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Natural History of Antibodies to Deamidated Gliadin Peptides and Transglutaminase in Early Childhood Celiac Disease

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ABSTRACT

Introduction: Gliadin proteins play a key role in the pathogenesis of celiac disease; however, as a screen for celiac disease, anti-gliadin antibody testing has been replaced by the more sensitive and specific serological assays for transglutaminase autoantibodies (TGAA). A new generation of anti-gliadin antibody assays has been developed to detect synthetic, deamidated homologous gliadin peptides (DGP) with high sensitivity and specificity.

Methods: Sera were collected prospectively from children with an increased risk for celiac disease as part of an ongoing study at Denver, and studied for the development of celiac autoimmunity. We investigated the high-performance DGP antibody assay in 50 TGAA-positive children both before the development of celiac autoimmunity and following the institution of a gluten-free diet to determine the relationship of DGP antibodies to TGAA. TGAA were measured by an in-house radioassay.

Results: DGP antibodies and TGAA parallel each other over the period of years children were studied. DGP antibodies resolved sooner than TGAA in subjects on a gluten-free diet. DGP antibodies appeared earlier than TGAA in 9 children.

Conclusions: Measuring DGP antibodies may be more useful than TGAA in monitoring children on a gluten-free diet. DGP antibodies can precede the appearance of TGAA in some at-risk children. ***JPGN 45:293–300, 2007.*** **Key Words:** Celiac disease—Deamidated gliadin peptide—Gliadin antibody—Natural history—Pediatric—Gluten-free diet—Transglutaminase.

INTRODUCTION

Celiac disease is estimated to be prevalent in 1:133 of the general population (1), and in Denver it has been estimated to occur in 1:104 children (2). The number from Denver is based on long-term prospective studies of children at genetic risk for celiac disease because they have type 1 diabetes (4% to 6% risk) (3–5), have a family member with type 1 diabetes (4%) (6) or celiac disease (5% to 10%) (7,8), or carry the high-risk HLA-DR3/DR2 (3%) (9). One advantage of monitoring such a population is that prospective data and sera can be collected before actual transglutaminase autoantibody (TGAA) seroconversion occurs to study the natural history of celiac disease.

Gliadin proteins play a key role in the pathogenesis of celiac disease; however, as a screen for celiac disease, anti-gliadin antibody testing has been replaced by the more sensitive and specific serological assays for TGAA. A new generation of immunoglobulin (Ig) A and IgG assays has been developed to detect synthetic, deamidated homologous gliadin peptides with high sensitivity and specificity (10,11). We investigated the high-performance deamidated gliadin peptide (DGP) antibody assay in children monitored prospectively because of an increased risk for celiac disease, both before the development of celiac autoimmunity and after the institution of a gluten-free diet (GFD) to determine the relationship of DGP antibodies to TGAA.

Transglutaminase catalyzes selective crosslinking or deamidation of site-specific glutamine residues of gliadin peptides into glutamic acid (12,13). This causes the introduction of negatively charged residues into specific

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positions of the gliadin peptides, enhancing major histocompatibility complex class II binding and T cell reactivity (14). Therefore, transglutaminase-modified gliadin peptides could exhibit an enhanced binding affinity, leading to increased immunogenicity. Further work into B cell epitopes has shown that selective deamidation of gliadin peptides also enhances antibody reactivity, consistent with previously established deamidation motifs. Such DGP performed better than nondeamidated gliadin peptides by being able to distinguish anti-gliadin antibody–positive individuals as having celiac disease or not (15,16). In addition, 1 group showed that children with celiac disease reacted with a limited number of deamidated gliadin epitopes (17). On the basis of these findings, an enzyme-linked immunosorbent assay (ELISA) using synthetic DGP of fewer than 30 amino acids was designed by INOVA Diagnostics, underscoring the fact that celiac disease patients recognize a highly restricted set of gliadin peptides. An assay that includes the use of these DGP is now approved by the U.S. Food and Drug Administration for use in the diagnosis of celiac disease.

