Intraepithelial γδ+ Lymphocytes

A Comparative Study Between Celiac Disease, Small Intestinal Bacterial Overgrowth, and Irritable Bowel Syndrome

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Background: Intraepithelial lymphocytes (IELs) phenotyping has emerged as a useful test in intestinal pathology. In celiac disease (CD), a permanent and marked increase of γδ+ IELs has been described. However, there is a lack of knowledge about this peculiar IELs population in other intestinal pathologies.

Aim: To analyze the percentage of IELs, specifically γδ+ IELs subset, present in duodenal mucosa biopsies from patients with CD and compare it with those obtained from patients with small intestinal bacterial overgrowth (SIBO) or irritable bowel syndrome (IBS).

Methods: Twelve patients with untreated CD, 8 patients with SIBO, and 10 patients with diarrhea-predominant IBS were evaluated. All subjects underwent upper endoscopy for mucosal biopsy and jejunal aspirate. From 2 small bowel biopsies, intraepithelial cells were isolated and labeled with the following monoclonal antibodies CD103-PE (phycoerythrin), CD3-FITC (fluoresecein isothio-cynate), CD-7R-PE, CD45RO-APC (allo-phycocyanin), and TcR γδ-FITC. Flow cytometry analysis was performed on a standard FACScan. Total and IELs subset counts were expressed as percentage.

Results: Mean total IELs percentage was 16.7 ± 6% in IBS, 25.4 ± 17% in SIBO, and 26 ± 13% in CD patients ($P = 0.2$). CD and SIBO patients, had significantly higher percentages of γδ+ IELs (15.7 ± 13% and 14.6 ± 8%) than IBS subjects (4.1 ± 2.5%, $P < 0.05$). There was no difference between CD and SIBO ($P = 0.6$).

Conclusions: An increased density of γδ+ IELs is typical, but not specific for CD. A similar increase was observed in subjects with SIBO. Our findings suggest that this unique T-cell population might have a key role against intestinal bacterial infections.

Received for publication April 28, 2006; accepted September 17, 2006.

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Key Words: celiac disease, small bacterial overgrowth, intraepithelial lymphocytes

(*J Clin Gastroenterol* 2007;41:671–676)

Intraepithelial lymphocytes (IELs) constitute a peculiar lymphoid compartment of heterogeneous populations with unique features that differentiate them from lymphocytes present in the lamina propria and elsewhere.[1,2] There are 2 major populations of IELs, one bears the αβ+ T-cell receptor (> 90%) and the other has the γδ+ T-cell receptor (< 10%).[3] The αβ+ cells seem to increase in response to antigenic challenge or stress, whereas the γδ+ cells have a less clear role, though they may be involved in regulating the immune response to a limited diversity of antigens.[1–4]

Previous studies have shown that IELs are increased in the small bowel mucosa of untreated patients with celiac disease (CD), which has lead to consider this finding as the earliest lesion presented by these patients, as it is even seen when the villous architecture is preserved.[5–9] Nevertheless, increased IELs counts have also been described in other small bowel disorders, including small intestinal bacterial overgrowth (SIBO),[10] tropical sprue,[11] microscopic colitis,[10] inflammatory bowel disease,[12] irritable bowel syndrome (IBS),[13] allergic enteritis,[14] autoimmune disorders,[15] viral infections,[11] and nonsteroidal inflammatory related enteropathy.[10,16]

Recently, genetic and immunohistologic tests have been developed to facilitate the diagnosis of CD, especially in those cases where clinical presentation and histologic findings are uncertain.[6,8,17] Thus, the analysis of the IELs populations (IELs phenotyping) using immunohistochemistry and/or flow cytometry (FCM) has emerged as a useful diagnostic tool in intestinal pathology. In CD for example, a systematic, permanent, and marked increase of γδ+ IELs has been consistently described.[18–23] However, there is a lack of knowledge about this peculiar IELs population in other intestinal pathologies.

