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ORIGINAL ARTICLE

Cell Density Counts of the Intestinal Intraepithelial Lymphocytes in the Celiac Patients

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ABSTRACT

Background: Increased number of intestinal intraepithelial lymphocytes (IELs) is a key histological finding in the diagnosis of celiac disease (CD); however, the number of IELs in celiac patients and healthy subjects may vary from one region to another. Additionally, there are some seronegative celiac patients with a borderline histology. **Objective:** To determine the number of the $CD3^+$ and $CD8^+$ IELs T-cells in the celiac patients and healthy subjects (controls) in Isfahan. **Methods:** The duodenal biopsies were obtained from the celiac patients (n=15) and the controls (n=19). The total number of IELs/100 epithelial cells (ECs) were counted using the hematoxylin-eosin (H&E) staining method, and that of $CD3^+$ and $CD8^+$ IELs/100 ECs were counted using the immunohistochemistry (IHC) staining method. **Results:** This study defined the upper normal limit for each variable as mean + 2SD. Accordingly, the upper normal limits of the total IELs, $CD3^+$ IELs, and $CD8^+$ IELs/100 ECs were calculated as 37 (95% confidence intervals, CI: 33–41), 22 (95% CI: 19–25) and 12 (95% CI: 10–14), respectively. In 3 clinically CD diagnoses, the total IELs counts/100 ECs were below the upper normal limit, and the histopathological and serologic assays were negative. Nevertheless, the $CD8^+$ IELs T-cells counts/100 ECs showed borderline values. Interestingly, these patients responded to a gluten-free diet (GFD). **Conclusions:** The study findings suggest that in the clinically diagnosed celiac disease, IELs count/100 ECs below the upper normal limit as well as negative histopathological and serologic assays and the cell density counts of the $CD8^+$ IELs T-cells/100 ECs could be a useful parameter for CD diagnosis and make a decision to put them on a GFD.

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INTRODUCTION

Celiac disease (CD) is a chronic, immune-mediated, gluten-induced gut disorder manifesting itself with a series of clinical symptoms in people with genetic susceptibilities. The immune system may react to gliadin and glutenins from wheat, barley, and rye (1). CD occurs in 1% of adults and children in the United States and Europe. It is one of the most common gastrointestinal diseases worldwide (2). In Iran, however, where this study has been conducted, the prevalence of CD has been reported as 1 per 104 to 167 adults (3,4). Intestinal intraepithelial lymphocytes (IELs) comprise phenotypically heterogeneous lymphoid subsets and are abundant lymphocyte populations in the body. More than 70% of IELs are $CD3^+$ T-cells (90% $CD8^+$ and 10% $CD4^+$), and most of which express the TCR-α/ß (5). Increased number of IELs is a key histological finding in CD as well as in other gut-associated diseases, such as enteropathy-associated T-cell lymphoma (6). Increased number of IELs within the intestinal mucosa is the most sensitive pathologic change observed in celiac patients (7); however, available literature suggest a wide range of 5-60 IELs per100 epithelial cells (ECs) in celiac patients from different countries. For example, Ferguson *et al*. reported 40 IELs/100 ECs as the upper normal limit (8), whereas in a more recent study in Sweden, Veress *et al*. showed 20 IELs/100 ECs on the hematoxylin-eosin (H&E) stained sections and 25 IELs/100 ECs on the immunohistochemistry (IHC) staining method, as the upper normal limit (9). A study in the United Kingdom reported 22 IELs/100 ECs as the upper normal limit (10). Another study counted IELs in the tip of the villi and confirmed the increased number of IELs as a diagnostic marker for CD (11). These studies used diverse settings for sample selection and methodology; therefore, a cut-off level may vary according to the IELs subsets, genetic backgrounds, environmental pathogeneses, dietary habits, and the socioeconomic situation. It has been hypothesized that the number of IELs in healthy subjects may vary in each region of the world. Therefore, it is worthwhile to determine the normal number of IELs in each region, and then to measure the subtypes of IELs in patients suspected to CD. This study aimed to determine the frequency of the $CD3^+$ and $CD8^+$ IELs T-cells in the celiac patients and healthy subjects (controls) in Isfahan, Iran.

