A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease¹–³

Carlo Catassi, Elisabetta Fabiani, Giuseppe Iacono, Cinzia D’Agate, Ruggiero Francavilla, Federico Biagi, Umberto Volta, Salvatore Accomando, Antonio Picarelli, Italo De Vitis, Giovanna Pianelli, Rosaria Gesuita, Flavia Carle, Alessandra Mandolesi, Italo Bearzi, and Alessio Fasano

ABSTRACT

Background: Treatment of celiac disease (CD) is based on the avoidance of gluten-containing food. However, it is not known whether trace amounts of gluten are harmful to treated patients.

Objective: The objective was to establish the safety threshold of prolonged exposure to trace amounts of gluten (ie, contaminating gluten).

Design: This was a multicenter, double-blind, placebo-controlled, randomized trial in 49 adults with biopsy-proven CD who were being treated with a gluten-free diet (GFD) for ≥2 y. The background daily gluten intake was maintained at ≤5 mg. After a baseline evaluation (t₀), patients were assigned to ingest daily for 90 d a capsule containing 0, 10, or 50 mg gluten. Clinical, serologic, and histologic evaluations of the small intestine were performed at t₀ and after the gluten microchallenge (t₁).

Results: At t₀, the median villous height/crypt depth (Vh/Cd) in the small-intestinal mucosa was significantly lower and the intraepithelial lymphocyte (IEL) count (×100 enterocytes) significantly higher in the CD patients (Vh/Cd: 2.20; 95% CI: 2.11, 2.89; IEL: 27; 95% CI: 23, 34) than in 20 non-CD control subjects (Vh/Cd: 2.87; 95% CI: 2.50, 3.09; IEL: 22; 95% CI: 18, 24). One patient (challenged with 10 mg gluten) developed a clinical relapse. At t₁, the percentage change in Vh/Cd was 9% (95% CI: 3%, 15%) in the placebo group (n = 13), −1% (−18%, 68%) in the 10-mg group (n = 13), and −20% (−22%, −13%) in the 50-mg group (n = 13). No significant differences in the IEL count were found between the 3 groups.


KEY WORDS Gastroenterology, celiac disease, gluten toxicity, small-intestinal morphometry, gluten-free diet, gluten threshold in gluten-free food

INTRODUCTION

Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten—the major protein fraction contained in the cereals wheat, rye, and barley—in genetically susceptible persons. CD is a life-long disorder affecting 0.5–1% of the general population worldwide. The standard treatment of CD involves the consumption of a diet completely devoid of gluten proteins, a so-called gluten-free diet (GFD). In the long term (1–2 y), a GFD is associated with clinical, serologic, and histologic remission (1). However, it is almost impossible to maintain a diet with a zero gluten content because gluten contamination is very common in food. “Hidden” gluten (used as a protein filler) may be found in commercially available products, such as sausages, soups, soy sauces, and ice cream. Even products specifically targeted to dietary treatment of CD may contain tiny amounts of gluten proteins, either because of the cross-contamination of originally gluten-free cereals during their milling, storage, and manipulation or because of the presence of wheat starch as a major ingredient.

The potential toxicity of trace amounts of gluten is still unclear. We previously showed in treated CD patients that the 4-wk ingestion of 100–500 mg gliadin/d (roughly equivalent to 200–1000 mg gluten) is able to cause measurable changes in the architecture of the small-intestinal mucosa (2). Only limited data are available on the toxicity of lower doses of gluten (3–6). This is an important issue because the daily ingestion of contaminating gluten in apparently well-treated CD patients is most likely to range from 5 to 50 mg.

Establishing a safe threshold of gluten consumption for CD patients is a matter of major public health importance, particularly in light of the recent reports concerning the high prevalence

¹ From the Center For Celiac Research, University of Maryland School of Medicine, Baltimore, MD (CC and AF); the Department of Pediatrics, Università Politecnica delle Marche, Ancona, Italy (CC, EF, and GP); the Department of Gastroenterology, Children Hospital, Palermo, Italy (GI); the University Department of Gastroenterology, “La Sapienza” University, Rome, Italy (AP); the Gastroenterology Unit, Università Politecnica delle Marche, Ancona, Italy (RG and FC); the Department of Pathology, Università Politecnica delle Marche, Ancona, Italy (AM and IB).

² Supported in part by the Italian Celiac Society. Purified gluten was kindly supplied by Bruno Jarry while working for the Tate & Lyle Group (United Kingdom).

³ Reprints not available. Address correspondence to A Fasano, Musculoskeletal Biology Research Center, University of Maryland School of Medicine, 20 Penn Street, Room 345, Baltimore, MD 21201. E-mail: afasano@mbrc.umbc.edu.

