# Immunohistochemical stains for CD3 and CD8 do not improve detection of gluten-sensitive enteropathy in duodenal biopsies

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Patients with gluten-sensitive enteropathy usually have increased numbers of duodenal intraepithelial lymphocytes even if the villous architecture is normal. Some authors advocate the use of CD8 and CD3 immunohistochemical stains to improve detection of intraepithelial lymphocytosis, yet the added value of immunohistochemistry when biopsies appear normal remains unproven. The purpose of this study was to evaluate the utility of CD3 and CD8 immunostains in detecting intraepithelial lymphocytosis among duodenal biopsies originally interpreted to be normal based on routine evaluation. We identified 200 duodenal biopsies from 172 patients, all of which were accompanied by a clinical question of gluten-sensitive enteropathy. Five well-oriented villi from each biopsy were assessed. Intraepithelial lymphocytes present in hematoxylin and eosin (H&E)-stained slides were counted and compared with the number of CD3 and CD8 immunopositive cells present in the villous epithelium. Results were expressed as the mean number of intraepithelial lymphocytes or immunopositive cells present per 20 villous tip enterocytes. Review of H&E-stained slides revealed a mean of $2.1 \pm 0.1$ intraepithelial lymphocytes, compared with $3.2 \pm 0.1$ CD3-positive and $2.1 \pm 0.1$ CD8-positive intraepithelial cells ($P = < 0.001$ and 1, respectively), although none of the cases displayed sufficient numbers of intraepithelial lymphocytes to be considered abnormal (ie, $\geq 12/20$ enterocytes) by any method. The number of intraepithelial lymphocytes detected by H&E evaluation or immunohistochemistry did not correlate with results of serologic studies for markers of gluten sensitivity. We conclude that immunostains for T cell markers do not improve detection of gluten-sensitive enteropathy when H&E-stained sections are normal.
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Gluten-sensitive enteropathy is an immune-mediated chronic inflammatory disorder of the small intestine triggered by a combination of environmental and genetic influences. Gliadin, a derivative of gluten, stimulates production of cytokines when presented to CD4-positive T lymphocytes by human leukocyte antigen (HLA)-DQ2 and DQ8 molecules.[1] Some of these cytokines activate plasma cells to produce antigliadin antibodies and antibodies to tissue transglutaminase (TTG), an enzyme that modifies gliadin for binding to HLA molecules, whereas others promote lymphocytic infiltration of the epithelium, thereby producing mucosal injury.[2–5] The disorder manifests at any age with variable symptoms that include diarrhea, abdominal pain, weight loss, malabsorption, and anemia. Left untreated, patients may develop complications, including osteoporosis, malnutrition, neurologic disorders, infertility, and malignant neoplasms such as enteropathy-associated T-cell lymphoma, B-cell lymphoma, and adenocarcinoma of the small intestine.[6,7]

Histologic changes of gluten-sensitive enteropathy are usually limited to the duodenum and proximal jejunum. Biopsies display a spectrum of features ranging from increased numbers of intraepithelial T lymphocytes with normal villous architecture to severe villous shortening and crypt hyperplasia that produce a flat mucosal surface.[8–11] Intraepithelial lymphocytosis with preserved villous architecture occurs in individuals with 'latent' gluten sensitivity

and asymptomatic relatives of patients who have the disease, but it is also believed to represent an early histologic feature of gluten-sensitive enteropathy. Most of the intraepithelial lymphocytes have a CD4-negative/CD8-positive phenotype and a few are CD4-negative/CD8-negative, whereas lymphocytes in the lamina propria are mostly CD4-positive and negative for CD8.[12]

Some authors advocate use of immunohistochemical stains for T lymphocyte markers to improve detection of gluten-sensitive enteropathy, especially when the villous architecture is normal, although there are no data supporting their use among histologically normal biopsies.[13,14] Despite a lack of supporting evidence, we have noted the increased application of CD3 and CD8 immunostains to normal small bowel biopsy specimens in our consultation material from other laboratories. We have also seen more clinician-driven requests for these stains in our own institution. We performed this study to determine whether CD3 and CD8 immunohistochemical stains improved detection of intraepithelial lymphocytosis among patients with histologically normal duodenal biopsies in whom gluten-sensitive enteropathy is a clinical consideration.

