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RAPID COMMUNICATION

Duodenal intraepithelial T lymphocytes in patients with functional dyspepsia

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Abstract

AIM: To quantify the intraepithelial lymphocytes (IELs) and to document the membrane expression of CD4, CD8, TCRγδ and adhesion and/or activation-associated molecules (CD103, CD28, CD44, CD69, HLA-DR, CD95/Fas) in the duodenal mucosa of patients with functional dyspepsia (FD) in order to provide arguments for an immunological process in FD.

METHODS: Twenty-six FD patients according to Rome Ⅱ criteria (20 were *H pylori* negative) were studied and compared to 12 healthy adults. IELs were isolated from five duodenal biopsy samples, then quantified by microscopy and flow cytometry while the membrane phenotypes were determined by cytofluorometry.

RESULTS: Duodenal histological examination was normal. In *H pylori* negative patients, the number of IELs was not different from that in healthy controls. Median percentage expression of CD4, CD8, or TCRγδ and CD103, CD44, CD28, CD69 on CD3+ IELs, among the adhesion/activation associated molecules tested, was not different from that in healthy controls. In contrast, the median percentage expression of CD95/Fas [22 (9-65) *vs* 45 (19-88), $P = 0.03$] and HLA-DR expressing CD3+ IELs [4 (0-30) *vs* 13 (4-42), $P = 0.04$] was significantly lower in the *H pylori* negative FD group than in healthy controls, respectively. The number of IELs was significantly greater in *H pylori* positive FD patients than in healthy controls [median ratio for 100 enterocytes 27.5 (6.7-62.5) *vs* 10.8 (3-33.3), $P = 0.02$] due to a higher number of CD8+ CD3+ IELs.

CONCLUSION: In *H pylori* negative FD patients, the phenotypic characterization of IELs suggests that we cannot exclude a role of IELs in FD.

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Key words: Functional dyspepsia; Intraepithelial T lymphocytes; Gut; CD95/Fas; HLA-DR

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INTRODUCTION

Functional dyspepsia (FD) is one of the most common functional gastrointestinal disorders in adults. This clinical syndrome is characterized by chronic or recurrent pain or discomfort centered in the upper abdomen without a cause that is identifiable by conventional diagnostic means[1]. Several mechanisms have been already proposed to explain its symptoms: a delayed gastric emptying found in about 40% of the patients, an impaired proximal gastric accommodation to meals promoting either proximal gastric distension during food intake and/or an early migration of the components of the meal distending the antrum[2,3], a gastric hypersensitivity with a decreased pain threshold during distension that may account for early and/or delayed dyspeptic symptoms in about 40% of the patients[4,5]. However, a poor correlation exists between these findings and symptoms. The pathophysiology of FD is probably multifactorial and not completely understood. In irritable bowel syndrome (IBS), recent findings have pointed out the possible role of an immune dysfunction in the onset of symptoms: a low grade mucosal inflammatory process, undetectable with conventional mucosal histology, exists, particularly in post-infectious IBS[6,7]. An immune activation with the release of several mediators, including cytokines, nitric oxide, histamine and protease which interfere with the function of enteric nerves, has been

observed in the intestinal mucosa of IBS patients[8]. Moreover, immune-mediated activation of mastocytes and macrophages in contact with pain-sensitive endings seems to be involved in the development of hypersensitivity in experimental models of visceral pain[9]. As symptoms of IBS and FD often overlap with time, we could speculate that an immune dysfunction may be also involved in the onset of FD in adults.

One of the key clinical features in FD is that symptoms are often meal-related, being either induced or exacerbated by food[10]. The intestinal mucosal barrier consists of intestinal epithelial cells and intestinal intraepithelial lymphocytes (IELs). By their strategic location, IELs are likely to be important in the preservation of mucosal integrity including maintenance of oral tolerance to both particulate and soluble antigens[11-14] and the vast majority of IELs are of T-cell type and more than 70% are CD4-/CD8+ T-cells[15-17].

The present study was carried out to investigate changes in number and phenotype of single cell isolated duodenal IELs, especially phenotypic markers of the IEL activation process, by cytofluorometry, in symptomatic FD patients compared with healthy control adults.