Received May 26, 2006.
Accepted for publication August 28, 2006.
of the disease worldwide (7, 8). The recent National Institutes of Health Consensus Conference position on CD estimated that as many as 3 million people in the United States are affected by CD. These findings, together with the recently approved Food Allergen Labeling and Consumer Protection Act, created a vacuum in terms of health care policy, food safety, legislative guidelines, and industry-related legal liability that needs to be filled to allow the Food and Drug Administration’s governmental mandate to implement the new bill by 2006. The “gluten threshold” topic is currently under evaluation by the Codex Alimentarius, the WHO/FAO commission that is in charge of setting food standards at the international level. To address the aforementioned issues we undertook a prospective, double-blind, placebo-controlled multicenter trial to investigate the toxicity of gluten traces in the celiac diet, with the cooperation and the sponsorship of the Italian Celiac Society (Associazione Italiana Celiachia; AIC). We report herein the final results of this study.

SUBJECTS AND METHODS

Study design

This was a prospective, multicenter, placebo-controlled, double-blind, randomized trial performed between the years 2001 and 2004. The patients were adults with biopsy-proven CD who had consumed a GFD for ≥2 y and were in apparent good health. Patients were excluded if they 1) were younger than 18 y, 2) were poorly compliant with the GFD, 3) were positive for anti-tissue transglutaminase (tTG) antibodies or had a villous height/crypt depth (Vh/Cd) <1.5 at baseline (t₀), or 4) associated conditions, such as selective immunoglobulin A (IgA) deficiency or other autoimmune diseases. Patients that qualified for the study were interviewed and gave their informed consent. Control subjects (only for comparison of the morphometric values at t₀) were adults who were negative for serologic CD markers and for Helicobacter pylori (urea breath test) and were undergoing upper endoscopy for diagnostic purposes.

The patients qualifying for the trial underwent a screening and a dietary interview (t₁). They were asked to maintain a strict GFD during the study period, ie, avoidance of any possible source of gluten contamination (such as restaurant meals). The only cereal-based food they were allowed to eat was the special gluten-free products consumed by these subjects was collected and analyzed in paraffin wax. The sections (5 μm) were stained with hematoxylin and eosin and immunostained by using anti-human CD3 antibody (DAKO, Glostrup, Denmark) to enhance diagnostic accuracy in counting intraepithelial lymphocytes (IELs). Only well-oriented sections were examined; when necessary, the samples were dissected again until they were of good quality. The specimens were examined in batches by 2 pathologists with long-standing experience in morphometric analysis (IB and AM), who were blinded to subject assignment. The morphometric analysis of the sections was performed on ≥10 well-oriented villi, arranged like fingers, and 10 well-oriented crypts, arranged perpendicular to the muscularis mucosa, by a computerized image analyzer (IBAS-AT; Kontron, Munich, Germany). The following variables were evaluated: Vh/Cd and IEL count. The number of IELs was calculated by computing their mean value out of 1000 enterocytes and expressed as a percentage of enterocytes in the area analyzed (2). The IEL count was performed in well-oriented villi through the overall length of the villi, including the villous tip. The overall architecture of the small-intestinal mucosa was also evaluated according to the Marsh-Oberhuber classification (10).

Analysis of background gluten intake

The consumption of gluten-free flour was measured in a different sample of 46 adults with CD (30 women, 16 men; mean age: 36 y; range: 19–63 y) who had been consuming a GFD long term (>2 y). These subjects were asked to report the type and the amount of all the special gluten-free products consumed during 30 consecutive d in a diary. A random sample of 42 gluten-free products consumed by these subjects was collected and analyzed for gluten contamination by enzyme-linked immunosorbent assay (Ridscreen Gliadin; R-Biopharm AG, Darmstadt, Germany) with a sensitivity limit of 3 ppm of gluten.

Statistical analysis

The sample size was selected to detect a mean effect size of 0.3 for changes in morphometric variables before and after the microchallenge, which was expressed as a percentage of baseline values among the 3 groups of patients at a significance level of 0.05.
5% and a power of 85%; one-way analysis of variance was used for the data analysis. The number of patients required was 39 (n = 13 for each group). Considering a 20% dropout rate, 49 subjects were recruited. The normality of the distribution of the examined variables was assessed by using the Shapiro-Wilk test. Nonparametric methods were used for the statistical analysis of the data because the morphometric variables were not normally distributed. A nonparametric analysis of variance (Kruskal-Wallis test) was performed to evaluate the treatment groups (placebo, 10 mg/d, and 50 mg/d) at t₀. Wilcoxon’s test was used to compare pooled CD patients with control subjects at t₀. The correlation between Vh/Cd and IELs was analyzed by Spearman’s coefficient. Changes in morphometric variables before and after the microchallenge were expressed as percentages of the t₀ values. The comparisons between the 3 groups were performed by the Kruskal-Wallis test. Because this test has an asymptotic efficiency of 96% with respect to the one-way parametric analysis of variance (11), the number of patients recruited guaranteed a level of study power not lower than 80%. The results were expressed as medians and 95% CIs. A 5% probability was used to assess statistical significance.