Inasmuch as celiac disease is driven by the ingestion of gluten, we hypothesized that antibodies against DGP would be a better indicator of gluten ingestion and withdrawal than TGAA in patients with celiac disease. The aim of this study was to assess the appearance of DGP antibodies in relation to TGAA in children with evidence of celiac autoimmunity (either biopsy confirmed or shown by persistent TGAA positivity), using sera obtained from these children before TGAA seroconversion and after the development of autoimmunity.

PATIENTS AND METHODS

Sera were collected under the DAISY study (Diabetes AutoImmunity Study of the Young), led by Dr Marian Rewers (18,19), wherein children with a genetic risk for type 1 diabetes mellitus are monitored prospectively for the development of anti-islet autoantibodies. Individuals with type 1 diabetes are also at risk for celiac disease, given the high prevalence of HLA-DR3/DQ2 in both conditions, and TGAA develop in 1 of 3 of individuals with type 1 diabetes who have DR3 homozygosity (20). Therefore, children under the age of 18 years at genetic risk for celiac disease (having HLA-DR3+ from the general population, or having type 1 diabetes, or having a first-degree relative with diabetes or celiac disease) are monitored prospectively in a branch of the DAISY study, termed CEDAR (CEliac Disease Autoimmunity Research), with serial TGAA determinations, many from birth (21). Sera from 50 nondiabetic children in this study branch, found to have evidence of celiac autoimmunity by persistently high-titer TGAA (n = 16) or both TGAA and positive biopsy results (n = 34), were examined for this particular report. No children with TGAA positivity with negative biopsy were selected. None of these children had any known comorbid conditions that may give TGAA positivity besides celiac disease. Of these 50 children selected, 33 had DR3+ from the general population, and 17 had a family history of type 1 diabetes. A total of 440 serum samples were collected over the span of 12 years (0.5–17 years of age) in these children, which included sera from the time before the development of celiac autoimmunity and after diagnosis. The interval between serum sampling was dependent on the age of the patient at the time of enrollment, starting at 9 months of age, then at 15 and 24 months. Afterwards, serum was obtained yearly. In individuals enrolled after 2 years of age, serum was obtained yearly as well. In an individual with a positive TGAA (defined as TGAA index >0.05), samples were repeated in 3 to 6 months (20). A patient underwent evaluation and intestinal biopsy by a pediatric gastroenterologist when 2 blood samples drawn on separate visits were TGAA positive, or sooner if requested by the family (2) (Fig. 1). At the time of intestinal biopsy, TGAA was again measured. The children continued to receive a regular diet before undergoing intestinal biopsy, confirmed by dietary interview by a qualified dietitian, who monitored them at every visit. At upper gastrointestinal endoscopy, 4 samples from the distal duodenum were obtained. A single pathologist who was unaware of the clinical and laboratory findings interpreted the sample according to the system described by Marsh (22). A biopsy specimen with crypt hyperplasia and increased numbers of intraepithelial lymphocytes (Marsh score 2) or any degree of villous atrophy (Marsh score 3) was considered abnormal and consistent with celiac disease. Individuals receiving a diagnosis of celiac disease (biopsy confirmed) were instructed to maintain a GFD, with nutritional counseling and interview for dietary

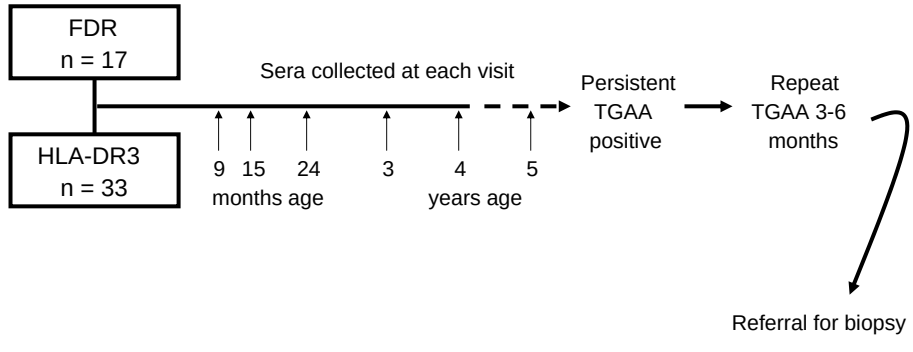

FIG. 1. Algorithm used to collect sera prospectively in children at risk for celiac disease. FDR = first degree relative of a child with type 1 diabetes. A child who was persistently TGAA positive was referred for intestinal biopsy. Sera collected in 50 selected children from this study were used retrospectively to measure DGP antibodies.

