Peripheral Blood Intracellular Cytokine Analysis in Children Newly Diagnosed with Inflammatory Bowel Disease

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ABSTRACT

Patterns of cytokine profiles have emerged for different forms of inflammatory bowel disease with a predominance of type 1 cytokines in patients with Crohn disease and type 2 cytokine expression in patients with ulcerative colitis. Most of these studies have involved older patients with long-standing disease or after various therapeutic interventions, and patterns of cytokine expression were hypothesized to be influenced by these factors. To evaluate for these possibilities, we studied 23 patients (15 boys) with newly diagnosed Crohn disease ($n = 14$) or ulcerative colitis. Their mean age at diagnosis was 13.1 ± 2.9 y (mean $\pm$ SD). Healthy control subjects ($n = 9$) were previously obtained. Peripheral blood intracellular cytokine analysis was performed within 24 h using a modification of Becton Dickinson's FastImmune Cytokine system. Multiparametric flow cytometry and phenotyping of lymphocytes was performed. T-cell populations were defined as type 1 being CD69⁺, CD3⁺, and interferon- γ ⁺ and type 2 being CD69⁺, CD3⁺, and IL-4⁺. The median percent of type 1 T cells from normal subjects (2.8%) was similar to that of ulcerative colitis subjects (1.8%, $p > 0.20$) but greater than that of Crohn disease subjects (0.55%, $p = 0.05$). The median percent of type 2 lymphocytes in normal subjects

(1.8%) was greater than that of ulcerative colitis subjects (0.35%, $p = 0.02$) but was similar to that of Crohn disease subjects (1.1%, $p > 0.20$). Serial determinations showed the median percent of type 2 T cells increased in ulcerative colitis patients as remission was induced. Reduced activated peripheral type 1 T cells of newly diagnosed, untreated children are similar to interferon- γ expression in mucosa of adults with postoperative recurrence. Reduced type 2 cytokine expression patterns in subjects with ulcerative colitis are similar to lamina propria T-cell expression levels in adults and improve with disease remission. (*Pediatr Res* 51: 328–332, 2002)

Abbreviations

CD, Crohn disease
UC, ulcerative colitis
Th, T helper
IFN- γ , interferon- γ
IBD, inflammatory bowel disease
PCDAI, pediatric Crohn disease activity index
FACS, fluorescence-activated cell sorter

It is generally accepted that there exists functionally polarized CD4⁺ class II MHC-restricted Th cell responses that develop under the influence of both environmental and genetic factors (1, 2). The classification is based on cytokine expression in which Th1-like cells are CD4⁺ T cells that produce IFN- γ but not IL-4, and Th2 or Th2-like cells are CD4⁺ T cells that express IL-4 but not IFN- γ (1). The cytokines produced by Th1 cells are involved with cell-mediated immunity and

phagocyte-dependent protective responses whereas Th2 cytokine expression facilitates antibody production, eosinophil activation, and phagocyte-independent protective responses (1). In addition to normal protective responses, aberrant responses of Th1 and Th2 cells are involved in the pathogenesis of a number of diseases including IBD (3). For instance, Th1 cytokine expression predominance in patients with CD has been reported (4–6) whereas increased Th2 cytokine expression profiles have been shown in UC (6–8).

Most of the studies have involved adult patients with long-standing or previously treated disease, both factors that might influence cytokine profiles. For instance, cytokine expression profiles for CD have been reported to be altered during differ-

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ent stages of CD, such as after resection (9). In addition, children can differ from adults in their ability to produce cytokines (*e.g.* IL-2, IFN- γ , IL-4) even after maximal stimulation (10). Thus in this study, we sought to evaluate cytokine profiles in circulating T lymphocytes from children newly diagnosed with IBD before the initiation of therapy to determine whether the cytokine profiles in this group of patients differed from adult reports.