MATERIALS AND METHODS

Patients. In this case-control study, 15 celiac patients were referred to the Poursina Hakim Endoscopy Department, Isfahan, Iran, from January 2015 to December 2016. CD was diagnosed, based on the criteria specified by the American College of Gastroenterology (ACG), the presence of clinical exhibitions, CD-related antibodies, including celiac-specific serum IgA endomysial antibody (EMA) and tissue-type transglutaminase IgA (tTG-IgA), as well as Marsh histopathological classification (12).
The inclusion criteria were also as follows: only patients aged 15 years or over who presented characteristic signs such as chronic diarrhea, anemia, weight loss, nausea, vomiting, abdominal distension and pain, hypoalbuminemia, stunting of growth, malabsorption, and signs of nutrient or vitamin deficiency. Patients who met the inclusion criteria were enrolled successively, and their written informed consent was obtained, and were asked them to be on a gluten-free diet (GFD). No patient was treated with a GFD before the serological, immunological, and histopathological examinations.

The exclusion criteria were as follows: patients with an allergic disease, parasitosis, infection conditions, inflammatory bowel disease (IBD), previous gastrointestinal surgery, unknown disease of the gastrointestinal region, regular use of non-steroidal anti-inflammatory drugs (NSAIDs) (at least, one regular dose of any NSAIDs per week over the last four weeks), immune deficiency syndrome, cancer, allograft transplantation, and those, who were reluctant to donate a blood sample and undergo endoscopy.

Controls. There were 19 controls who suffered from the symptoms of dyspepsia/heartburn without diarrhea, and underwent small intestinal endoscopy and showed a normal histology. They had also negative EMA and tTG assays. None of the controls had a family member/relative with celiac disease. Furthermore, none of the controls had any autoimmune or allergic diseases. Five milliliters of the venous blood were drawn from each subject; sera were separated and kept at -20° C for further assays.

Sampling and Staining. The duodenal biopsy specimens were taken from the second part of the duodenum and placed into a container of 10% fresh buffered formalin, whereupon they were processed and then embedded in paraffin. Since the histopathologic changes can be patchy in CD, four biopsies were obtained to maximize the probability of finding any histopathologic changes. Tissues were sectioned at 5 µm and mounted onto slides pre-coated with Poly L-Lysin (PLL) using a Microtome Cryostat (HM 500 OM, Microm Heidelberg) operated at -22ºC.

H&E Staining Method. One slide underwent the H&E staining method. In brief, after heat-fixation of the sections, while they were still warm, they were dewaxed twice in xylene for 10 minutes, and rehydrated via 100% ethanol into distilled water. Counterstaining was carried out by light staining of the slides with undiluted Lillie Mayer's hematoxylin (alcohol containing counterstain). The slides were mounted using Entelan and viewed under a microscope fitted with an eye-piece graticule for cell density counts of IELs.

IHC Staining Method. In brief, other sections of biopsies, after heat-fixation, while they were still warm, were dewaxed twice in xylene for 10 minutes, and rehydrated via 100% ethanol into distilled water prior to antigen retrieval. Antigen retrieval was carried out using 10 mM Tris and 1 mM ethylenediaminetetraacetic acid (EDTA) at 90° C in a warm bath for 20 minutes. Before incubation with primary antibody, the sections were incubated with 10% goat serum to block the nonspecific bindings for 30 minutes and then with 3% H_2O_2 to block the endogenous peroxidase activity. Next, the sections were stained with primary antibodies, including monoclonal mouse anti-human CD3 (Clone F7.2.3, IgG1, Dako Company) at 1/50 dilution at room temperature for 1 hour, or monoclonal mouse anti-human CD8 (Clone C8/144B, IgG1, Dako Company) at 1/500 dilution at room temperature for 1 hour. The sections were subsequently incubated with polyclonal goat anti-mouse Ig G conjugated to horseradish peroxidase (HRP) at room temperature for 30 minutes and developed with 3, 3'-diaminobenzidine tetrahydrochloride substrate (DAB). Subsequently, the slides were rinsed in phosphate-buffered saline (PBS), two fast dips in 0.6% acid-alcohol were applied, were washed for 30 seconds in tap water, were blued in Scott's solution for 10 seconds, and a quick dip in distilled water was applied to remove excess salts, respectively. Dehydration involved 2 minutes in each of two 50%, 70%, 95%, and 100% ethanol baths, and the slides were cleared for 10 minutes in each of the two xylenes. Counterstaining was carried out by light staining of the slides with undiluted Lillie Mayer's hematoxylin (alcohol containing counterstain). The slides were mounted using Entelan and viewed under a microscope fitted with an eye-piece graticule for cell density counts of IELs.