MATERIALS AND METHODS

Subjects

This prospective study concerned 26 consecutive dyspeptic patients, 22 women and 4 men, median age of 44 years (range: 18-69) referred to the Gastroenterology Department of the Rouen University Hospital for upper gastrointestinal endoscopy over a one year period of time. Median duration of symptoms was 4 years (range: 1-35 years). No patient had any history of post-infectious FD. Patients were assessed independently by two gastroenterologists in order to confirm the diagnosis of FD according to Rome Ⅱ criteria and to look for the following exclusion criteria to participate in the study: (1) predominant symptoms of gastro-esophageal disease (heartburn and/or acid regurgitation), (2) associated abdominal pain and transit disturbances suggestive of irritable bowel syndrome according to Rome Ⅱ criteria, (3) documented immunodeficiency state and medical history of inflammatory bowel disease, (4) clinical history of lactose intolerance, (5) consumption of non-steroidal anti-inflammatory drugs within two weeks before the study. In order to exclude a celiac disease leading to symptoms mimicking FD, both anti-gliadin and anti-transglutaminase serum antibodies were systematically dosed and normal in all patients.

In eligible dyspeptic patients, research of *H pylori* was systematically performed both by histological examination of two antral biopsies and by C^{13} urea breath test. Twenty patients were *H pylori* negative dyspeptics (both tests negative). In the remaining 6 patients (*H pylori* positive patients), both histological examination of antral biopsies and C13 urea breath test led to the diagnosis of *H pylori* infection. The control group consisted of 12 healthy non obese adult volunteers, 9 women and 3 men, median age of 42 years (range 19-76 years). None of them took non-steroidal anti-inflammatory drugs for at least two weeks prior to the study and all were *H pylori*-negative.

All patients and healthy volunteers received oral and then written information before inclusion. The study was approved by the local Ethical Committee of the Rouen University Hospital (CCPPRB of Haute-Normandie, France). Written consent was obtained from all participants.

Endoscopic duodenal and antral sampling

An upper gastrointestinal endoscopy was performed in all patients and healthy controls. Seven samples were taken in the duodenum, two for conventional histology and five from different sites in the second duodenum for IEL phenotype analysis. Two additional antral biopsy samples were obtained for *H pylori* detection. Duodenal biopsy specimens for IEL phenotype analysis were placed in calcium and magnesium free Hanks' balanced salt solution (Eurobio, Les Ulis, France) supplemented with antibiotics (penicillin: 100 U/mL, streptomycin: 100 μg/mL) and 4% (vol/vol) fetal calf serum before processed within the first 6 hours following endoscopy.

Flow cytometric membrane characterization of duodenal IELs

Duodenal biopsy samples were treated with 1 mmol/L dithiothreitol (DTT), (Sigma, Saint-Louis, Missouri, USA) for 30 min at 37℃, and 1 mmol/L EDTA for 45 min with continuous agitation at 37℃ to remove the epithelial layer. Single cell suspensions were pelleted from the supernatant, and washed once with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.02% sodium azide (1200 r/min, 10 min, at room temperature). For quantitative analysis, duodenal IELs were microscopically counted in a haemocytometer with their number expressed per 100 epithelial cells. IEL viability checked by trypan-blue exclusion was over 95% in all experiments and the stability of IEL over times was constant from one patient to another. As DTT/EDTA treatment has been previously reported to cause loss of β7 integrin expression[18], lymphocytes collected from the supernatant before this treatment were used for CD103 labelling. The following mouse monoclonal antibodies (Mab) used in this study are shown in Table 1.

Ten microlitres of each Mab stock solution was added to 100 μL of cell suspension (approximately 10^5 cells), then incubated at room temperature for 30 min in the dark before washing once with PBS, 0.5% BSA and 0.02% sodium azide. Flow cytofluorometry data were acquired in a flow cytometer (FACScalibur, Becton-Dickinson) and listmode files were analyzed using the Cell-Quest software (version 3.3, Becton-Dickinson). Cell suspensions were initially visualized in the forward scatter/side scatter (FSC/SSC) profile. Irrelevant isotype-matched control Mab (FITC-labelled IgG1 and PE-labelled IgG2, Becton Dickinson) was used to exclude non-specific binding and to determine the optimal setting of fluorescence quadrants. A gate was drawn around the CD3+ population and used thereafter, excluding most epithelial cells and debris from analysis.