RESULTS
Forty-nine subjects were enrolled in the microchallenge study: 37 women and 12 men with a median age of 30.5 y (range: 19.8–55.4 y) and a median BMI of 21.9 (range: 16.8–30.1). After t₀, 7 of the 49 patients had to be excluded because of abnormal small-intestinal histology (4), development of thyroid carcinoma (1), or gastric polyp confirmed by gastroduodenoscopy (1) or because the subject refused randomization (1). Three other cases did not complete the microchallenge because of change of residence (1 challenged with 50 mg), poor adherence to the protocol (1 challenged with 10 mg), or development of symptoms (1 challenged with 10 mg). Thirty-nine patients completed the study protocol: 30 women and 9 men with a median age of 30.6 y (range: 19.8–55.4 y) and who had consumed a GFD for a median of 10.1 y (range: 2.1–28.0 y). Of these 39 subjects, 13 were challenged with placebo, 13 with 10 mg gluten/d, and 13 with 50 mg gluten/d.

The control subjects were 20 adults (n = 13 women and 7 men) with a median age of 34.5 y (range: 24.0–60.0 y) and a final diagnosis of functional dyspepsia (n = 13) or gastroesophageal reflux (n = 7).

Clinical and serologic evaluation
One patient challenged with 10 mg gluten/d showed typical signs of relapse (vomiting, diarrhea, and abdominal distension) after 6–8 wk of microchallenge but refused to repeat the t₁ evaluation. The comparison between the baseline and the postchallenge findings in the 39 patients completing the microchallenge did not show any significant changes in the clinical outcome between the 3 groups. The results of the serologic CD markers are shown in Figure 1. In all patients, the IgA anti-tTG and IgG AGA values were in the normal range, both at baseline and after the microchallenge. No significant difference was detected between the 3 groups at baseline (Kruskal-Wallis test; P = 0.30 and P = 0.45, respectively). After the microchallenge the IgA anti-tTG did not show any significant change (P = 0.12), while the IgG-AGA showed a significant decrease in the group challenged with 50 mg of gluten in comparison with the placebo (P = 0.04).

Small-bowel biopsy results
The results of the small-intestinal morphometric measures in CD patients and control subjects at baseline are shown in Table 1. The Vh/Cd ratio was significantly lower in CD patients than in control subjects, and the IEL count was significantly higher in CD patients than in control subjects. In the pooled CD group (n = 39), the correlation between the Vh/Cd and the IEL count was

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Intestinal morphometric evaluation of the subjects before and after the gluten microchallenge1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control subjects (n = 20)</td>
</tr>
<tr>
<td>Villous height (μm)</td>
<td>372.7 (339.5, 385.4)</td>
</tr>
<tr>
<td>Crypt depth (μm)</td>
<td>135.1 (124.8, 149.7)</td>
</tr>
<tr>
<td>Vh/Cd</td>
<td>2.9 (2.5, 3.1)</td>
</tr>
<tr>
<td>IEL count (×100 enterocytes)</td>
<td>22 (18, 24)</td>
</tr>
</tbody>
</table>

1 All values are medians; 95% CIs in parentheses. CD, celiac disease; Vh/Cd, villous height/crypt depth; IEL, intraepithelial lymphocyte.
2 The Wilcoxon test was used for the comparison between groups.
3 Significantly different from the control subjects, P < 0.05.
weak and not statistically significant at either baseline ($r = -0.31; 95\% \text{ CI: } -0.56, 0.02; P = 0.06$) or after the microchallenge ($r = -0.29; 95\% \text{ CI: } -0.55, 0.03; P = 0.07$).