## Materials and methods

### Case Selection

We prospectively evaluated all duodenal biopsy samples submitted to the Department of Pathology and Laboratory Medicine of Weill Cornell Medical College during a 6-month period from 2011 and 2012. Cases accompanied by a clinical question of gluten-sensitive enteropathy were considered for inclusion in the study. Partial or complete villous shortening, increased intraepithelial lymphocytes based on evaluation of H&E-stained sections, and the presence of fewer than five well-oriented villi were considered to be the exclusion criteria. The final study group consisted of 200 duodenal mucosal biopsies from 172 patients. Pertinent data, including age, sex, clinical signs and symptoms, and endoscopic findings were obtained from the patients' medical records and direct conversations with their gastroenterologists. Results of serum anti-TTG (IgA) and antigliadin peptide (IgG and IgA) antibody titers obtained at the time of biopsy were recorded, as were the results of HLA-DQ2 and HLA-DQ8 subtype analyses. Pathologic findings in any concomitant biopsies from the remaining gastrointestinal tract were also noted. Permission for the study was obtained from the institutional review board at the Weill Cornell Medical College, NY, New York.

### Assessment for Intraepithelial Lymphocytes

H&E-stained slides prepared from routinely processed (10% buffered formalin) paraffin-embedded tissue sections obtained from the duodenum were evaluated. The number of intraepithelial lymphocytes present in each of five well-oriented villous tips was determined and expressed as the mean number of intraepithelial lymphocytes/20 enterocytes. Immunohistochemical stains for CD3 and CD8 (Leica Microsystems Inc., 1:100 and 1:75, respectively) were performed on 4-μm thick paraffin-embedded tissue sections using standard techniques and immunopositive cells in each of five villous tips were counted. The mean number of CD3 and CD8 immunopositive cells/20 enterocytes was recorded. A mean of at least 12 intraepithelial lymphocytes (or 12 immunopositive cells) per 20 villous tip enterocytes was considered increased and possibly reflective of gluten sensitivity, as previously described.[15]

### Statistical Analyses

A linear mixed-effects statistical model was used to estimate the average counts obtained from review of H&E-stained sections and immunohistochemically stained slides. This model accounts for variations that result from evaluation of multiple samples from the same patient and adjusts correlations that are measured by different staining methods performed on the same sample. Differences between means were evaluated using simultaneous tests for general linear hypotheses and $P$-values were adjusted for multiple comparisons using the Bonferroni method.[16] $P$-values $< 0.05$ were considered statistically significant.

## Results

### Clinical Features of Study Patients

The study group included 55 men and 117 women who ranged in age from 9 months to 92 years (mean: 49, median: 51 years). Six (3%) patients were less than 18 years of age. The presenting symptoms and endoscopic findings of the study patients are summarized in Table 1. One patient had a history of gluten-sensitive enteropathy and was restricted to a gluten-free diet at the time of endoscopy. Three patients had established Crohn's disease. Fifty-two (30%) patients underwent serologic testing for IgA anti-TTG antibodies (reference range: 0–19 units) at the time of biopsy, including seven (13%) who had elevated titers (mean: 35.9, median: 31, range: 20–56 units). Antigliadin peptide antibodies were measured in 26 (15%) patients (reference range: 0–19 units), including three (12%) who had elevated titers. Two of these patients had elevated IgA titers (34 and 88 units), and one had an elevated IgG titer (53 units). Only three patients were tested for HLA-DQ2/DQ8 subtypes and all were negative.

## Pathologic Features of Study Cases

One hundred and sixty-five (83%) duodenal biopsies from 140 patients were interpreted to be normal following evaluation of H&E-stained sections.