FACScan settings were routinely standardized with beads (Calibrite, Becton Dickinson). Results were obtained

Table 1 Monoclonal antibodies used in this study

Clone	Specificity	Isotype	Label	Source
SK7	CD3	IgG1	PE	Becton-Dickinson, Le Pont de Claix, France
SK7/SK1/ 2D1/SK3	CD3/CD8/ CD45/CD4	IgG1/IgG1/ IgG1/IgG1	FITC/PE/ Per CP/APC	Becton-Dickinson, Le Pont de Claix, France
Ber-ACT8	CD103 (αE subunit of the αEβ7 integrin)	IgG1	FITC	Becton-Dickinson, Le Pont de Claix, France
CD28.2	CD28	IgG1	FITC	Becton-Dickinson, Le Pont de Claix, France
G44-26,	CD44	IgG2b	FITC	Becton-Dickinson, Le Pont de Claix, France
11F2	TCR-γδ	IgG1	FITC	Becton-Dickinson, Le Pont de Claix, France
L78	CD69	IgG1	FITC	Becton-Dickinson, Le Pont de Claix, France
CR3/43	HLA-DR	IgG1	FITC	Dako, Glostrup, Denmark
UB2	CD95/Fas	IgG1	FITC	Immunotech, Marseille, France

as percentages of total CD3+ lymphocytes and absolute cell counts determined using calibrated fluorescent beads (FlowCount fluorospheres, Coulter Immunotech, Marseille, France).

Statistical analysis

Variance comparisons between groups were performed using the "non-parametric" Mann-Whitney test. Significance was expressed as P values, and $P < 0.05$ was considered statistically significant.

RESULTS

Duodenal histology

The weight of biopsy specimens was not different between those obtained in dyspeptic patients and healthy controls (data not shown, $P > 0.05$). Duodenal specimens were considered normal after conventional histological examination in all patients, whatever *H pylori* status. All healthy controls exhibited also normal duodenal histology.

Number and membrane phenotypes of duodenal IELs

The number of IELs did not differ between *H pylori* negative dyspeptic patients and control subjects, when this number was expressed as the median ratio for 100 enterocytes determined microscopically [12.5% (2.5-50) *vs* 10.8% (3-33.3), $P > 0.05$] or as a median absolute number of CD3+ IELs using cytofluorometry [122.4 × 10^3 (190.2 × 10^3 - 509 × 10^3) *vs* 53.6 × 10^3 (7.5 × 10^3 - 756.6 × 10^3, $P > 0.05$)]. At variance, duodenal IEL counts were significantly higher ($P = 0.02$) in *H pylori* positive patients

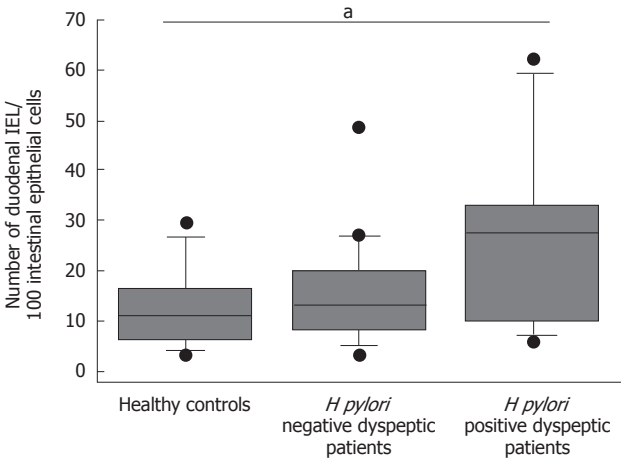

Figure 1 Box- and -wiskers plots of duodenal IEL counts expressed as number of IELs per 100 duodenal epithelial cells (Ec) in healthy controls, *H pylori* negative and positive dyspeptic patients. [a]$P < 0.05$ for *H pylori* positive *vs* controls.

than in healthy controls, when they were determined microscopically as the median ratio for 100 enterocytes [27.5 (6.7-62.5)] or as a median absolute number using flow cytometry [468.3 × 10^3 (275 × 10^3-630 × 10^3)] (Figure 1).

Median percentages of CD3+ lymphocytes in *H pylori* negative dyspeptic patients and healthy controls were 96% (84%-99%) and 93% (80%-96%) respectively ($P > 0.05$). Neither the percentages of CD3+, CD4+, CD8+ and CD3+ TCRγδ IELs nor the percentages of CD103, CD44, CD28 or CD69 expressing CD3+ IELs differed between *H pylori* negative dyspeptic patients and healthy controls. However, *H pylori* positive dyspeptic patients exhibited increased percentages of CD8 expressing CD3+ IELs compared with healthy controls[88% (76%-94%) *vs* 77% (54%-87%), respectively, $P = 0.01$] (Table 2).

The median percentages of CD95/Fas [22% (9%-65%) *vs* 45% (19%-88%), $P = 0.009$] (Figure 2) and HLA-DR expressing CD3+ IELs [4% (0%-30%) *vs* 13% (4%-42%), $P = 0.04$] (Figure 3) were significantly lower in the *H pylori* negative FD group than in healthy controls respectively.