METHODS

Patients. Twenty-seven consecutive patients were enrolled in the study of which samples from 23 subjects were available for study. The mean ($\pm$ SD) age of the subjects studied was 13.1 ± 2.9 y, with a range in ages from 7.1 to 18.7 y. Among the subjects, there were 14 diagnosed with CD and nine with UC. Diagnosis was established by using the usual clinical, radiologic, endoscopic, and histologic criteria with sites of involvement determined either by endoscopic visualization or barium examination of the jejunum and ileum in CD subjects. As shown in Table 1, most UC patients had pancolitis at the time of diagnosis, as is common in children (11). Most of the children diagnosed with CD had colonic or ileocolonic involvement. The mean time of onset of symptoms to time of diagnosis was approximately 14 mo for subjects with CD and 5 mo for subjects with UC ($p = 0.18$). The mean ($\pm$ SD) PCDAI score at the time of diagnosis was 38.4 ± 14.2 points, and a subject with a PCDAI score ≤ 10 was considered to have inactive disease in this study (12, 13).

Patient specimens. After informed consent or assent was obtained from the subjects and their parents or guardians, peripheral blood (5–10 mL) was obtained in a heparinized tube for analysis from subjects at the time of diagnosis before the initiation of therapy and during clinical remission. Samples with any clot formation were discarded. Volunteer normal control samples were from adult patients and had been collected before the current study according to Institutional Review Board guidelines established at the University of Nebraska Medical Center. Approval for the study was obtained from the Institutional Review Board of Children's Hospital (Omaha, NE, U.S.A.) and from The Institutional Review Board for the Protection of Human Subjects at the University of Nebraska Medical Center.

Detection of intracellular cytokines. Blood specimens were analyzed as previously described (14) using a modification of Becton Dickinson's FastImmune Cytokine Application Note 1 (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) within 24 h of collection. Briefly, cells were washed in PBS and resuspended at 2×10^6 /mL in RPMI 1640 + 2 mM L-glutamine, but without serum. Activation was performed in the presence of phorbol 12-myristate 13-acetate (25 ng/mL), ionomycin (1 mg/mL), and brefeldin A (10 mg/mL) in 37°C, 5% carbon dioxide for 4–5 h (Becton Dickinson). After incubation, staining for surface antigen-specific fluorescent-conjugated MAb CD3 perCP and CD69-FITC was performed. Next, a FACS lysing solution was added to fix external epitopes and assist in permeabilization. After permeabilization

Table 1. Details of patients

IBD subtype	Subject	Sex	Age at diagnosis	Time of symptoms to diagnosis (mo)	Disease location	PCDAI at diagnosis	Drugs
Crohn disease	1	M	12 y, 3 mo	24	Stomach + colon	35.0	P, M
	2	M	9 y, 11 mo	1	Colon alone	12.5	M, E, P, S, MTX
	3	M	12 y, 7 mo	6	Stomach + colon	42.5	P, A
	4	M	13 y, 2 mo	1	Colon alone	20.0	A, E
	5	M	11 y, 11 mo	3	Colon alone	32.5	A, E
	6	M	7 y, 1 mo	2	Colon alone	42.5	S, F, P
	7	F	14 y, 7 mo	48	TI alone	60.0	P, I, F, M, MTX
	8	F	11 y, 0 mo	24	TI + any colon	40.0	M, P, I
	9	F	17 y, 0 mo	2	TI + any colon	37.5	M, I
	10	M	13 y, 1 mo	9	TI + any colon	50.0	P, I, M
	11	M	13 y, 4 mo	12	TI alone	42.5	P, I, M
	12	F	16 y, 10 mo	60	TI + any colon	25.0	M
	13	M	17 y, 2 mo	1	Colon alone	37.5	M, P, E, I
	14	F	18 y, 8 mo	5	Colon alone	62.5	P, I, M
Ulcerative colitis	15	M	13 y, 6 mo	1	Pancolitis		P, M
	16	M	13 y, 9 mo	3	Pancolitis		P, M, S, E, I
	17	F	15 y, 11 mo	4	Left-sided colitis		E, M, P, I
	18	M	13 y, 5 mo	1.5	Pancolitis		P, I
	19	M	9 y, 11 mo	1.5	Pancolitis		M, P, I
	20	F	9 y, 0 mo	2	Pancolitis		P, M, E, F
	21	M	9 y, 9 mo	5	Pancolitis		M
	22	M	13 y, 3 mo	24	Pancolitis		M
	23	F	14 y, 6 mo	3	Pancolitis		P, M

Abbreviations used: M, male; F, female; TI, terminal ileum; Drugs used to induce or prescribed at time of remission (Drugs): P, prednisone; M, mesalamine; S, sulphasalazine; E, mesalamine enema; F, metronidazole; I, 6-mercaptopurine/azathioprine; MTX, methotrexate.