Owing to the fact that histologic abnormalities and increased total IELs applying conventional staining (hematoxylin and eosin) do not allow to distinguish

between CD and other enteropathies, the aim of our study was to analyze the percentage of IELs, specifically γδ+ IELs subset, present in duodenal mucosa biopsies from patients with CD and compare it with those obtained from patients with SIBO or IBS. We used FCM analysis, because this technique allows simultaneous analysis of the different T-cell surface markers providing a more accurate quantification and an easier determination of IELs subset.[22,23]

MATERIAL AND METHODS

Subjects

During the period from January to August 2004, consecutive subjects who were referred for upper gastrointestinal endoscopy evaluation in which duodenal mucosal biopsies and duodenal aspirates were required were invited to participate in this study. All subjects had previous appropriate clinical workup including symptomatic evaluation, physical examination, hematologic/biochemical studies, stool studies for ova and parasites, D-xylose, and liver function tests. Subjects with present or past history of malignancies, immunodeficiency or under immunosuppressive therapy, coagulation disorders, cirrhosis, gastrectomy (total or partial), short bowel syndrome, lactose intolerance, pancreatic disease, and inflammatory bowel disease were excluded. All participants gave written informed consent, and the Institute's Ethics Committee approved the study.

Methods

Subjects were prospectively evaluated in the Department of Gastroenterology and Endoscopy at the *Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán."* They underwent upper gastrointestinal endoscopy for small bowel mucosal biopsies and duodenal aspirate. Five biopsy specimens were taken from the distal part of the duodenum; 3 of these were stained with hematoxylin-eosin and studied under light microscopy and graded according to the Marsh classification.[24] The 2 remaining biopsies were sent to the Department of Microbiology of the Medicine School at *Universidad Nacional Autónoma de México* for FCM analysis (see below). Aspirate from the duodenum was obtained during the endoscopy and collected in a sterile plastic tube. Specimens were cultured for aerobic and anaerobic microbes on blood agar plates with 4% defibrinated horse blood in aerobic and anaerobic atmosphere of 10% CO_2 and N_2, and for selective cultivation of Gram-negative strains on Drigalski agar under aerobic conditions. The incubation time of the plates was at least 48 hours. Identification of the microorganisms was based on colony characteristics and Gram staining. Quantization was performed by counting the number of colony-forming units.[25] Cultures were considered positive if the total count of colonic microbes was $\geq 10^5$ colony-forming units/mL (cfu/mL), and growth of upper respiratory tract flora was not regarded as positive.[25,26] On the other hand, cultures were considered negative if no microbial growth was observed in the duodenal aspirate. In addition, a 5 mL blood sample was taken and the serum tested with a new generation human recombinant protein-based IgA tissue transglutaminase antibody ELISA commercial kit (Aeskulisa tTG-IgA, Wendelsheim, Germany). The cut-off value provided by the manufacturer was 15 U/mL. Values above this cut-off point were considered as positive.

Subsequently, and according to histologic findings, duodenal aspirate cultures and serologic tests, subjects were classified in 3 groups:

(a) CD patients: All subjects had histologic findings indicative of CD (Marsh classification I-IV) and positive tTGA-IgA. In addition, these subjects must have had negative duodenal aspirate cultures. Also, a clinical response was later observed in all individuals after a gluten-free diet.
(b) SIBO: All subjects must have had colony counts $\geq 10^5$ cfu/mL of anaerobes or Gram-negative bacteria in the duodenal aspirate and negative tTGA-IgA.
(c) IBS: These subjects had abnormal bowel habits (loose or watery stools, more than 3 bowel movements a day, and/or urgency) in the absence of endoscopic, histopathologic, laboratory, and microbiologic abnormalities. In addition, all subjects must have had a normal duodenal mucosa histology, negative duodenal aspirate cultures, and negative tTGA-IgA. All subjects fulfilled diarrhea-predominant IBS diagnostic criteria according to Rome II.[27]

For our specific aim, only subjects who meet strict criteria (all of them) for one of the 3 aforementioned groups were included for further analysis. For example, subjects with overlap between entities were excluded.

