Two different doses of gluten show a dose-dependent response of enteropathy but not of serological markers during gluten challenge in children with coeliac disease

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In order to study dose-dependency in histopathological reactions and in changes of serological markers of mucosal relapse, gluten challenge was performed with two defined amounts of gluten in 54 children with earlier enteropathy. Gluten was provided in the form of powder and the patients were randomly allotted to either 0.2 (group A, n = 27) or 0.5 (group B, n = 27) grams per kg body weight per day. At the start and after 4 wk of challenge a small intestinal biopsy was performed. Biopsy specimens were evaluated, in accordance with defined criteria, graded and summarized in an enteropathy score. Blood was sampled at the start and after 2 and 4 wk of challenge. Serum levels of anti-gliadin antibodies (AGA) and anti-endomysium antibodies (EmA) were measured. Within 4 wk of challenge, 24 out of 27 patients in group A and all patients in group B had relapsed. After increasing the gluten dose to 0.5 g/kg/d during the subsequent 4 wk, the three non-relapsing patients also relapsed.

Conclusion: The severity of mucosal inflammation was significantly higher for group B (p = 0.04) indicating a dose-related severity of the enteropathy. No significant difference was found for maximum AGA level, or in the proportion of patients that converted to pathological values for AGA or EmA.

Key words: Coeliac disease, gluten challenge, gluten dose, short term

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An important issue in current research on coeliac disease is the possible gluten dose-dependency for symptoms and signs and clinical tests used in diagnosis of the disease. Particularly relevant for our understanding is whether the dose is a determinant of the clinical expression or whether other factors are involved. In Sweden, the incidence of coeliac disease tripled from the mid-1970s to the mid-1990s (1), whereas it decreased in Britain (2). The consumption of gluten during infancy is comparatively high in Sweden. It has been discussed whether the high gluten content of baby foods causes coeliac disease in individuals who otherwise would not have acquired the disease (3, 4). However, screening programmes from different parts of Europe indicate that the prevalence of coeliac disease is similar in regions with different incidences (5), thus suggesting that impact of the high gluten content is more a conspicuous clinical manifestation of the disease than its cause.

The ESPGHAN diagnostic criteria for coeliac disease were changed in the early 1990s (6) to allow the gluten challenge in children over the age of 2 y to be omitted. In countries where classical coeliac disease is prevalent, most children are below the age of 2 y at the first small intestinal biopsy and many therefore still follow the old criteria. The gluten challenge procedure is poorly standardized, however. The ‘optimal’ intake of gluten is not known, thus reflecting the lack of standardization of gluten intake in clinical and experimental research. The few studies published on gluten consumption in normal children in the Nordic countries indicate a range from 0.14 to 0.42 g/kg/d (7–8). There are also a few published studies on the subject of dose-dependent effects of gluten on the small intestinal mucosa under experimental conditions in coeliac disease. With up to 84 h of exposure to 12 g of gluten, the small intestinal mucosa of patients reacted with an increase in intraepithelial lymphocytes (IEL) and a decrease in epithelial height (9). With 4 wk on a daily dose of 0.1 or 0.5 g of gluten a similar trend towards enteropathy was found, with an increase in IEL and a decrease in villous height/crypt depth ratio, but the
alterations did not allow an established diagnosis of coeliac disease (10).

Results vary due to lack of standardization of at least two factors: time and gluten dose. Some clinicians modify ESPGHAN’s old criteria and perform a challenge that is monitored by measuring the concentration of serological markers of the disease. For these reasons, in this study we also included data on both serological markers and small intestinal mucosa histopathology.

The aim of this investigation was to study dose-dependency in relapse rate, histopathological response in the small intestine and levels of serum markers for coeliac disease with two different doses of gluten in children with coeliac disease. The study also aimed at ensuring that a sufficient response was obtainable for conclusive diagnostic information during a 4 to 8-wk-long gluten challenge.

Patients and methods

Study protocol

The children were recruited to this study at the time of second biopsy, after having been on a gluten-free diet for at least 12 mo. They all had severe inflammation in the intestinal mucosa with total or subtotal villous atrophy at their first biopsy with clinical recovery on gluten-free diet and no sign of cow’s milk intolerance, soy allergy, giardiasis or other explanation for the enteropathy. Mean age at the first biopsy was 17 (±12) mo.

