The Normal Range of Duodenal Intraepithelial Lymphocytes

Siavosh Nasseri-Moghadam MD*, Azadeh Mofid MD*, Mehdi Nouraie MD PhD*, Behnoosh Abedi MD*, Akram Pourshams MD*, Reza Malekzadeh MD*, Masoud Sotoudeh MD**

Background: Increased duodenal intraepithelial lymphocytes is a key histological finding in celiac disease. Available studies suggest a wide normal range (10 – 40 intraepithelial lymphocytes /100 epithelial cells, EC). We assessed the normal range of distal duodenal intraepithelial lymphocytes.

Methods: Biopsies from the normal-looking distal duodenum from individuals referring for endoscopy for reasons other than intestinal pathologies were stained with hematoxylin-eosin and immunohistochemistry (immunohistochemistry, CD45). At least 1000 cells from the tip and body of the villi were assessed. Villous height to crypt depth ratio was calculated.

Results: Fifty individuals were enrolled. Four were excluded (inadequate biopsies). Mean intraepithelial lymphocyte count was similar in villous tip and body. In the whole villous, upper limit of normal (mean+2SD) was 35 intraepithelial lymphocytes/100EC (95%CI: 31 – 39) in immunohistochemistry and 34 intraepithelial lymphocytes/100EC (95%CI: 29 – 37) in H&E staining. The villous height to crypt depth ratio was 3.9. Hematoxylin-eosin and immunohistochemistry had excellent agreement.

Conclusion: Intraepithelial lymphocyte counts less than 35/100EC in IHC and 34/100EC in hematoxylin-eosin staining can be considered normal. Counts between 36-39 (immunohistochemistry) and 35 – 37 (hematoxylin-eosin) are borderline and more than 39 (immunohistochemistry) and 37 (hematoxylin-eosin) are increased. The hematoxylin-eosin staining method seems adequate for clinical purposes. Regional differences in the normal upper limit for intraepithelial lymphocytes as well as changes over time should be considered when interpreting duodenal biopsies.

Keywords: Celiac disease ● duodenum ● intra-epithelial lymphocytes ● normal range

Introduction

Intraepithelial lymphocytes (IEL) are normal inhabitants of the intestinal epithelium.\(^1\) Increased numbers of IEL (intraepithelial lymphocytosis) are seen in conditions such as celiac sprue, refractory sprue, enteropathy associated T-cell lymphoma, microsporidiosis, and protein loosing enteropathy due to food allergy in children.\(^2,3\) Recently it has also been described in patients harboring Helicobacter pylori (HP) in their stomach,\(^4\) as well as those taking non-steroidal anti-inflammatory drugs (NSAIDs).\(^5\)

Celiac sprue is common in various parts of the world. Its prevalence is one per 104 to 167 adults in Iran,\(^6,7\) and it is considered to be the most common cause of chronic diarrhea as well.\(^8\) Prompt diagnosis is sometimes problematic, especially in oligo-symptomatic or asymptomatic cases.

Increased numbers of IEL is the earliest and probably the most sensitive pathologic change seen in celiac disease.\(^9\) Studies addressing the normal range for IEL are few and some date back to several decades ago. For instance Ferguson et al. reported 40 IEL per 100 nucleated cells as the
upper limit of normal in 1971,\textsuperscript{10} while in a more recent study, Veress et al. found 20 IEL/100 epithelial cells (EC) on hematoxylin-eosin (H&E) stained sections and 25 IEL/100EC on immunohistochemistry (IHC) as the upper limit of normal in Sweden.\textsuperscript{11} Other investigators have found different numbers. Mahadewa et al. reported 22 IEL as the upper limit of normal.\textsuperscript{12} Biagi et al. counted IEL in the tip of the villi and stated that it was sufficient for making a diagnosis of increased IEL.\textsuperscript{13}

These studies have diverse sample selections and methodologies, therefore a uniform conclusion is lacking. We hypothesized that normal IEL numbers may be related to environmental exposures and ethnic background and differ from region to region and along time. If this holds true, the diagnosis of celiac disease according to IEL number in the distal duodenum should be made considering these differences. Therefore, we determined the normal range for IEL of the distal duodenal mucosa on H&E stained sections and compared it with IHC among Iranians.