Definition of Marsh Classification and Quantification of the Duodenal IELs. All biopsy samples were examined by a gastro-intestinal disease-experienced pathologist, who was blinded to the patients' clinical and laboratory data. Marsh I is the infiltrative type characterized by a normal villous architecture, a normal height of the crypts and an increase in IELs number. Marsh II is the hyperplastic type characterized by a normal villous architecture, an increase in IELS number and crypt hyperplasia. Marsh III signals the so-called 'destructive' type of the CD lesion, and is characterized by a mild villous atrophy to villous flattening and crypt hypertrophy (12). The H&E stained sections as well as positively stained CD3 and CD8 T-cells were enumerated per 389 µm of the mucosa, using a calibrated linear microscopic graticule (389 µm, x25 objective lens) aligned along the muscularis mucosae from two successive intestinal segments per each subject. The average of ten positively IELs counts was calculated per 100 ECs in the whole villus. The average of 500 ECs counts was also calculated.

Statistical Analysis. Statistical analysis was performed using the SPSS 19 statistical package (SPSS, Chicago, IL, USA). Data were expressed as mean values ± SD of the mean, and statistical comparisons are performed using the Student's t-test. The upper normal limit for the number of the total, the $CD3^+$, and $CD8^+$ IELs T-cells/100 ECs was defined as "mean of the control group + 2 standard deviations". Consequently, values below the upper normal limit with 95% confidence intervals (CI) were defined as normal; those between the upper normal limit and the mean of patient group were defined as borderline; and those higher than the mean of the patient group with 95% CI were considered increased. Pearson's correlation coefficients were used to measure the linear relationship between the variables.

Ethical Considerations. This study was carried out in accordance with the ethical guidelines of the 1975 declaration of Helsinki as reflected in a prior approval by the Institution's Human Research Committee. Accordingly, the Ethical Committee of Isfahan University of Medical Sciences, and the Iranian Celiac Association approved the study protocol, and the participants gave their written informed consent once the researchers had explained to them the study aims and protocol.

RESULTS

Patients and controls.

Fifteen untreated celiac patients (5 males, 10 females, age range: 15-62 years, the mean age 33.6 years, Table 1) and 19 controls (9 males, 10 females, age range: 16-65 years) were studied. Early histopathological examination of the duodenal biopsy samples showed normal villous morphology with IELs infiltration in 11 out of 15 celiac patients (Marsh I), and a flat mucosa in 4 out of 15 celiac patients (Marsh III, Table 1 and Table 2).

Results of H&E and IHC staining.

H&E staining results indicated that the frequencies of the duodenal total IELs/100 ECs were significantly higher in the celiac patients than in the controls (40 ± 11, ranging from 25 to 55 and 19 ± 9, ranging from 9 to 35, respectively, $p<0.0001$). In addition, IHC staining results demonstrated significantly higher frequency of the duodenal $CD3^+$ IELs T-cells/100 ECs in the celiac patients than in the controls (31 ± 12, ranging from 15 to 57, and 10 ± 6, ranging from 3 to 22, respectively, $p<0.0001$) (Figure 1 A, B and Figure 2). IHC staining results also revealed that the frequency of the duodenal $CD8^+$ IELs T-

Table 1. Baseline demographic, Marsh classification and findings of the serological assays in the celiac patients.