The results of the gluten microchallenge are shown in Figure 2 and Table 2. No significant difference in the morphometric indexes was found at baseline between groups. After the microchallenge, the Vh/Cd index improved in 11 of 13 subjects (85%; 95% CI: 55%, 98%) in the placebo group, in 6 of the 13 subjects (46%; 95% CI: 19%, 75%) in the 10-mg group, and in only 2 of the 13 subjects (15%; 95% CI: 2%, 45%) in the 50-mg group; the difference between the 50-mg group and the placebo group was significant. A significant improvement in the median percentage variation between $t_1$ and baseline (percentage change) of the Vh/Cd index was observed in the placebo group but not in the 10-mg and 50-mg groups. Conversely, at $t_1$, the Vh/Cd index showed a significant decrease in the group challenged with 50 mg gluten/d. The Kruskal-Wallis test showed a significant difference in the percentage change in Vh/Cd between the placebo and the 50-mg group ($P = 0.029$). An increase in IELs was observed in only 5 of the 13 subjects (38%; 95% CI: 14%, 68%) in the placebo group, in 6 of the 13 subjects (46%; 95% CI: 19%, 75%) in the 10-mg group, and in 8 of the 13 subjects (61%; 95% CI: 32%, 86%) in the 50-mg group. No significant differences in the percentage change in IEL counts were found between the 3 groups.

**Analysis of background gluten intake**

The daily consumption ($\bar{x} \pm SD$) of commercially available gluten-free products was 332 ± 98 g (range: 177–574 g) in 46 adults being treated for CD. The median gluten content of the 42 gluten-free products analyzed was 5.2 ppm (ppm = mg/kg) (range: 0–50 ppm). On the basis of these values, we estimated...
that the background gluten intake from the GFD followed by our patients during the microchallenge study was <5 mg/d.

**DISCUSSION**

The morphometric analysis of the small-intestinal mucosa, particularly the evaluation of the Vh/Cd index and the IEL count, is the reference method for the quantitative assessment of gluten-induced damage in CD. The normal values found in our control subjects agree with previous data for both Vh/Cd and the IEL count (5). The IEL count is considered to be the most sensitive index of the celiac enteropathy (12). However, this paradigm has been investigated mainly in active CD but does not necessarily apply to CD patients receiving long-term dietary treatment. In our study, the analysis of the effects of the gluten microchallenge indeed suggested that the Vh/Cd index reflected more closely the potential toxicity of 10 mg gluten/d, which remained a “gray”

**TABLE 2**

Results of the 3-mo gluten microchallenge in patients with celiac disease

<table>
<thead>
<tr>
<th>Group</th>
<th>Placebo (n = 13)</th>
<th>10 mg gluten/d (n = 13)</th>
<th>50 mg gluten/d (n = 13)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marsh-Oberhuber grading of small-bowel histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>0.263</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>After microchallenge</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>0.433</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Intestinal morphometric results at baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villous height (μm)</td>
<td>354 (315, 437)</td>
<td>318 (306, 366)</td>
<td>380 (266, 396)</td>
<td>0.431</td>
</tr>
<tr>
<td>Crypt depth (μm)</td>
<td>150 (134, 165)</td>
<td>161 (124, 187)</td>
<td>131 (110, 159)</td>
<td>0.197</td>
</tr>
<tr>
<td>Vh/Cd</td>
<td>2.2 (2.1, 3.0)</td>
<td>2.0 (1.8, 3.4)</td>
<td>2.4 (2.2, 3.2)</td>
<td>0.273</td>
</tr>
<tr>
<td>IEL count (× 100 enterocytes)</td>
<td>26 (23, 45)</td>
<td>29 (23, 46)</td>
<td>27 (17, 48)</td>
<td>0.968</td>
</tr>
<tr>
<td>Morphometric changes after the microchallenge (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villous height</td>
<td>5 (5, 13)</td>
<td>9 (9; 19)</td>
<td>−17 (−30, 23)</td>
<td>0.198</td>
</tr>
<tr>
<td>Crypt depth</td>
<td>−2 (−16, 9)</td>
<td>3 (−28, 21)</td>
<td>3 (−17, 24)</td>
<td>0.693</td>
</tr>
<tr>
<td>Vh/Cd</td>
<td>9 (3, 15)</td>
<td>−1 (−18, 68)</td>
<td>−20 (−22, −13)</td>
<td>0.029</td>
</tr>
<tr>
<td>IEL count</td>
<td>−4 (−22, 42)</td>
<td>0 (−14, 47)</td>
<td>12 (−8, 93)</td>
<td>0.514</td>
</tr>
</tbody>
</table>

1 n = 39. Vh/Cd, villous height/crypt depth; IEL, intraepithelial lymphocyte.
2 0 = normal, 1 = infiltrative lesions (increased IEL count), 2 = hyperplasic lesions (increased IEL count and increased crypt depth).
3 Fisher’s exact test.
4 All values are medians; 95% CIs in parentheses.
5 Kruskal-Wallis test.
6 Significantly different from placebo, P < 0.05.
TOXICITY OF GLUTEN TRACES IN CELIAC DISEASE

area. For ethical reasons we had to limit the duration of the microchallenge to 3 mo, but it is well known that mucosal deterioration may become manifest after a longer gluten challenge (19). Reactions to gluten are not only influenced by the quantity but also the quality of the ingested protein, which may change according to cereal variety (20) and food processing (raw versus cooked grain, fermentation, etc) (21).