**Materials and Methods**

**Patients**

Consenting patients who referred to the Shariati hospital upper endoscopy unit on Saturday and Monday afternoons (working days of two of the authors, SNM and AP), during the period of October 1, 2004 to March 1, 2005 were enrolled successively if they met the inclusion criteria (Table 1).

Exclusion criteria were as follows: history of chronic diarrhea (defined as loose or frequent stools for more than four weeks), malabsorption, iron deficiency anemia, osteoporosis, regular use of NSAIDs (at least one regular dose of any NSAIDs per week over the last four weeks), or if any of these criteria were found on detailed history, physical examination, or initial laboratory evaluation.

The laboratory tests included complete blood count (CBC), serum iron, total iron binding capacity (TIBC), serum ferritin, serum albumin, serum calcium, phosphorus, triglyceride, cholesterol, and stool examination for occult blood, ova, and parasites.

A questionnaire covering demographic data and patients’ symptoms and medication use was filled in. Five milliliters of venous blood were drawn, sera of which were separated and kept at -20°C for further IgA anti-tissue transglutaminase (t-TG, rabbit anti-human IgA, GD71/72, Genesis, Cambridgeshire, UK) and total serum IgA measurement. In addition, the sera were checked for anti-HP IgG antibody (GD01, Genesis, Cambridgeshire, UK).

**Sampling and staining**

The patients then underwent upper gastrointestinal (GI) endoscopy performed on left lateral position with local pharyngeal anesthesia. In addition to recording all gross findings, four biopsies were taken from the second portion of the duodenum, oriented on filter paper, fixed in 10% formalin and paraffin blocked. As the histopathologic changes can be patchy in Celiac disease (CD)\textsuperscript{14} we obtained four biopsies to maximize the chances of finding any histopathologic changes. Four-micrometer thick sections were then prepared. One slide was stained with H&E and another was used for IHC. The IHC sections were fixed on poly L-Lysine inoculated slides and stained with stigmatized monoclonal antibody for leukocyte common antigen (LCA, CD45) \{Monoclonal Mouse Anti-Human CD45, Leukocyte Common Antigen, Clones 2B11+PD7/26, Code N./Code/Code-Nr. M 0701, DakoCytomation, Denmark\}. Background was stained with hematoxylin (Figures 1 and 2). A

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**Table 1. Inclusion criteria for enrollment**

- 16 years or older
- Consenting to participate in the study
- Endoscopy being done for any reason except to evaluate malabsorption or intestinal disorders
- Absence of signs or symptoms suggestive for malabsorption or intestinal disorder

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**Figure 1.** Villous tip in H&E stained sections (a). Villous body in H&E stained sections (b). (original magnification is 400)
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A sample was considered adequate when it was well-oriented and was not lacerated during processing.

Sample review

An expert pathologist reviewed the H&E slides to exclude those having gross evidence of malabsorption e.g., villous atrophy and crypt hyperplasia. He also screened for infiltration of leukocytes other than lymphocytes in the epithelium, as the former stain for LCA and can be mistaken for lymphocytes on IHC. The height of the villi and the depth of the crypts were measured using a graded ocular lens and the ratio was determined.

The epithelium covering areas of stroma with lymphocyte aggregations were not used for IEL counting in order to avoid false overestimation. We tried to carefully distinguish the dense triangular nuclei of goblet cells from the cytoplasm-rich lymphocytes in H&E slides to avoid mistaking them for IEL.

In the IHC slides, well-defined completely positive stained rings between epithelial cells were recognized as lymphocytes and the brown granules in the epithelium were not counted. Only the lymphocytes above the basal membrane were assumed as IEL. At least 500 – 600 nucleated cells in the villous tip and 500 – 600 nucleated cells in villous body were counted in a continuous length of the epithelium in well-oriented villi. Therefore, we counted at least 1000 – 1200 epithelial cells and intervening lymphocytes in a total villous to maximize our accuracy. The same procedure was done for IHC slides separately. The IHC stained sections were studied blindly and without any information about the H&E results. Then we calculated the mean number of lymphocytes per 100 epithelial cells in the tip of the villous, in the body of the villous, and in the complete villous for H&E and IHC slides separately (six groups overall). Villous height to crypt ratio was determined semi quantitatively comparing IHC and H&E sections.