Number of patients	Sex	Age (years)	Marsh classification	tTg IgA (U/ml)	tTG IgG (U/ml)	EMA
1	F	26	I	10	1	+
2	F	42	I	1	1	+
3	F	34	I	13	13	+
4	M	22	I	380	40	+
5	F	19	I	1	60	+
6	M	28	I	ND	ND	+
7	F	35	III	ND	ND	+
8	M	15	III	13	1	+
9	F	52	I	25	1	+
10	F	62	I	83	5	+
11	M	27	I	ND	ND	+
12	F	46	III	2	3	+
13	F	46	I	ND	ND	+
14	F	29	III	1	49	+
15	M	21	I	12	19	+

tTG: Tissue-type transglutaminase IgA (tTG-IgA); EMA: IgA endomysial antibody; F: Female; M: Male; ND: Not determined.

cells/100 ECs in the celiac patients was significantly higher than that in the controls (23 ± 10, ranging from 13 to 45, and 6 ± 3, ranging from 3 to 12, respectively, P<0.0001) (Figure 1 C, D and Figure 2). Moreover, frequencies of the total, $CD3^+$, and $CD8^+$ IELs T-cells/100 ECs were correlated with each other (Figure 3).

Table 2. Marsh classification of the duodenal biopsies.

Marsh classification	Age (years)	H&E staining IELs/100 ECs	IHC staining	
			$CD3^+$ IELs /100 ECs	$CD8^+$ IELs /100 ECs
Marsh 0 (No CD) (n=19)	16-65 (36.68 ± 14.76) (31)	9-35 (18.94 ± 9.33) (16)	3-22 (9.89 ± 5.97) (7)	3-12 (6.43 ± 2.85) (5.5)
Marsh I (n=11)	19-62	25-55	15-46	13-35
Marsh III (n=4)	15-46 (33.6 ± 13.44) (29)	26-55 (39.64 ± 10.93) (40)	21-57 (30.81 ± 12.66) (26)	13-45 (23.2 ± 10.14) (21)

H&E: Hematoxylin and eosin, ECs: Epithelial cells, CD: Celiac Disease, Below of the ranges are mean ± SD and median, respectively.

Table 3. Suggested normal, borderline and pathological values of the IELs.

	Normal	Borderline	CD
Total IELs/100 ECs	≤37	38–40	>40
CD3$^+$ IELs/100 ECs	≤22	23–31	>31
CD8$^+$ IELs/100 ECs	≤12	13–23	>23

Cutoff values of the duodenal IELs in CD.

The upper normal limit of the total, CD3$^+$, and CD8$^+$ IELs T-cells counts/100 ECs were defined as cell density counts of the controls + 2SD. Values ≤ the upper normal limit with 95% CI were defined as normal; those between the upper normal limit and the mean of patient group were defined as borderline; and those ≥ the mean of patient group with 95% CI were considered CD (Table 3).