DISCUSSION

While FD is highly prevalent in the general population, very little is known about the immunopathology of the disease and its underlying mechanisms. In order to check for a possible immune mediated mechanism, by analogy with recent findings in irritable bowel syndrome[7], our study was conducted to quantify and characterize duodenal IELs (particularly membrane expression of adhesion or activation-associated molecules) both in *H pylori* negative and positive dyspeptic patients by comparison with healthy controls. The results of this study suggest that in FD, besides motor or sensory dysfunctions previously demonstrated, duodenal mucosal immune changes exist. In a series of *H pylori* negative patients with FD defined according to Rome Ⅱ criteria, CD95/Fas and HLA-DR, two activation markers, were expressed by a significantly smaller percentage of duodenal CD3+ IELs

Table 2 Immunophenotype of duodenal intraepithelial lymphocytes in *H pylori* negative (*Hp*-) and positive (*Hp*+) dyspeptic patients and healthy control adults

IEL	Hp- dyspeptic patients (n = 20) Median % (range %)	Hp+ dyspeptic patients (n = 6) Median % (range %)	Healthy control adults (n = 12) Median % (range %)	*P*
CD3+	93 (80-98)	94.5 (89-99)	96 (84-99)	[a,c,e]$P > 0.5$
CD3+ CD4+	8 (3-34)	8 (3-15)	10 (2-22)	[a,c,e]$P > 0.5$
CD3+ CD8+	78 (58-96)	86 (75-87)	77 (53-87)	[a,c]$P > 0.5$ [b]$P < 0.01$
CD3+ TCRgd+	22 (1-36)	10 (10-30)	24 (8-60)	[a,c,e]$P > 0.5$
CD3+ CD103+	91 (58-96)	81 (75-87)	90 (80-99)	[a,c,e]$P > 0.5$
CD3+ CD44+	95 (50-99)	94 (84-100)	94 (65-100)	[a,c,e]$P > 0.5$
CD3+ CD28+	2 (1-11)	5 (1-10)	7 (1-9)	[a,c,e]$P > 0.5$
CD3+ CD69+	97 (68-100)	99 (96-100)	96 (91-98)	[a,c,e]$P > 0.5$

[a]$P > 0.5$, *Hp- vs* control; [c]$P > 0.5$, *Hp- vs* Hp+; [e]$P > 0.5$, *Hp+ vs* control; [b]$P < 0.01$, *Hp+ vs* control.

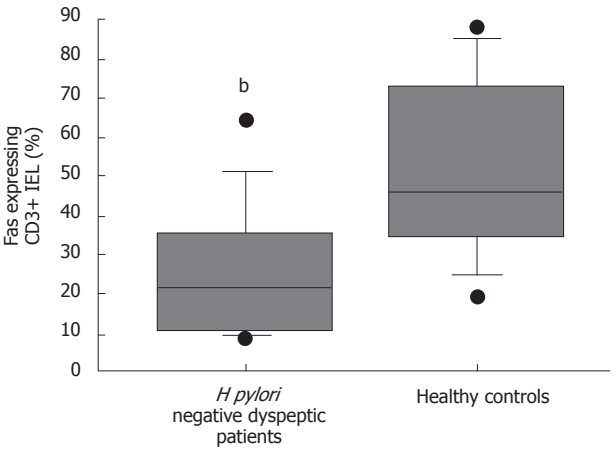

Figure 2 Box- and -wiskers plots of percentages of Fas expressing CD3+ duodenal IELs in healthy controls and *H pylori* negative dyspeptic patients. [b]$P < 0.01$ *vs* controls.

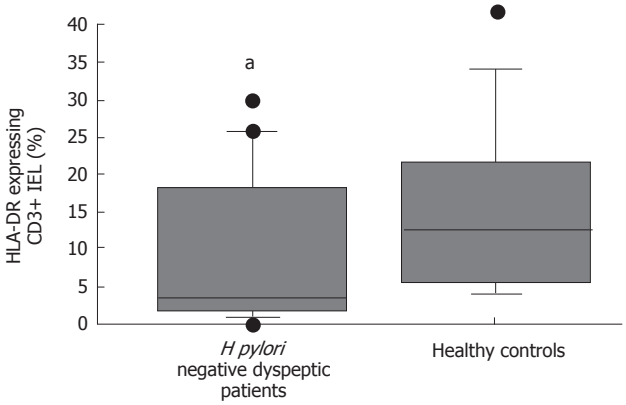

Figure 3 Box- and -wiskers plots of percentages of HLA-DR expressing CD3+ duodenal IELs in healthy controls and *H pylori* negative dyspeptic patients. [a]$P < 0.05$ *vs* controls.