During a 12-mo period, 57 consecutive children (36 girls and 21 boys) with suspected coeliac disease under routine diagnostic investigation were included from the paediatric departments of the Queen Silvia Children’s Hospital, Gothenburg, and Ryhov County Hospital, Jönköping. Fifty-four completed the study (33 girls (61%) and 21 (39%) boys). The mean age at challenge was 32 (±12) mo.

Two different gluten doses were chosen in the range indicated in studies of gluten consumption in infants (7–9). The patients were allotted to receive either 0.2 (n = 27 group A, 10 boys and 17 girls) or 0.5 (n = 27 group B, 11 boys and 16 girls) g/kg/d of gluten. The age and sex distribution did not differ between the two groups. The mean and median ages were 31 and 30 mo for group A and 33 and 30 mo for group B. The condition of the small intestinal mucosa at the start of the study is described in Table 2. Gluten powder was introduced in increasing amounts during the course of 3 d, after which the allotted amount of gluten was added daily to the pre-existing gluten-free diet of the patient, and the powder was processed with the rest of the food as required, – baked, boiled, fried or heated. From a dietician, each family received a collection of recipes with a number of exchangeable commodities suited to make the gluten powder palatable. The amount of gluten that each patient actually consumed was recorded in a special diary. If the patient suffered intense symptoms or if the gluten intake was much reduced due to symptoms, the biopsy was performed ahead of schedule. The challenge was terminated if the mucosa appeared at inspection in low magnification immediately after biopsy. If the mucosa appeared normal, the challenge continued until the histopathological evaluation was reported. The challenge was planned for a fixed duration of 4 wk, but 10 patients had a shorter duration than 4 wk due to symptoms. In patients who did not relapse during the first 4 wk, the challenge was extended by another 4-wk period, followed by a repeat biopsy. During the second 4-wk period, all patients received 0.5 g/kg/d of gluten.

Table 1. Study design.

<p>| Table 2. The change of distribution of patients within the four signs of small intestinal inflammation during gluten challenge. |</p>
<table>
<thead>
<tr>
<th>Sign of inflammation</th>
<th>Time</th>
<th>Gluten group</th>
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<tbody>
<tr>
<td>V/C ratio</td>
<td>Start A 27</td>
<td></td>
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<tr>
<td></td>
<td>Start B 24 3</td>
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<td></td>
<td>End A 5 9 6 7</td>
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<td></td>
<td>End B – 8 6 13</td>
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<tr>
<td>IEL</td>
<td>Start A 23 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Start B 20 7</td>
<td></td>
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<tr>
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<td>End A 2 5 12 8</td>
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<tr>
<td></td>
<td>End B – 1 13 13</td>
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</tr>
<tr>
<td>SED</td>
<td>Start A 27</td>
<td></td>
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<tr>
<td></td>
<td>Start B 27</td>
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<tr>
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<td>End A 3 5 9 10</td>
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<tr>
<td></td>
<td>End B – 2 6 19</td>
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<tr>
<td>INFL</td>
<td>Start A 19 8</td>
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<td></td>
<td>Start B 14 13</td>
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<td></td>
<td>End A 2 4 19 2</td>
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<td></td>
<td>End B 1 19 7</td>
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Patients with extended challenge followed this schedule for another 4 wk with day 35 = day 7, etc.
Three of the patients were subsequently excluded. One patient, belonging to group A, was excluded when the biopsies were read blindly post-challenge. The biopsy of this patient did not fulfill the criteria of relapse. The patient had 4 wk of gluten exposure and was never offered an extended challenge with a biopsy-proven relapse. Thus, the diagnosis of coeliac disease was not established. Two patients, belonging to group B, were excluded for reasons related to compliance. They had returned to the gluten-free diet due to severe symptoms within 7 d of challenge without an immediate biopsy. The parents refused further challenge. The results of the remaining 54 children are presented in this paper.

Biopsy specimens
Small intestinal biopsy was performed with a single-port Watson capsule or a Stortz multiple biopsy capsule placed at the ligament of Treitz under fluoroscopic control. The biopsies were fixed in formaldehyde and embedded in plastic before being cut into 1µm thick sections, stained and evaluated under a light microscope. All specimens were examined consecutively a few days after the biopsy of each patient. Finally, when all patients had completed the challenge, they were read serially and on the same occasion by one experienced pathologist (W.R.). The outcome of this examination is presented in this report.