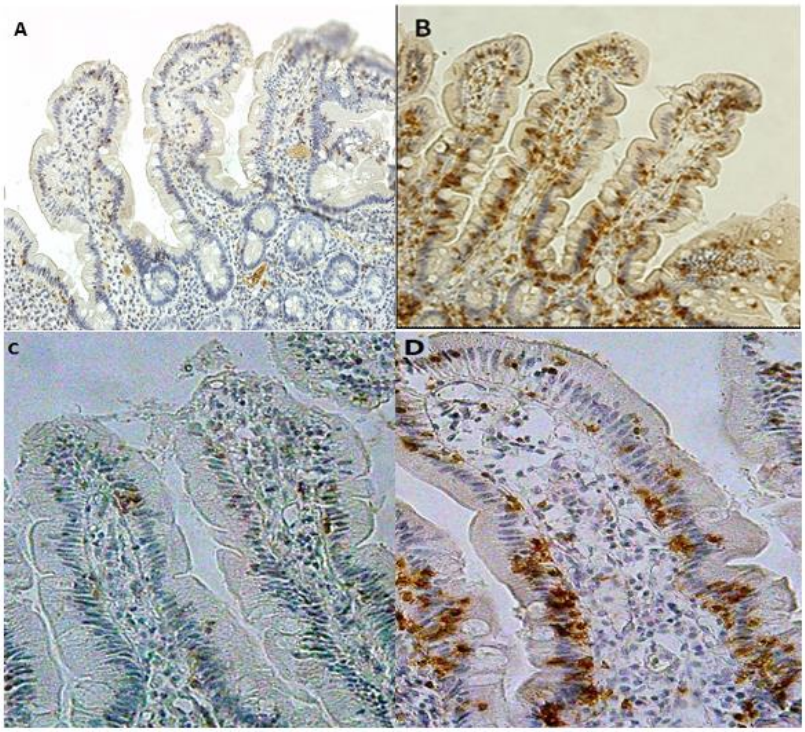

Figure 1. IHC staining results. The data showed the frequency of CD3$^+$ IELs T-cells in the duodenum of a healthy subject (A) and in a celiac patient (B) as well as the frequency of CD8$^+$ IELs T-cells in the duodenum of a healthy subject (C) and in a celiac patient (D). (Original magnification A and B×100, C and D×400).

Accordingly, the upper normal limit for total, CD3$^+$, and CD8$^+$ IELs/100 ECs was calculated as 37 (95% CI: 35–40), 22 (95% CI: 14–30), and 12 (95% CI: 3–23), respectively. For 3 clinically diagnosed celiac patients, though the total IELs counts/100 ECs were below the upper normal limit and negative histopathological and serologic results, CD8$^+$ IELs T-cells count/100 ECs showed borderline values, and interestingly these patients responded to a GFD.

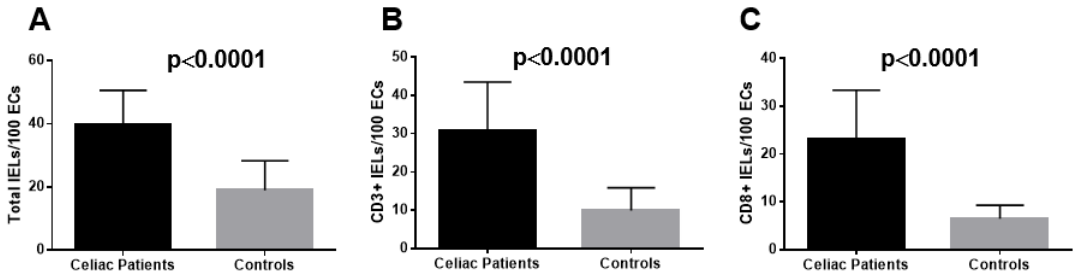

Figure 2. IHC staining results showed the cell density counts of the total, CD3$^+$, and CD8$^+$ IELs T-cells/100 ECs in the celiac patients and controls.

Comparison between H&E and IHC staining methods.

The study results indicated a positive correlation between the findings of two H&E and IHC staining methods, using the total cell counts of the CD3$^+$ and CD8$^+$ IELs T-cells (Figure 3).

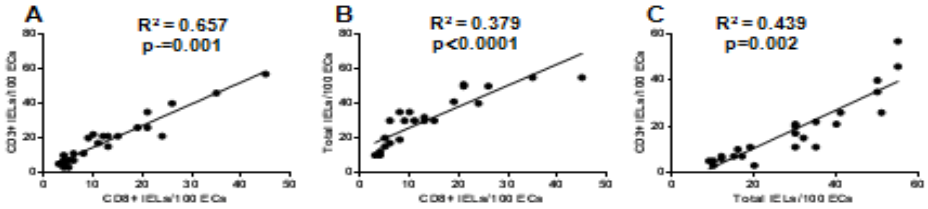

Figure 3. Positive correlation between the findings of H&E and IHC staining methods using the total cell counts of the CD3$^+$ and CD8$^+$ IELs T-cells (The dots in the graphs are representative of a combination of the controls and patients' samples).