FCM Analysis

Tissue collection: Isolation of intraepithelial cells and FCM analysis were performed by 2-blinded researchers (K.A. and A.I.C.R.). After small bowel biopsies were taken, epithelial and intraepithelial cells were isolated from 2 biopsy specimens. In brief, biopsies were shaken for 30 minutes in a Hanks balanced salt solution supplemented with 5% fetal calf serum, 1 mM dithiothreitol, and 1 mM ethylenediamine tetraacetic acid. The suspension of released cells was then washed and labeled by 4-color staining procedure using the following monoclonal antibodies: anti-CD103-PE (phycoerythrin), CD3-FITC (fluoresecein isothio-cynate), CD-7R-PE, CD45RO-APC (allophycocyanin), and TcR γδ-FITC. Expression of IELs surface markers was analyzed by 4-color flow cytometric analysis (Epics Altra, Beckman-Coulter, Fullerton, CA), and data analyzed using an Expo 32 Altra software program (Beckman-Coulter, Fullerton, CA). IELs were selected from the whole population of epithelial cells by the expression of $CD3^+$ (a T-cell marker),[22,23] and their intraepithelial localization was confirmed by the expression of $CD103^+$.[28] Thereafter, the 3 following IELs subsets were analyzed according to expression of: (1) TcR γδ+ (a population that has been considered specific for CD)[18–23];

(2) CD45RO+ (a marker associated with memory or previously activated T cells)[29]; and (3) $CD3^-CD7^+$ (a phenotype suggestive of natural killer cells, which are decreased in CD).[22,23]

Statistical Analysis

Total and subsets of IELs were expressed as densities (percentage) of total cells in the epithelium (mean ± standard deviation). Differences in densities of total IELs, TcR γδ+, CD45RO+, and $CD3^-CD7^+$ between groups were analyzed using χ^2, Mann-Whitney, and Kruskall-Wallis tests when appropriate (SPSS 10, Chicago, IL and NCSS-200, Kaysville, UT). A P value < 0.05 was accepted as statistically significant. According to previous studies, the sample size of at least 5 patients per group provides > 80% power to detect a difference in γδ+ IEL densities of 28%, which was considered clinically relevant.[23]

RESULTS

Subjects

A total of 30 subjects were included in our study, 21 were female and 9 male (mean age 52 ± 17 y). Twelve patients had CD (without treatment at the time of duodenal mucosa biopsy), 8 had SIBO, and 10 subjects were classified as having IBS. Demographics and histologic findings are shown in Table 1.

(a) *CD*: In CD patients, upper endoscopic evaluation was performed as part of the clinical workup for a variety of symptoms, including chronic diarrhea (6 cases), iron-deficiency anemia (4 cases), and abdominal pain (2 cases). Four patients (33%) had another associated autoimmune diseases: 2 had autoimmune hypothyroidism, one primary Sjögren syndrome, and another systemic erythematous lupus. Nine patients (75%) had marked villous flattening and/or atrophy (Marsh's classification stage III or IV).

(b) *SIBO*: These patients underwent upper endoscopy for chronic diarrhea (n = 5), and abdominal pain (3 cases). The following microbes were cultured: *Escherichia coli* (n = 3), *Pseudomona sp* (n = 2), *Bacteroides sp* (n = 2), and *Enterococci* (n = 1). One subject had chronic, nonspecific inflammatory changes in the mucosal biopsy, 2 had type II Marsh's classification changes, and 3 had marked villous flattening (Table 1).

(c) *IBS*: All these subjects had normal results of their previous clinical work up and had abdominal pain or discomfort associated with changes in the frequency and form of stool, which was relieved with defecation.

Total Epithelial Cells and Total IELs

The mean total epithelial cells isolated for further FCM analysis was similar between groups (Table 2, $P = 0.94$). There was no correlation between the total number of epithelial cells isolated and the severity of villous atrophy ($r = -0.028$, $P = 0.882$, Fig. 1).

The mean percentage of total IELs was 26 ± 13% in CD patients, 25.4 ± 17% in SIBO patients, and 16.7 ± 6% in IBS subjects. No significant differences were observed among subgroups ($P = 0.20$).