Statistical analysis

Mean, median, range, standard deviation, and interquartile range for each of the six aforementioned groups were calculated. We examined the normal distribution of IEL in each group by one sample Kolmogrov-Smirnov test. The upper limit of IEL was defined as "mean+2SD" in each group, 95% confidence interval (CI) was calculated and the numbers falling within the upper limit of the 95% CI were considered as borderline. Anything higher than the upper limit of the 95% CI was accounted as abnormal.15 We also calculated 99% confidence interval for "mean+3SD".

We compared the two staining methods by paired t-test for the difference in IEL count and "least products regression model" to estimate the fixed and random errors.16 The latter method of analysis is proposed as the current best choice for agreement studies in quantitative variables. We used the SPSS software version 12.5 (SPSS Incorp. Chicago, IL) for statistical analysis.

The study was approved by the ethics committee of the Digestive Disease Research Center affiliated to Tehran University of Medical Sciences.

Results

Fifty individuals (27 females) were enrolled. The reason for undergoing endoscopy was heartburn in 48 individuals and looking for esophageal varices in two. Median age was 40 years (range: 17 – 53 years). Four cases were excluded because the samples were either inadequately oriented or were distorted during processing (one on H&E stain and three on IHC). Forty-six cases had adequate duodenal biopsies with both staining methods. All sera were negative for IgA anti-tissue transglutaminase and none showed IgA deficiency. Thirty-nine patients (of the 46 with adequate biopsies, 84.7%) had positive HP serology. Mean IEL on IHC and H&E stains were not different between HP positive and negative patients.
The H&E stain

Mean IEL count per 500 – 600 nucleated cells in the tip of the villous was 19 (range: 7 – 38), in the body of villous was 18 (range: 5 – 38) and in the whole villous was 19 (range: 6 – 38). Upper normal limit for the number of IEL per 100 EC (Mean+2SD) was 34 in villous tip (95% CI: 30 – 38), 34 in villous body (95% CI: 29 – 38) and 34 in the whole villous (95% CI: 29 – 37), (Table 2). The number of IEL ± 3SD and 99% CIs are also shown in Table 2. As depicted in Table 3, the upper limit for IEL in the whole villous on H&E stained sections is 34, and 35 – 37 IEL/100EC can be considered as borderline and counts above 37 is pathologic.

The IHC stain

Mean IEL count per 500 – 600 nucleated cells in the tip of the villous was 23 (range: 9 – 52), in the body of villous was 21 (range: 8 – 34) and in the total villous was 21 (range: 9 – 38). Upper normal limit for the number of IEL per 100 EC (mean+2SD) was 41 in villous tip (95% CI: 35 – 45), 34 in villous body (95% CI: 30 – 38) and 35 in the whole villous (95% CI: 31 – 39), (Table 2).

For the IHC stain, the upper limit of normal in the whole villous was 35 IEL/100EC. And 36 – 39 was borderline and any count above 39 IEL/100EC counted as pathologic (Table 3).

Comparison of the two staining methods and different villous sites

The number of IEL/100EC in the whole villous was slightly higher in IHC than H&E stains (21 vs. 19, P=0.006). There was no difference between the number of IEL in the tip and body of the villous with either staining methods. Figures 3 – 5 show the agreement between the two methods of staining in the “least product regression model”. As shown in these figures, the two methods showed excellent agreement with regression lines of 41 to 48 degrees. Mean normal value for “villous height” to “crypt depth” ratio was 3.9 (SD: 0.8, range: 2.0 – 5.3).

