatashi, N., Liu, Z. and Toyota, T.** 1998. Secretion imbalance between tumour necrosis factor and its inhibitor in inflammatory bowel disease. Gut **43:** 203–209.
28. **Ogura, Y., Inohara, N., Benito, A., Chen, F.F., Yamaoka, S. and Nunez, G.** 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J. Biol. Chem. **276:** 4812–4818.
29. **Parronchi, P., Romagnani, P., Annunziato, F., Sampognaro, S., Becchio, A., Giannarini, L., Maggi, E., Pupilli, C., Tonelli, F. and Romagnani, S.** 1997. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. Am. J. Pathol. **150:** 823–832.
30. **Plevy, S.E., Landers, C.J., Prehn, J., Carramanzana, N.M., Deem, R.L., Shealy, D. and Targan, S.R.** 1997. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. J. Immunol. **159:** 6276–6282.
31. **Romagnani, P., Annunziato, F., Baccari, M.C. and Parronchi, P.** 1997. T cells and cytokines in Crohn's disease. Curr. Opin. Immunol. **9:** 793–799.
32. **Roman, L.I., Manzano, L., De La Hera, A., Abreu, L., Rossi, I. and Alvarez-Mon, M.** 1996. Expanded CD4+CD45RO+ phenotype and defective proliferative response in T lymphocytes from patients with Crohn's disease. Gastroenterology **110:** 1008–1019.
33. **Ruemmele, F.M., Dionne, S., Levy, E. and Seidman, E.G.** 1999. Dexamethasone inhibits IFN gamma-induced MHC class II expression of intestinal epithelial cells independently of the TGF-beta1 regulatory pathway. Aliment. Pharmacol. Ther. **13:** 595–601.
34. **Sallusto, F. and Lanzavecchia, A.** 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. **179:** 1109–1118.
35. **Selby, W.S. and Jewell, D.P.** 1983. T lymphocyte subsets in inflammatory bowel disease:peripheral blood. Gut **24:** 99–105.
36. **Targan, S.R., Hanauer, S.B., van Deventer, S.J.H., Mayer, L., Present. D.H., Braakman, T., DeWoody, K.L., Schaible, T.F. and Rutgeerts, P.J.** 1997. A short term study of chimeric monoclonal antibody cA2 to tumor necrosis factor for Crohn's disease. New Eng. J. Med. **337:** 1029–1035.
37. **Yamamoto, M., Yoshizaki, K., Kishimoto, T. and Ito, H.** 2000. IL-6 is required for the development of Th1 cell-mediated murine colitis. J. Immunol. **164:** 4878–4882.