TABLE 1. Demographic and Histologic Findings Among Groups

	CD (n = 12)	SIBO (n = 8)	IBS (n = 10)
Age (mean ± SD)	45 ± 5	55 ± 4	43 ± 3
Male:female (n)	3:7	3:5	3:9
Histologic findings (n, %)			
Normal	0 (0%)	2 (25%)	100 (100%)
Nonspecific changes	0 (0%)	1 (12%)	0 (20%)
Marsh classification			
Stage I	2 (16%)	0 (0%)	0 (0%)
Stage II	1 (8%)	2 (25%)	0 (0%)
Stage III	7 (58%)	3 (37%)	0 (0%)
Stage IV	2 (16%)	0 (0%)	0 (0%)

IELs Subsets

As shown in Figure 2, both patients with CD and SIBO had significantly higher percentages of the γδ+ IELs subset in the duodenal mucosa (15.7 ± 13% and 14.6 ± 8%) compared with IBS subjects (4.1 ± 2.5%, $P = 0.002$). However, there was no difference between CD and SIBO patients ($P = 0.6$). Regarding the $CD3^-CD7^+$ IELs subset, low percentage values were observed in all groups (Table 2). However, no significant differences were observed among groups. Also, the percentage of IELs that expressed $CD45^+$ was similar among groups (Table 2).

DISCUSSION

Until now, the presence of a marked and persistent increase in the γδ+ IELs subset in the duodenal mucosa had been considered a specific finding for CD. In the present study, we show that duodenal mucosal biopsies obtained from SIBO subjects also presented an increase in both the total percentage of IELs and the γδ+ IELs.

IELs are in the front line of a constant conflict between the mucosal immune system and the tremendous number of foreign antigens present in the gut lumen.[1,30] Considering that IELs have a number of important immunologic functions, such as cytotoxic activity and secretion of cytokines playing a crucial role for combating microbial and protozoa infections, is predictable that total intraepithelial lymphocytosis is a somewhat nonspecific finding present in small bowel pathologies, such as SIBO.[10–16] As in other studies, we have confirmed this finding even when 75% of the subjects with CD and 37% of SIBO patients had villous flattening. The mean percentage of total IELs detected in proximal small bowel biopsies in our CD patients was similar to that reported by Camarero et al[22] using the same technique (26 ± 13% and 20.3 ± 7.4%, respectively) in subjects with severe villous atrophy. The ability to detect an increase in total and IELs subsets, regardless the presence of severe mucosal changes is an advantage of using FCM for IEL phenotyping. This is because FCM allows a more objective and easier quantification, taking into account

TABLE 2. Mean Total Number of Epithelial Cells, Percentage of Total IELs, and IELs Subsets in the 3 Groups (Mean ± SD)

	CD Mean ± SD	SIBO Mean ± SD	IBS Mean ± SD	*P*
Total epithelial cells isolated (no.)	19,521 ± 8428	18,884 ± 8910	18,706 ± 6044	0.947
Percentage of total IELs	26 ± 13	25.4 ± 17	16.7 ± 6	0.20
IELs subsets				
γδ+	15.7 ± 13	14.6 ± 8	4.1 ± 2.5	0.002*
$CD3^-$ $CD7^+$	5.6 ± 5	5.2 ± 5	8.2 ± 6	0.48
CD45RO	26.5 ± 23	26 ± 25	15.7 ± 14	0.26

*Kruskal-Wallis test.

only those cells that are positively stained with the $CD3^+$ marker, a specific marker for T cells.