Discussion

Histologic structure of the gut is well known, but the details useful for management of some diseases have remained controversial. Celiac disease has been the focus of interest over the past two decades as a disease with a spectrum of clinical presentations, ranging from asymptomatic to overt malabsorption. It has been shown that it is much commoner than previously thought. Available serologic tests have added much to our understanding and diagnostic capabilities for this disease. But histologic evaluation of the small bowel is still central for verification of the diagnosis and further follow-up. Increased IEL are considered as the earliest histological change.9, 22, 23 In addition, gluten challenge in patients with symptoms suggestive for malabsorption and intraepithelial lymphocytosis has been shown to change the histopathologic findings towards higher Marsh stages in almost a third of the patients.24 Therefore establishing the normal range for IEL is

Table 2. Characteristics of IEL count per 100 EC with different staining methods and at different sites.

<table>
<thead>
<tr>
<th>Staining Method</th>
<th>Mean (min-max)</th>
<th>SD</th>
<th>Mean+2SD</th>
<th>95% CI</th>
<th>Mean+3SD</th>
<th>99% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&amp;E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tip</td>
<td>19(7–38)</td>
<td>7.7</td>
<td>34</td>
<td>30–38</td>
<td>42</td>
<td>36–47</td>
</tr>
<tr>
<td>Body</td>
<td>18(5–38)</td>
<td>7.8</td>
<td>34</td>
<td>29–38</td>
<td>42</td>
<td>35–46</td>
</tr>
<tr>
<td>Whole villous</td>
<td>19(6–38)</td>
<td>7.5</td>
<td>34</td>
<td>29–37</td>
<td>41</td>
<td>35–46</td>
</tr>
<tr>
<td>IHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tip</td>
<td>23(9–52)</td>
<td>9</td>
<td>41</td>
<td>35–45</td>
<td>50</td>
<td>43–56</td>
</tr>
<tr>
<td>Body</td>
<td>21(8–34)</td>
<td>6.8</td>
<td>34</td>
<td>30–38</td>
<td>41</td>
<td>35–43</td>
</tr>
<tr>
<td>Whole villous</td>
<td>21(9–38)</td>
<td>6.9</td>
<td>35</td>
<td>31–39</td>
<td>42</td>
<td>37–47</td>
</tr>
</tbody>
</table>

Table 3. Recommended normal values for IEL at different sites.

<table>
<thead>
<tr>
<th>Staining Method</th>
<th>Upper normal limit</th>
<th>Borderline</th>
<th>Pathologic</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&amp;E</td>
<td>Tip ≤34 35–38 &gt;38</td>
<td>Body ≤34 35–38 &gt;38</td>
<td>Villous ≤34 35–37 &gt;37</td>
</tr>
<tr>
<td>IHC</td>
<td>Tip ≤41 42–45 &gt;45</td>
<td>Body ≤34 35–38 &gt;38</td>
<td>Villous ≤35 36–39 &gt;39</td>
</tr>
</tbody>
</table>

H&E=hematoxylin-eosin staining, IHC=immunohistochemistry.
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of great importance.

Considering the high exposure of the gut to environmental antigens and its variable response to them according to the genetic and ethnic backgrounds, it sounds logical that this normal range may differ in different areas according to genetic background and environmental exposures. Studies looking at this issue are scanty with varying methodology. Table 4 summarizes the available studies on this subject. As seen in Table 4, the reported normal ranges differ substantially, ranging from 10 to 40 IEL/100EC.

Most of the discrepancy seems to be due to the different methodologies used, different sample sizes, and probably different populations studied.

Currently the normal upper limit for IEL in the distal duodenum is considered to be 20/100 enterocytes. We tried to minimize methodological flaws as much as possible, therefore 50 patients were enrolled, 46 of whom had adequate biopsy samples for histological evaluation. To increase the accuracy of the cell counts, we studied 1000-1200 nucleated cells. We also compared H&E and IHC for CD45 (LCA) on 4-µm thick sections in order to evaluate the accuracy of the H&E, using meticulous statistical methods. As mononuclear cells other than lymphocytes may stain with CD45, we screened the slides meticulously before CD45 staining for presence of cells other than lymphocytes. We also examined the villous height to crypt depth ratio semi-quantitatively as well as checking the patients’ sera for H. Pylori.