adherence within the first 3 months, then with yearly visits. In this group of children, 30 elected to use a GFD, and 20 continued to use a regular diet. Of the 30 receiving a GFD, biopsy confirmed celiac disease in 27, and the other 3 had persistently high titers of TGAA and the families opted for a GFD without biopsy. Of the 20 electing to continue with a regular diet, biopsy confirmed celiac disease in 7, and the remainder had not undergone biopsy at the time of this report. In addition, 116 sera from healthy control children (individuals not known to have celiac disease) were studied. One quarter of the control individuals had a family history of type 1 diabetes, and the remainder were DQ2 positive. All of the control individuals were previously TGAA negative when measured at a single time point. Sera were analyzed by use of the DGP ELISA and TGAA radioimmunoassay as described below. Statistical analysis was performed by GraphPad Prism Version 4.0 for the Mann-Whitney test and GraphPad InStat for the Fisher exact test. This study was performed in accordance with the Colorado Multiple Institutional Review Board and the Health Insurance Portability and Accountability Act.

Deamidated Gliadin Peptide ELISA

The INOVA QUANTA Lite Celiac DGP Screen for measuring both IgG and IgA antibodies to DGP (cutoff 20 units) was performed according to the manufacturers' instructions, with use of a serum dilution of 5 μL in 500-μL buffer, and incubating 100 μL diluted serum per well. This assay measures only antibodies to DGP. Separate ELISAs were also run for DGP IgA alone and for DGP IgG alone.

Transglutaminase Autoantibody Radioimmunoassay

Measurement of TGAA was performed by an extensively validated human recombinant transglutaminase (TG) IgA radioimmunoassay performed in a 96-well fluid-phase format (20,23) with ^{35}S-labeled TG. The antibody is precipitated with goat anti-human IgA agarose and measured with use of a Top Count β-counter. The cutoff for positivity (0.05 index) was established on the basis of 3 × 100th percentile of 184 healthy control individuals. Total IgA was not measured in this population because these 50 children were preselected for persistent TGAA positivity.

COMPETITIVE BINDING ASSAYS

Sera from 3 children who were both TGAA and DGP antibody positive were studied: The sera were serially diluted to obtain 50% attenuation in the signal for TGAA (dilutions 1:5) and also for DGP (dilution 1:1). By use of a dilution of 1:5, sera were first preincubated with DGP by incubating 200 μL of diluted sera in a DGP-coated ELISA well for 30 minutes and transferring the sera to a different DGP-coated well. This was repeated × 4 wells; then the remaining sera were used to detect TGAA by radioimmunoassay. To study possible overlap of DGP antibodies to TGAA, a dilution of 1:1 sera was first preincubated with guinea pig TG in increasing concentrations × 1 hour before the sera were tested with the DGP ELISA kit.

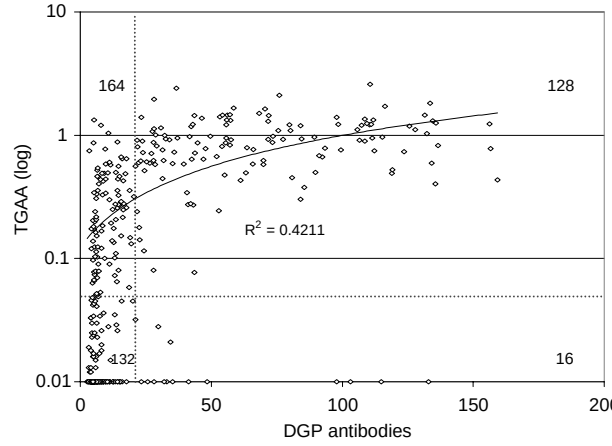

FIG. 2. Overall correlation of DGP antibodies (x-axis) and TGAA (y-axis) in 440 serum samples from all time points in the 50 children monitored with celiac disease. DGP antibodies are rarely positive when TGAA is negative.