Few studies have shown that intraepithelial lymphocytosis could also be present in diarrhea-predominant IBS patients.[13,31,32] In this study, IBS patients had a mean total percentage of IELs of 16%. Wahnschaffe et al[31] using $CD3^+$ immunohistochemistry found that 23% of patients with diarrhea-predominant IBS had abnormally high counts of IELs 23% (median 23 IELs per 100 enterocytes) in the duodenal mucosa. Also, it has been reported that subjects who develop postinfectious IBS, have a modest increase in proximal jejunum mucosal lymphocytes.[32] However, whether intraepithelial lymphocytosis is an indicator for a luminal factor that triggers a low-grade inflammatory response, an "immunologic memory" that persists after earlier antigenic provocations (ie, postinfectious IBS), or a characteristic of an "hyperactive gut" remains to be elucidated.[13]

Some studies suggest that the role of γδ+ IELs in CD is in surveillance and repair of damaged epithelial tissue, whereas others consider it possible that these cells are cytotoxic for epithelial cells in the presence of gluten.[1,2,30,33] As in other studies, we found that patients with CD had significantly higher percentages of the γδ+ IELs subset.[18–23] To our knowledge this is the first study

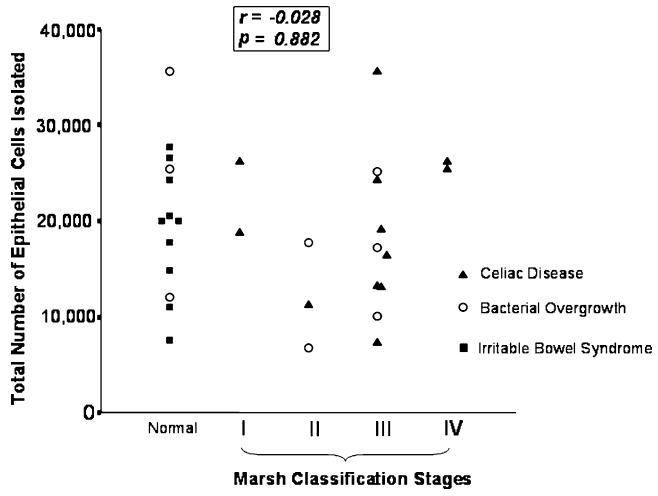

FIGURE 1. Correlation between total number of epithelial cells isolated and histologic findings among groups.

to evaluate the γδ+ IELs population in either subjects with SIBO or IBS. The most relevant finding was the fact that patients with SIBO also had significantly higher percentages of the γδ+ IELs subset when compared with IBS, but similar to CD patients. Hence, we show that an increase in this population of IELs in the proximal small bowel is nonpathognomonic for CD.

Because of their location, γδ+ IEL are one of the first lines of defense against mucosal microbes.[1,2,30,33] Interestingly, recent studies show that several bacteria and its components, induce the production of self-glycosphingolipids in antigen-presenting cells, which are subsequently presented on the cell surface and recognized by specific αβ T-cell receptors.[2,30] It is possible that a similar mechanism may also have a role in eliciting the responses of γδ+ T cells. Other studies have demonstrated that either *Escherichia coli* or *Salmonella* infections produce phosphoantigens that activate human γδ+ T cells.[34,35] Engstrand et al,[36] using immunoperoxidase staining on gastric biopsy specimens from patients infected with *Helicobacter pylori*, observed increased numbers of γδ+ IEL in most cases. It has also been shown that the generation of γδ+ T lymphocytes in peripheral blood cell is part of a normal immune response to bacterial infection in *Campylobacter jejuni* gastroenteritis.[37] These findings suggest unique functions of γδ+ T lymphocytes in antimicrobial immunity and it is obvious that this special population of lymphocytes has a key role against intestinal bacterial infections. In this perspective, it seems that the increase of the γδ+ IELs population observed in our patients with SIBO is a logical finding in response to abnormal amounts of microbes in the proximal small bowel. If the γδ+ IELs subset decreases after antibiotic therapy remains to be elucidated.

Regarding the $CD3^-/CD7^+$ IELs subset, We found that the percentage of this population was low among the 3 groups. It has been shown that this subset displays a suggestive natural killer cells phenotype with possible cytotoxic activity.[22,23] Previous studies have found that this subset is drastically reduced in the duodenal mucosa of CD patients (mean percentage of 4.9% ± 7.9), and this is independent of dietary gluten.[38] Our patients with CD and SIBO had similar mean percentages (5.6% and 5.2%, respectively) of this lymphocyte subset. Furthermore, the mean percentage of this population was not different in the IBS group