compared with healthy controls, whereas the number of duodenal IELs was similar in patients with FD and healthy controls. In healthy controls, individual ratios of CD95/Fas expressing CD3+ IEL demonstrated a large range of variations (19%-88%) consistent with other reports[19,20]. Fas/FasL interaction seems closely related to the lymphocyte activation process and the expression of CD95/Fas by intestinal IELs supports the hypothesis that intestinal IELs are activated cells[21-23]. Fas-FasL system has been shown to mediate intestinal lymphocyte apoptosis not only in normal intestine where antigenic challenge continuously activates IELs[20,24-26] but also in some pathological conditions such as celiac disease[27]. Lower ratios of CD95/Fas and HLA-DR-expressing duodenal IELs in dyspeptic patients may reflect the presence of an altered population of primed lymphocytes[28]. With respect to the small sample of patients and due to the inclusion criteria, comparisons of immunopathologic parameters in relation to age, mode of onset, length of history, or predominant symptoms were not performed in the present study.

Duodenal IEL counts, determined either microscopically or by flow cytometric counting for CD3+ cells, did not differ between *H pylori* negative dyspeptic patients and healthy controls. In these two groups, values were very close to those previously reported by others, i.e. 10-20 IELs per 100 villous enterocytes[29-33]. Recent studies have shown that the upper limit of IEL number in the proximal small intestine is 25 IELs/100 epithelial cells[34,35]. In the absence of villus architectural changes, an increased number of IELs can be related to reactions to intraluminal antigens such as *H pylori*[35] and is considered as the result of an immune response analogous to the response to gluten in patients with celiac disease[36-38]. This is confirmed in our study with the demonstration that *H pylori* positive patients had higher IEL counts than healthy controls and *H pylori* negative patients. This quantitative difference in the number of IELs between our two sub-groups of

dyspeptic patients is an indirect argument to validate that the patients considered in this study as *H pylori* negative dyspeptics were really negative. Indeed, this negative status can be discussed as we did not perform *H pylori* serology (including CagA status) in all patients.

We have confirmed that, like in healthy controls, duodenal IELs in dyspeptic patients are predominant CD3+ CD8+ lymphocytes (> 70% of small bowel IELs)[39-41]. According to a previous study, a significant difference was seen in patients with *H pylori* gastritis who had an increased of CD8+ CD3+ IEL counts compared with healthy controls[42]. The distribution of CD3+ CD4+ IELs was not different between dyspeptic patients and healthy controls. TCR-γδ CD3+ lymphocytes have been investigated because of their potential role in immune-mediated hypersensitivity *via* recognition of a variety of both non peptidic and peptidic antigens without requirement for antigen processing/presentation[12]. The median TCR-γδ+ CD3+ percentages are similar to those of normal gut[30,40-44]. Adhesion molecules CD44 and CD103 have also been investigated since they are recognized as co-stimulatory molecules which may regulate interactions between IELs and epithelial cells[45,46]. No difference in their membrane expression was observed between IELs from dyspeptic patients and healthy subjects. In this study, CD44, which belongs to a family of proteins involved in T cell activation and lymphocyte homing to the gut was expressed by the majority of CD3+ IELs as previously reported[13,19,47], and CD103, which is up-regulated in epithelial T lymphocytes and contributes to binding and/or signaling between IELs and epithelial cells was also expressed by most IELs, which is in agreement with previous reports[13,41,48]. Ratios of CD3+ IELs expressing the co-stimulation molecule CD28 were low in both dyspeptic and healthy individuals, which is in agreement with previous reports on freshly isolated small intestinal IELs[13,19,49,50]. As reported in previous studies, most CD3+ IEL expressed CD69, suggesting a "quasi-activated" phenotype in both dyspeptic and healthy adults[13,46,48,51]. Nevertheless, in our study, we could not exclude that differences between duodenal IELs from dyspeptic patients and healthy controls could be partly due to an uncontrolled diet in order to prevent the onset of symptoms following ingestion of some specific nutrients by FD patients. Indeed, the immune responsiveness following oral administration of an antigen is dose-dependent, and depending on dose feeding could lead to clonal anergy or clonal deletion[52]. Shut-down effects of antigen-activated IEL migration to the antigen-sensitized mucosa could alter the population of these antigen-reactive T-cells in the mucosa[53].

Considering the small number of investigated patients and the great scattering of individual values for the two activation markers found to be altered, our findings warrant future studies to determine whether *H pylori* negative functional dyspepsia is, at least partly, immune-mediated and to ascertain a role of IELs in FD.

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