**Table 1** Clinical features of the study patients

| Clinical and endoscopic findings | Number of patients |
|---|---|
| *Indications for endoscopy (n = 172)* | |
| Abdominal pain | 47 (27%) |
| Iron deficiency anemia | 31 (18%) |
| Diarrhea | 31 (18%) |
| Dyspepsia | 16 (9%) |
| Reflux | 16 (9%) |
| History of gastritis | 10 (6%) |
| Weight loss | 7 (4%) |
| Dysphagia | 6 (4%) |
| Abdominal distention | 5 (3%) |
| Vitamin B12 deficiency | 2 (1%) |
| Gluten-sensitive enteropathy | 1 (1%) |
| | |
| *Endoscopic appearance of duodenal mucosa (n = 172)* | |
| Normal | 128 (74%) |
| Inflamed or eroded | 24 (14%) |
| Scalloped, flattened, or atrophic folds | 12 (7%) |
| Nodular | 8 (5%) |

Thirty-four (17%) biopsies from 31 patients displayed features of peptic injury, including foveolar metaplasia and plasma cell-rich inflammation in the lamina propria with, or without, neutrophils. One patient had active enteritis associated with *Giardia lamblia* infection.

The mean number of villous intraepithelial lymphocytes detected in H&E-stained sections was $2.1 \pm 0.1$ (range: 0.4–5.8, median: 2) per 20 enterocytes. Immunostains for CD3 demonstrated a mean of $3.2 \pm 0.1$ (range: 0.2–9.6, median: 3) immunopositive cells per 20 villous tip enterocytes compared with a mean of $2.1 \pm 0.1$ (range: 0.6–6, median: 1.8) for CD8 in the same region (Figure 1). Evaluation of CD3-stained slides yielded significantly higher counts compared with H&E-stained sections ($P < 0.001$), but the difference was not clinically relevant as all means were well within the range of normal ($\leq 12/20$ enterocytes). The mean number of CD8-positive intraepithelial cells was statistically similar to the mean number of intraepithelial lymphocytes observed in H&E-stained slides ($P = 1$). There were no differences between patients with normal serum anti-TTG levels and antigliadin antibodies and those with elevated antibody titers with respect to mean numbers of intraepithelial lymphocytes as determined by any method (Table 2).
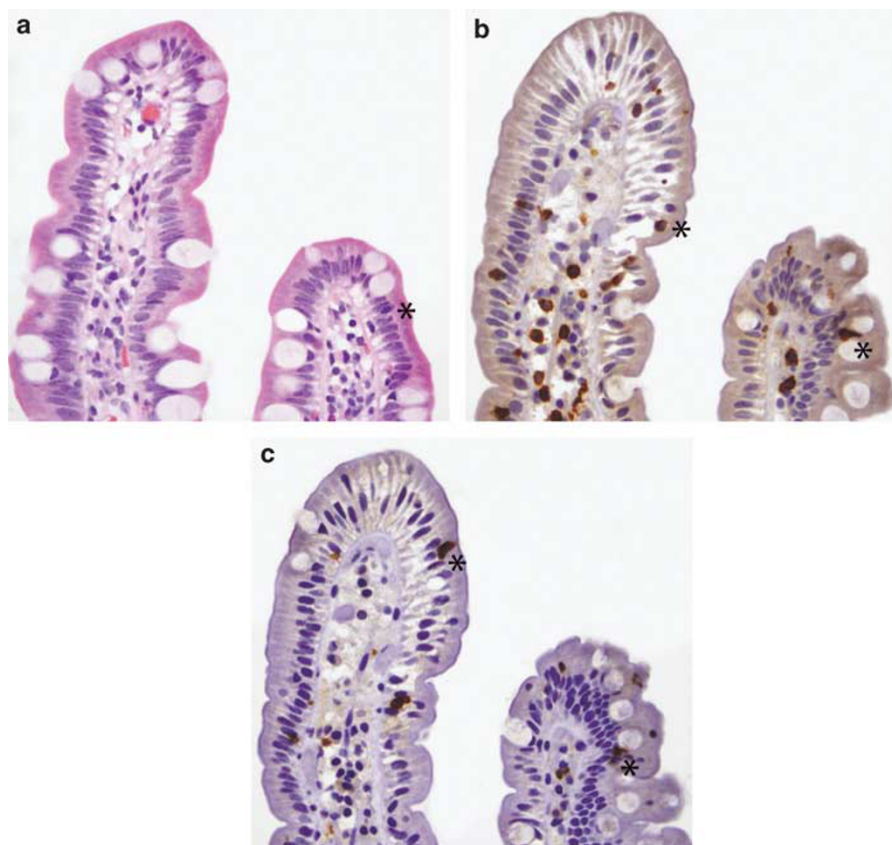