RESULTS

In this group of children with evidence of celiac autoimmunity (persistent TGAA positivity), the combined DGP (IgA and IgG) ELISA kit performed better than the DGP IgA- or IgG-only kit and gave positive results in 45 of 50 children in at least 1 time point during their course of follow-up. Of these 45 combined DGP antibody positive sera, 8 were IgG positive alone, and only 1 was IgA positive alone. Therefore, the IgG antibody kit performed better than the IgA, but the combined IgA and IgG kits performed best. The remainder of the data reported are for the combined DGP (IgA and IgG) ELISA kit only, unless otherwise specified. Figure 2 shows the overall correlation of DGP (IgA and IgG combined) antibodies and TGAA in 440 serum samples from all time points in the 50 children followed up.

The approximate 99th percentile of normal control individuals for TGAA is 0.05, and until relatively high levels of TGAA were present the DGP assay was usually "negative," although 16 serum samples at some time during follow-up had DGP antibodies and were negative for TGAA. DGP antibodies were positive at the time of initial appearance of TGAA in 39 of 50 children, and 9 of these had DGP antibodies that actually preceded the appearance of TGAA (average 1.2 years earlier). In 5 of 50 children, DGP positivity never developed at any time point measured, and in 4 of these children, TGAA was positive for only 1 or 2 consecutive measurements before they became negative after the consumption of a gluten-free diet. The fifth child had recently shown seroconversion to TGAA positive (DGP negative) but at the time of this report had not yet had a follow-up visit to confirm celiac autoimmunity. In these 5 children neither the individual IgA nor IgG antibodies were

positive. Only 2 of 116 healthy control individuals (with no known previous autoimmune disease) were positive for DGP antibody (98% specificity), and in 1 sample, both DGP IgA and IgG were separately detected, whereas in the other, both IgA and IgG were separately negative.

Of the 30 children receiving a GFD, 26 were DGP negative at the last follow-up. When it is considered that 7 were DGP negative in the sera immediately before GFD (although 2 were positive earlier), of 23 children who were DGP positive, all but 4 were DGP negative while receiving a GFD ($19/23 = 83\%$ seroconversion to negative with GFD). Meanwhile, only 10 were TGAA negative while receiving GFD ($10/30 = 33\%$ seroconversion to negative with GFD). Of the 20 children continuing with a regular diet, 15 remained DGP positive and 19 remained TGAA positive at last follow-up. Of the 5 children who were DGP negative at last follow-up, 1 of those 5 children has recently shown TGAA seroconversion to positive and was never DGP positive, and has not had a follow-up visit to confirm celiac autoimmunity. The other 4 children had low positive (<25 units) DGP antibodies for only 1 or 2 time points before becoming negative again. No children were DGP positive while TGAA negative. There was no statistically significant difference in the response to GFD or to regular diet according to the Fisher exact test.

There was a significant decrease in both DGP antibodies and TGAA in children receiving GFD when measured before GFD (or first TGAA positivity in those continuing with a regular diet) compared with the antibodies at last follow-up (Fig. 3). The average time between the 2 measurements was 3.7 years (±1.7 years) and 3.1 years (±1.8 years) for children receiving a GFD and a regular diet, respectively. The average change in both antibodies over this time period is shown in Table 1.

Figure 4 shows representative graphs of the relationship of the appearance and persistence of DGP antibodies and TGAA in 4 children continuing to receive a regular diet. There is a remarkable relationship between the 2 antibodies that tends to persist while the child remains on a regular diet.