Mucosal morphology
In the histopathological evaluation, four signs of small intestinal mucosa inflammation were recorded. Three of these were graded semiquantitatively (1–4), namely: number of intraepithelial lymphocytes (IEL), degree of surface epithelium damage (SED) and degree of inflammatory infiltrate in the mucosa (INFL). The fourth sign, villous to crypt ratio (V/C), was graded quantitatively as follows: Grade 1 V/C = 1/1, grade 2 V/C = 1/2, grade 3 V/C = 1/3. Each grade of the four signs scored one point. The sum of the points, ranging from 4 to 16, of these four signs was a measure of the small intestinal inflammation and was termed enteropathy score (ES).

Criteria for relapse
For the diagnosis of relapse of coeliac disease, the following three criteria had to be met: (i) ES should increase by at least three points; (ii) inflammatory changes should involve at least three different signs; and (iii) unequivocal inflammation should exist upon routine evaluation (corresponding to at least 8 points in enteropathy score level in this study).

Serological markers
Serum anti-gliadin antibody (AGA) of IgA type was analysed with a solid phase enzyme-linked immunoas-say, producing a yellow-stained product when positive, and measured spectrophotometrically as absorbance at 420 nm. The result is expressed as arbitrary units (AU) (Pharmacia Upjohn) (11). The cut-off value for pathological AGA has been ≥25 AU for all ages in this study.

Serum endomysium antibodies (EmA) were measured with an indirect immunofluorescence method (Electra Box). The results are considered positive (pathological) when a serum sample dilution of 1/5 or more produces immunofluorescence (12) and expressed as pathological or normal.

Gluten powder
The gluten powder used in this study is the same product as is commercially used in bakeries to improve baking properties. Gluten powder was produced in a process where wheat is milled and sifted. The wheat flour was mixed with water at 30°C. Gluten will then precipitate as small threads. Contamination was removed by further washing. Separation of the water-soluble starch and the precipitated protein fraction was performed in a centrifuge (hydrocyclone). The remaining gluten fraction was repeatedly washed, pressed into a warm air stream and finally whipped into powder by rotating knives. The powder contains at least 75% gluten by weight, usually 80–85%, according to the manufacturer’s specification (Ceralia AB, Sweden). In this study we have assumed 80% gluten by weight for calculations of amount of gluten ingested.

Statistics
The results are presented as mean with standard deviation in parentheses. The change over time of individuals was analysed with Wilcoxon’s signed rank test and differences between groups were analysed with the Mann-Whitney U test. For comparison of proportions between groups we used the Fisher exact test. Multiple regression analysis was used to determine combined effects of several variables. Adjustments for confounding variables in correlation analyses were done by Spearman partial correlation coefficient. All statistical tests were two-tailed with a minimum confidence level of 0.05 for significance.

Ethics
This study was approved by the ethics committee of the medical faculties of the universities of Göteborg and Linköping.

Results
Small intestinal mucosa morphology
Fifty-one patients (94%) relapsed within the first 4 wk of challenge. Three patients of group A with normal mucosa all progressed to villous atrophy after an
additional 4-wk challenge with 0.5 g gluten per kg per day. The number of relapses in group A, 24/27, was not significantly \( (p = 0.24) \) different compared to group B, 27/27. There was a significant change of ES from the start to the end of the challenge for both gluten dose groups. The mean ES of group A went from 4.4 to 11.3 \( (p < 0.001) \) and the ES of group B from 4.9 to 13.5 \( (p < 0.001) \) (Fig. 1). The mean change of ES was significantly different between the two gluten dose groups \( (p = 0.037) \). The distributions of the separate signs are presented in Table 2.

**AGA**
At the start of the study, 4 out of 54 (7%) had serum concentrations of AGA above the cut-off value. The rise from normal to pathological values occurred within 2 wk in 38 of the remaining 50 (76%) and at the end of the first 4 wk in 44 of 50 (88%). Three patients with 8 wk of challenge all had AGA levels below cut-off after the first 4 wk. At the end of the complete period of challenge, 3 of 54 (6%) still had normal concentrations of AGA despite the pathology of the small intestinal specimens. There was no significant difference between the two groups of children in the maximum levels of AGA at either 2 wk \( [\text{mean value of group A 82 AU (±88) and group B 92 AU (±77) (} p = 0.30) \] ) or 4 wk \( [\text{mean value of group A 106 AU (±94) and of group B 118 AU (±83) (} p = 0.47) \] ) and an equal number of patients (21 in group A and 23 in group B) increased from normal to pathological values of AGA (Table 3). One patient had IgA deficiency and was normal in both AGA and EmA in all blood samples.