**Figure 1** All duodenal biopsies display normal villous architecture with occasional intraepithelial lymphocytes in H&E-stained sections (asterisk), as well as a sprinkling of mononuclear cells in the lamina propria. (**a**) Immunostains against CD3 demonstrate immunopositive cells in the villi (asterisk), but their numbers are not sufficiently increased to suggest gluten-sensitive enteropathy. (**b**) Occasional immunopositive cells (asterisk) are also present in sections stained for CD8 (**c**).

**Table 2** Mean intraepithelial lymphocytes and immunopositive cells in duodenal biopsies from study patients

| Clinicopathologic features | Mean intraepithelial lymphocytes/20 villous enterocytes | | |
|---|---|---|---|
| | H&E evaluation | CD3 immunostain | CD8 immunostain |
| *Pathologic findings* | | | |
| Histologically normal (n = 165) | 2.1 ± 0.1 | 3.2 ± 0.1 | 2.1 ± 0.1 |
| Features of peptic injury (n = 34) | 2.1 ± 0.5 | 3.3 ± 0.7 | 2.2 ± 0.6 |
| Giardiasis (n = 1) | 2 | 5.6 | 3.4 |
| *Serologic findings* | | | |
| Elevated anti-TTG serum antibodies (n = 7) | 2.4 ± 0.5 | 3.6 ± 0.7 | 2.2 ± 0.6 |
| Normal anti-TTG serum antibodies (n = 45) | 2.2 ± 0.5 | 3.1 ± 0.7 | 2.0 ± 0.6 |
| Elevated antigliadin peptide antibodies (n = 3) | 1.7 ± 0.3 | 3.5 ± 0.8 | 1.3 ± 0.4 |
| Normal antigliadin peptide antibodies (n = 23) | 2.1 ± 0.4 | 3.2 ± 0.7 | 2.4 ± 0.6 |

Abbreviation: H&E, hematoxylin and eosin.

Concomitant biopsies of the esophagus and stomach were obtained in 83 (48%) and 160 (93%) patients, respectively. Of these, 11 (6%) had *Helicobacter pylori*-associated gastritis, including two with peptic duodenitis. Twenty-seven (16%) patients had *Helicobacter pylori*-negative chronic gastritis, including four with features of duodenal peptic injury. Sixty-nine (40%) patients had chemical gastropathy, nine of whom also had peptic duodenitis. Forty-six (27%) patients underwent colonoscopy with biopsies of the terminal ileum and/or colon at the time of upper endoscopy. Twenty-seven of these patients had normal biopsies, three had mild active colitis, one had Crohn's ileocolitis, one had melanosis coli and the remainder had one, or more benign colorectal polyps.

## Discussion

The diagnosis of gluten-sensitive enteropathy is based on a combination of clinical features, serologic positivity for anti-TTG and/or antigliadin peptide antibodies, histologic findings, and a clinical and serologic response to gluten withdrawal.[17,18] The American Gastroenterological Association mandates that biopsies of the proximal small bowel be obtained to confirm the diagnosis of gluten-sensitive enteropathy.[19] Evaluation for genetically susceptible HLA subtypes is also useful, as the absence of DQ2 or DQ8 virtually excludes a diagnosis of gluten sensitivity in the United States.[20]