Figure 5 shows representative graphs of the relationship between DGP antibodies and TGAA in 4 children monitored prospectively both before and after the diagnosis of celiac disease and the institution of a GFD. The 3 types of patterns observed were disappearance of both antibodies, persistence of both antibodies, or resolution of DGP antibodies with persistence of TGAA.

Given the remarkable relationship (when monitored over time) between DGP antibodies and TGAA, we thought that it was important to rule out cross-reactivity between the 2 antibodies and also to exclude the possibility that a single antibody was recognizing both DGP and TG. Competitive binding assays were performed in which sera were preincubated with guinea pig TG in solution before being run on the DGP ELISA kit (Fig. 6). Conversely, sera were preincubated with plate-bound DGP before being run for TGAA

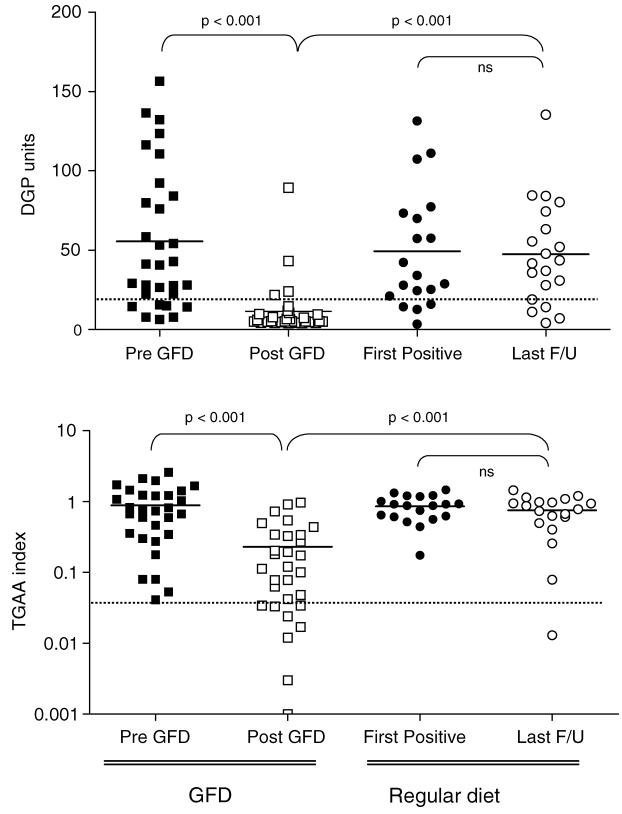

FIG. 3. Top, In children treated with GFD, DGP antibody levels significantly decreased (most resolved); however, children receiving a regular diet had persistently high levels of DGP antibodies. Bottom, TGAA levels also decreased significantly with GFD treatment, compared with children receiving a regular diet; however, the majority of children treated with GFD remained TGAA positive.

TABLE 1. *Change in DGP antibodies and TGAA in children receiving GFD vs regular diet*

	DGP antibody units (cutoff 20 units)	TGAA index (cutoff 0.05 index)
GFD		
Pre-GFD	55.5 units	0.88
At last follow-up	11.5	0.23
Average change in antibody level	Decreased by 44 units	Decreased by 0.65 units
Regular diet		
First TGAA positive	43.9 units	0.85
At last follow-up	49	0.75
Average change in antibody level	Decreased by 0.3 units	Decreased by 0.1 units