DISCUSSION

This study, for the first time, compared the frequency and phenotype of the duodenal IELs in the celiac patients and the controls in Isfahan, Iran. Moreover, this study used the IHC staining method to better count the number of the IELs, thereby improving the diagnostic value of the IELs counts in the celiac patients, particularly in patients with ambiguous clinical and pathological manifestations. This study showed that the cell density counts of IELs expressing the CD3 and CD8 markers were significantly higher in the celiac patient than in the non-celiac subjects. This partially confirms this notion that an increase in the cell density counts of the CD3$^+$ IELs T-cells is indicative of CD, and particularly an increase in the cell density counts of CD8$^+$ IELs T-cells further supports the possibility of the gluten intolerance. CD has been a focus of interest over the past two decades since being considered a disease with a spectrum of clinical reports, ranging from an asymptomatic condition to an overt malabsorption. Available serologic tests have added much to our understanding and diagnostic capabilities toward CD (13); however, histologic evaluation of the small intestine still play a key role in diagnosis and follow-

up. In particular, increased number of IELs is considered one of the earliest histological changes in CD (13,14). Therefore, definition of a normal range for IELs counts is extremely important. This study indicated that the cell density counts of IELs/100 ECs after counting of 500 ECs of the whole villous were 19/100 and 40/100 in the controls and celiac patients, respectively. This finding agrees with the findings achieved by Kakar *et al*., Nasseri-Moghaddam *et al*. and Walker *et al*., who reported the cell density counts of IELs/100 ECs 40, 34 and 33/100, respectively (15). However, Hayat *et al*. reported the mean frequency of IELs/100 ECs as 25/100 (16). Mahadeva *et al*. also reported the cell density counts of the duodenal IELs/100 ECs as 22/100 (10). As mentioned above, the reported normal ranges differ substantially, ranging from 22 to 40 IELs/100 ECs.

The differences observed between the studies may reflect the use of various methods, as well as different sample sizes and populations. Furthermore, the mean frequency of the IELs/100 ECs may be different in various parts of the world owing to different racial and environmental backgrounds. This study showed that the cell density counts of the $CD3^+$ IELs T-cells/100 ECs in the celiac patients was 31, and the upper normal limit of duodenal mucosa was 22/100. The cell density counts of the $CD3^+$ IELs T-cells/100 ECs in the celiac patients was reported as 60/100 in Dublin, Ireland (17), 40/100 in Newcastle, Australia (18), 26/100 in Nile Delta, Egypt (19), 16/100 in Haifa, Israel (20), and 38/100 in Paris, France (21). This study also showed that the cell density counts of the $CD8^+$ IELs T-cells/100 ECs were 23/100 in the celiac patients, and the upper normal limit for the duodenal $CD8^+$ IELs T-cells was 12/100 ECs. Several studies have indicated that the cell density counts of the duodenal $CD8^+$ IELs T-cells/100 ECs vary, ranging from 14, 20 and 24/100 (17, 19-21). The investigators have reported different cell density counts of the duodenal $CD8^+$ IELs T-cells/100 ECs. The present study demonstrated that measurement of the $CD8^+$ IELs T-cells could be useful in CD diagnosis and consequently administration of a GFD to the patients. Generally, patients with clinical symptoms of celiac, who do not show positive histopathological or serologic results, are considered Marsh I. In this study, 11 patients were Marsh 1. The cell density counts of the total, $CD3^+$, and $CD8^+$ IELs T-cells/100 ECs showed that, although the number of total and $CD3^+$ IELs T-cells/100 ECs in three of these patients was below their defined upper normal limit, the number of the $CD8^+$ IELs T-cells/100 ECs showed the borderline values. This indicates that the cell density counts of the $CD8^+$ IELs T-cells in the duodenal biopsy samples in the celiac patients could be a useful tool to differentiate the celiac from non-celiac patients and make a decision to put them on a GFD. The study limitations were the limited number of celiac patients and controls. Nevertheless, the study strength points are the novelty of considering the total number of IELs/100 ECs as well as the cell density counts of the $CD3^+$ and $CD8^+$ IELs T-cells/100 ECs in the controls and celiac patients in Isfahan city. In conclusion, the study findings suggest that in the clinically diagnosed CD IELs count/100 ECs below the upper normal limit as well as negative histopathological and serologic assays and the cell density counts of the CD8+ IELs T-cells/100 ECs could be a useful parameter for CD diagnosis and make a decision to put them on a GFD. However, the present study employed a limited number of patients and controls; therefore, a higher number of subjects are needed to examine the importance of cell density counts of the $CD3^+$ and $CD8^+$ IELs T-cells/100 ECs in CD diagnosis.

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