Although many patients with gluten-sensitive enteropathy have duodenal and/or jejunal biopsies that show variable villous shortening and crypt hyperplasia, some display only increased intraepithelial lymphocytes with preserved villous architecture, which may be difficult to distinguish from occasional lymphocytes that are normally present. Normal villi in the proximal small intestine contain 20–30 intraepithelial lymphocytes per 100 enterocytes and they tend to be more numerous on the lateral aspects of the villi compared with the tips.[18,21] In contrast, biopsies from patients with gluten-sensitive enteropathy typically contain increased numbers of lymphocytes that are evenly distributed in epithelium on the lateral aspects of villi as well as the villous tips.[15]

Methods used to assess intraepithelial lymphocytes vary. Some authors recommend evaluating the entire length of the villous for lymphocytic infiltration and consider numbers in excess of 20 intraepithelial lymphocytes per 100 enterocytes to be abnormally elevated.[21–23] Others suggest counting intraepithelial lymphocytes per 20 enterocytes in five villous tips and consider a mean of 12 or more to be increased and suggestive of gluten sensitivity.[15] Immunohistochemical stains for CD3 and CD8 may be utilized to more accurately and reproducibly assess the number of intraepithelial lymphocytes present in small-intestinal biopsies.[13,14] Settakorn, *et al*[14] evaluated small-intestinal biopsies from patients with at least one positive serologic marker of gluten sensitivity, gastrointestinal complaints, and normal villous architecture upon histologic evaluation. They found that immunostains for CD3 and CD8 aid detection of 'minimal morphologic change gluten sensitivity', as defined by at least 30 immunopositive cells per 100 enterocytes evenly distributed over the lateral aspects and tips of the villi. However, the authors did not report whether symptoms and/or serologies resolved following adherence to a gluten-free diet. It is not clear whether patients with 'minimal morphologic change gluten sensitivity' have gluten-sensitive enteropathy, irritable bowel syndrome, or another type of inflammatory condition.

Mino *et al*[13] compared proximal small bowel biopsies from patients with new onset gluten-sensitive enteropathy to those of patients with established gluten sensitivity treated by gluten withdrawal, as well as biopsies from individuals without gluten-sensitive enteropathy. They found that all patients with gluten-sensitive enteropathy had more than 20 intraepithelial lymphocytes per 100 enterocytes, regardless of whether they were adherent to a gluten-free diet. The authors also reported that patients with new onset gluten sensitivity had significantly more CD3-positive intraepithelial cells at the villous tips compared with patients who adhered to a gluten-free diet and

controls. They concluded that a 'top-heavy' distribution of CD3-positive intraepithelial lymphocytes in the tips of villi was a sensitive marker of gluten sensitivity and noted that immunostains detected more intraepithelial lymphocytes than evaluation of H&E alone.[13] We also found that CD3 immunostains detected significantly higher numbers of intraepithelial lymphocytes compared with H&E, but failed to demonstrate an added benefit of immunohistochemistry. Ancillary stains did not detect gluten-sensitive enteropathy when H&E-stained sections were interpreted to be normal.

The results of this study suggest that assessment of H&E-stained slides adequately detects mild histologic changes characteristic of gluten-sensitive enteropathy. Although immunohistochemical stains for T cell markers reveal slightly more numerous intraepithelial T cells than are evident in H&E-stained sections, they do not identify intraepithelial lymphocytosis (ie, 12 or more lymphocytes per 20 enterocytes) or gluten-sensitive enteropathy when H&E-stained sections are interpreted to be normal. We conclude that, immunohistochemical stains for CD3 and CD8 are of limited value in the evaluation of intestinal biopsy samples for gluten-sensitive enteropathy. Performance of 'up front' immunohistochemical stains for T cell markers do not enhance detection of intraepithelial lymphocytosis in routine duodenal biopsies and contribute to unnecessary health care costs. Rather, efforts should be directed at educating pathologists to recognize intraepithelial lymphocytosis and other subtle manifestations of gluten-sensitive enteropathy.

## Disclosure/conflict of interest

The authors declare no conflict of interest.

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