The effects of a low gluten intake in CD patients have been investigated in a limited number of studies. Ciclitira et al (22) analyzed the toxicity and time response of a gliadin dose (the major toxic fraction of gluten) in a single patient. They concluded that 10 mg produced no change, 100 mg a very slight measurable change, 500 mg a moderate change, and 1 g extensive damage to small-intestinal morphology. The same group also reported that the ingestion of 2.4–4.8 mg gluten/d caused no change in the jejunal biopsy morphometry of treated CD patients after either 1 or 6 wk (3, 23). Ejderhann et al (4) showed that a daily intake of 4–14 mg gliadin did not affect the morphology of the small-bowel mucosa in CD patients receiving long-term treatment with a GFD. Recent Finnish studies indicate that an intake of 20–36 mg gluten/d has no detectable effect on mucosal histology (5, 6). We previously showed that a 4-wk challenge with 100 mg gliadin caused deterioration of the small-intestinal architecture and that the histologic changes were more pronounced in patients challenged with 500 mg gliadin/d (2). Finally, a higher gluten intake (1–5 g/d), still lower than the normal gluten intake for the non-CD population in Western countries (10–20 g/d) (24), caused relapse of disease at a clinical, laboratory, and histologic level, both in children and in adults (25–27). On the basis of the evidence from the current study and the quoted literature, it appears that 50 mg gluten/d is the minimum dose required to produce measurable damage to the small-intestinal mucosa in CD patients.

Currently, different national positions hamper the implementation of uniform guidelines on the maximum level of gluten contamination (expressed as ppm) that can be tolerated in products that are marketed for the treatment of CD. This is a “hot” topic that was recently reviewed extensively (28, 29). In Northern European countries, up to 200 ppm gluten is permitted in food for CD patients, to use wheat starch as ingredient. Conversely, a more prudent value of 20 ppm has been adopted in North American and southern European countries. On the basis of their clinical and analytic data, Finnish experts recently advocated the intermediate limit of 100 ppm (30). The decision about what the threshold is depends, however, not only on the minimum toxic dose but also on the amount of gluten-free products consumed. Our results indicate that 200 ppm is not a safe threshold because the harmful gluten intake of 50 mg/d could be reached even with a moderate consumption (≥250 g/d) of nominally gluten-free products. A 100-ppm threshold, by allowing up to 10 mg gluten/100 g product, is also probably not suitable for generalized use, especially in countries such as Italy, where consumption of wheat substitutes is occasionally as high as 500 g/d (as shown by our data). The threshold of 20 ppm keeps the intake of gluten from “special celiac food” well below the amount of 50 mg/d, which allows a safety margin for the variable gluten sensitivity and dietary habits of patients.

In conclusion, this study confirmed that an abnormal small-bowel morphology persisted in a significant proportion of CD patients being treated with a GFD, most likely because of the persistent ingestion of trace amounts of gluten. The protracted intake of 50 mg gluten/d produced significant damage in the architecture of the small intestine in patients being treated for CD. However, the sensitivity to trace intakes of gluten showed large interpatient variability, a feature that should be accounted for in the implementation of a safe gluten threshold. These findings should be confirmed by further studies in larger numbers of CD patients. Finally, the relation between the intestinal damage induced by trace intakes of gluten and the long-term complications of CD remains to be elucidated.

The implementation of this study was made possible thanks to the kind cooperation of the members of the AIC, particularly the patients who volunteered for the gluten microchallenge. A warm thank you is due to the President of the AIC, Adriano Pucci, and to the former Director of the AIC bulletin Celiachia Notizie, Franco Lucchesi, who recently died. We thank Susan Phillips for revising the manuscript.

CC and AF were primarily responsible for the conception and design of the study, but all the co-authors contributed to the implementation of the study design, EF, GI, CF, RF, FB, UV, SA, AP, ID, GP, AM, and HB collected the data. EF, RG, and FC performed the statistical analysis. All of the authors contributed to the implementation of the study design, the interpretation of the results, and the writing of the manuscript. At the time of this study, CC was a member of the scientific advisory board of the AIC (a nonprofit organization of patients with CD). None of the authors had a financial or personal conflict of interest related to the funding of or the outcome of this research.

REFERENCES