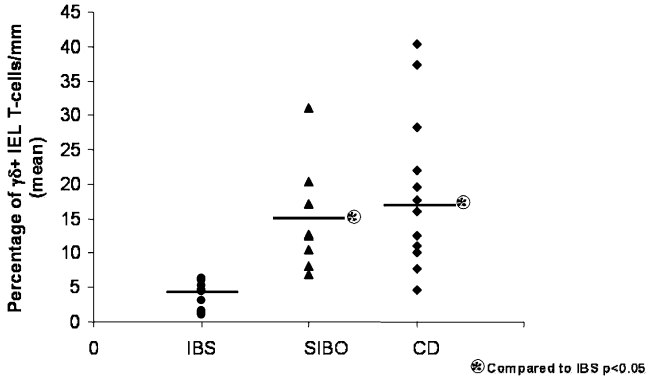

FIGURE 2. Percentage of intraepithelial γδ lymphocytes of total cells in the epithelium in the 3 groups (mean and individual data).

(8.2%). In addition, we did not find differences in the CD45RO+ IELs subset, a population that represents previously activated T cells ("memory") which provide help for antibody production.[29] Despite these findings, the biologic significance of this particular group of IELs and their function in the pathogenesis of small bowel diseases remains to be elucidated.

We have to acknowledge that the absence of a healthy control group limits our results, but it should also be recognized that it is difficult to perform such extensive and invasive evaluations in otherwise asymptomatic subjects. Despite this limitation, the findings we observed in CD subjects are consistent with those reported in previous studies, and we believe that the results obtained in SIBO and IBS subjects are reliable.

Several reports have described either a potential association between CD and IBS or SIBO and IBS.[39–42] However, the true association between these 3 entities remains controversial.[43–45] Aspiration and direct culture of the duodenum is regarded by many as the gold standard for the diagnosis of SIBO.[46] Nevertheless, the main difficulties with direct culture are a high risk of contamination by microflora, sampling restricted to the proximal bowel, and most of gut microbes cannot be cultured.[46] Recently, some studies using lactulose breath tests (LBT) have reported a prevalence of SIBO in up to 84% of IBS patients and in up to two-third of CD patients with persistent symptoms after gluten-free diet.[39,40] Even though the reliability of LBT has been criticized, particularly when used alone, it is difficult to ignore these findings.[47,48] Thus, we cannot rule out that CD and IBS subjects may have a more distal microbial proliferation (ie, jejunal and ileal), and an indirect test such as LBT would be helpful to address this condition. Although this situation could explain a similar percentage of total IELs among groups, our subset analysis shows that the percentage of the γδ+ IELs infiltrating the duodenal mucosa was significantly lower in IBS. In fact, the mean percentage of γδ+ IELs (4.1% ± 2.5%) is similar to those reported by other groups in healthy controls using FCM (6.7% ± 6%).[22,23] Using immunohisotchemistry analysis the normal limit for this peculiar population varies from 1.6 to 3.1 cells/mm of epithelium.[21] Whether the densities of γδ+ IELs in our IBS subjects represent the true absence of epithelial intestinal abnormalities or a lack of activation of this specific lymphocyte population remains to be studied.

Finally, recent studies have shown that IBS patients have mildly elevated bacterial counts ($\geq 5 \times 10^3$ but $< 10^5$ cfu/mL) in the duodenum, a clearly abnormal finding that does not meet the traditional criteria for SIBO ($\geq 10^5$ cfu/mL).[49] The clinical relevance of these mild alterations in small bowel bacterial flora in IBS remains unclear and requires further investigation. We should remark that in this study, we used strict definitions for a positive ($\geq 10^5$ cfu/mL) or negative (absence of bacteria) duodenal aspirates, and subjects with mild elevated bacterial counts were not included.

In conclusion, an increased density of γδ+ IELs population is typical, but not specific, for CD. A similar increase was observed in subjects with SIBO but not in subjects with IBS. Our findings add clinical evidence to the idea that this unique T-cell population has a key role against intestinal bacterial infections. However, further investigations are required regarding the specific function of IELs subpopulations in the pathogenesis of other small bowel diseases.

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