**Endomysium antibody**
At the start, 12 out of 54 patients (22%) had a positive EmA titre despite a normal small intestine mucosa. Subsequent to gluten challenge, 36 out of 54 turned from normal to positive titres. At the end of the 8-wk period, 6 out of 54 still had normal EmA titre, despite the occurrence of small intestinal pathology in all six of them. Table 3 shows the EmA levels related to gluten dose. There was no difference in the frequency of change to pathological EmA titres between the two study groups.

**Discussion**
In this study there was a gluten dose-dependency in severity of the enteropathy induced during 4 wk of gluten challenge. There was no significant dose-dependent difference in relapse rate at 4 wk of challenge. The responses of serological markers did not differ dose-dependently for either AGA or EmA.

Several groups have reported results from studies of gluten challenge, but objectives and designs have differed. In this study, two groups of patients were prescribed different doses of gluten. The gluten challenge was performed in the same manner in both groups in all other aspects. The groups were comparable in age, sex distribution and start levels of AGA and EmA. The amount of gluten prescribed in studies of gluten challenge varies from 0.1 to 1.0 g per kg body weight per day. The amounts we used were within the range commonly used in both studies and, most importantly, in the Scandinavian communities (8, 9).

However, some studies have compared results with even smaller doses of gluten (12). A relapse rate of 100% after 8 wk of gluten challenge, as in our study, is as high as reported by Valetta (13) and Troncone (14) in their studies with a median duration of 30 d and with naturally gluten-containing food with unspecified doses of gluten. In studies with large patient groups, relapse rates after 2 y challenge of between 95% and 82% have been reported (15, 16). Wauters (17) reported a relapse rate as low as 60% with doses of gluten powder as high as 0.75 g/kg/d (max. 20 g) over 3...
As an estimate from the results of several studies, dose-dependency of relapse rate is uncertain. However, the variation in relapse rate between different studies could also be related to different criteria for relapse or variations in controlling compliance. In our 4 wk study, where compliance was controlled and criteria for relapse were strictly defined within the normal gluten intake range, no significant dose-dependent variation in relapse rate was noted.

In this study, a dose-dependent difference in enteropathy was found. This information is not usually possible to obtain from clinical studies. Dose-dependent mucosal inflammation has been reported from studies of patients with coeliac disease under experimental conditions. Marsh demonstrated a time/dose-dependency for mucosal response to gluten after up to 84 h of exposure in patients with coeliac disease with respect to epithelial height, mitotic index of IELs and lamina propria volume (11). In another study, Catassi and co-workers compared mucosal changes induced by 0.1 and 0.5 g of gluten powder per day (micro challenge) during 4 wk and found a dose-dependent reduction of epithelial height and increase in number of inflammatory cells (12). Between the two groups with different gluten dose, the change of ES differed in two score points out of 12 after 4 wk. Within each dose-group the variation in ES was much larger and a substantial overlapping exists indicating that the span of individual sensitivity to gluten is greater than an average increase in gluten consumption of this study. Though there was a significant gluten dose-dependent difference in change of ES, from a practical point of view the difference in ES mattered only for those who did not relapse. Given the problem of symptoms triggered, starting with a gluten dose of 0.2 g/kg/d seems preferable to 0.5 g/kg/d.

The frequency of sero-conversion of AGA or EmA or the individual level of AGA did not differ between groups A and B. Changes in the cut-off level would not alter this lack of difference. The lack of correlation of serological markers to the amount of gluten consumed is interesting, considering the similar prevalences of coeliac disease found in screening programmes in countries with different gluten consumption. Variations in clinically detected coeliac disease are on the other hand noted between various regions with different gluten intake.

This short-term study shows dose-dependency to exist for the induction of enteropathy but not in relapse rate nor in the information obtained from AGA or EmA.

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References


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