According to our data, an upper normal limit for intraepithelial lymphocytes per 100 epithelial cells in the distal duodenum is 34 on H&E staining and slightly higher on LCA stains. The higher IHC value has been reported by Veress et al. as well. Although the IEL count on IHC is slightly higher than with the H&E method, but the agreement between the methods is very good. So for practical purposes, the H&E method seems to be adequate.

Our data also show that the height of the villous tip is roughly four times that of the depth of the crypt, which is in accord with Kakar et al’s findings, and is higher than that found by Hayat et al.

Our data did not show any difference between HP positive and negative patients regarding their
IEL count. This may be due to the high HP prevalence in our area and that the number of HP negative cases has been low in our series. Therefore this part of our data should be interpreted with caution.

Our data reveal that the upper limit of normal for IEL in the distal duodenum may differ in different areas. This difference may be due to the ethnic and environmental backgrounds. This should be considered when interpreting duodenal biopsies for IEL count and especially in the diagnosis of celiac disease. Therefore, it may be reasonable to find normal regional values and update it at intervals because environmental factors (e.g., HP) are dynamic and changing. A globally valid normal value may be less achievable.

**Table 4. Summary of studies addressing normal values for IEL.**

<table>
<thead>
<tr>
<th>Study</th>
<th>Staining method</th>
<th>Sample thickness</th>
<th>Site of sampling</th>
<th>n</th>
<th>Upper limit of normal</th>
<th>Tip/ crypt depth</th>
<th>length of assessed epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferguson et al. 10(1971)</td>
<td>H&amp;E</td>
<td>5 – 7 µm</td>
<td>Jejunal mucosa</td>
<td>*NR</td>
<td>40 IEL/100 EC</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Batman et al. 17 (1989)</td>
<td>IHC (CD45)</td>
<td>5 µm</td>
<td>Jejunal mucosa</td>
<td>10</td>
<td>20.5 IEL/100 EC</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Snijder et al. 18 (1996)</td>
<td>IHC (CD3)</td>
<td>NR</td>
<td>Small intestinal mucosa</td>
<td>15</td>
<td>5 IEL/high power field</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Goldstein et al. 19 (2001)</td>
<td>H&amp;E</td>
<td>NR</td>
<td>Duodenal mucosa</td>
<td>24</td>
<td>12 IEL/100 EC</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Hayat et al. 20 (2002)</td>
<td>H&amp;E</td>
<td>4 µm</td>
<td>Duodenal mucosa</td>
<td>20</td>
<td>25 IEL/100 EC</td>
<td>1.82</td>
<td>300 IEL plus EC</td>
</tr>
<tr>
<td>Veress et al. 11 (2004)</td>
<td>H&amp;E, IHC (CD3)</td>
<td>3 µm</td>
<td>Distal duodenal mucosa</td>
<td>18</td>
<td>25 IEL/100 EC</td>
<td>NR</td>
<td>At least 300 EC</td>
</tr>
<tr>
<td>Biagi et al. 13 (2003)</td>
<td>IHC (CD3)</td>
<td>*NR</td>
<td>Distal duodenal mucosa</td>
<td>17</td>
<td>4.6 IEL/20 EC</td>
<td>NR</td>
<td>At least 500 EC</td>
</tr>
<tr>
<td>Kakar et al. 5 (2003)</td>
<td>H&amp;E, IHC (CD3, CD8, CD20)</td>
<td>NR</td>
<td>Duodenal mucosa</td>
<td>46</td>
<td>40 IEL/100 EC</td>
<td>&gt;2.5</td>
<td>At least 300 EC</td>
</tr>
<tr>
<td>Mahadeva et al. 12 (2002)</td>
<td>H&amp;E</td>
<td>3 µm</td>
<td>Distal duodenal mucosa</td>
<td>626</td>
<td>22 IEL/100 EC</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Present study (2006)</td>
<td>H-E, IHC (CD45)</td>
<td>4 µm</td>
<td>Distal duodenal mucosa</td>
<td>46</td>
<td>34 IEL/100 EC (H&amp;E), 35 IEL/100 EC (IHC)</td>
<td>3.9</td>
<td>1000 – 1200 nucleated cells</td>
</tr>
</tbody>
</table>

*NR=not reported

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