Figure 3. Serial determinations of type 2 T cells in peripheral blood of UC subjects. Individual values and medians (horizontal lines) are shown with corresponding values indicated by connecting lines.

and control subjects. These results represent measurement of cytokine from more than lymphocytes (total mRNA of lymphocytes and macrophages). Patient details in the study by Mullin *et al.* (4) are not given, but in the study of Camoglio *et al.* (5), the mean duration of disease was greater than 6 y and the mean age of the patient was older than 32 y. In a study of surgical resection specimens obtained from adult patients, which evaluated enriched CD4⁺ T cells, IFN- γ secretion by both freshly isolated lamina propria and peripheral blood CD4⁺ T cells was found to be increased compared with CD4⁺ T cells from UC patients and control subjects (6). Thus, despite a number of variables to consider in cytokine analysis in IBD patients (17), there is a correlation between lamina propria CD4⁺ T cells and peripheral blood CD4⁺ T cells. In all of these studies most patients were receiving various medications. Of interest to our study on children with newly diagnosed IBD of short duration before the introduction of medications was the report of Desreumaux *et al.* (9). In their study, biopsy specimens were obtained from patients before a surgical resection and 3 mo after the resection. Reverse transcriptase-PCR analysis was applied to total mRNA isolated from biopsy specimens. Medications were discontinued before the surgery. In biopsy specimens obtained from mucosa with active disease before surgery, IFN- γ was higher than in biopsies obtained from mucosa in patients that relapsed postoperatively. The IFN- γ signals from mucosa obtained from those patients with postoperative recurrence were lower compared with control subjects and those patients without postoperative recurrence. There are certain analogies between the study of Desreumaux *et al.* (9) and the current study, which include new onset of disease and subjects without medications.

In UC, total mRNA analysis from biopsy specimens have revealed higher IL-4 expression levels in actively inflamed mucosa compared with noninflamed UC mucosa and that from non-UC inflamed mucosa (8) or showed a trend but no statis-

tical difference (4). In other studies of older patients with previously established CD or UC and taking various medications, decreased production of IL-4 from isolated lamina propria CD4⁺ T cells or mononuclear cells compared with control subjects was determined (6, 7). The opposing results from these studies may be on the basis that total mRNA detection techniques will detect IL-4 mRNA from cellular sources other than mononuclear cells and mRNA expression does not necessarily correlate with protein expression. In our study, we found the peripheral blood IL-4-producing cells were reduced only in UC patients at diagnosis. In those patients who underwent serial determinations from the time of diagnosis, when no medications had been given, to the time of remission after which various medications had been administered, the median percent of IL-4-producing cells rose. There are no adult cytokine studies of new-onset compared with long-standing UC to compare our results with that are analogous to the new-onset *versus* long-standing CD such as that described by Desreumaux *et al.* (9).

It is apparent that the age group of children evaluated in our study mounts an immunologic cellular response in both subtypes of IBD. Furthermore it is apparent that cytokine expression is not necessarily static. The cytokine alterations we describe in new-onset CD in children before therapy are similar to recurrence of CD in adults after resection. From this study, we suspect that medications or length of time of mucosal inflammation may be a factor in the intracellular cytokine expression profile. This may be an explanation of why medications such as infliximab are effective in some patients with UC (18) as well as in patients with CD (19). In addition, this may also be an explanation for the finding that other drugs such as cyclosporine with benefit in the acute phases of disease (20) do not have maintenance benefits (21) and why medications such as mercaptopurine become less effective with continued usage (22). As therapy for subtypes of IBD becomes more cytokine specific, it will be interesting to determine whether new-onset disease will respond differently than long-standing disease and which combinations or sequences of therapies should be used.

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