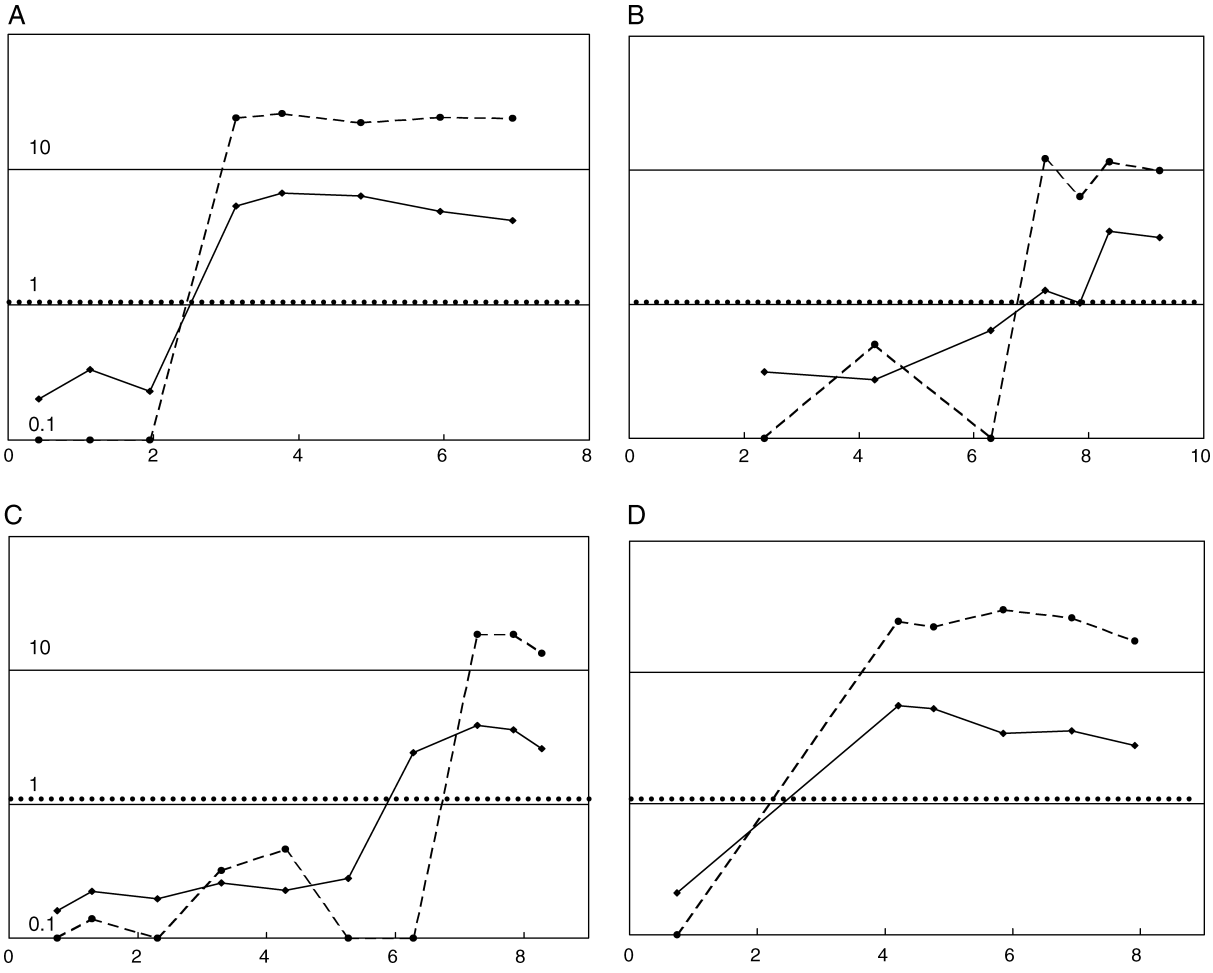

FIG. 4. Representative graphs of children receiving a regular diet over time (A–D); x-axis, age of patient; y-axis, antibody levels. Solid line = DGP antibody, dashed line = TGAA. Antibody levels are normalized to a value of 1, derived by the level of the antibody divided by the usual assay cutoff for positivity, indicated by dashed line on y-axis. Therefore, a value of 1 is the cutoff for a positive sample for each assay. The 2 antibodies parallel each other remarkably and persist while the child continues to receive a regular diet, as shown in all panels.

by radioassay (Fig. 7). We were unable to extinguish the signal for DGP by competition with TG, nor were we able to extinguish the signal for TG by competition with DGP. This suggests that the antibodies, although parallel in their natural history, are indeed 2 separate antibodies.

DISCUSSION

The purpose of this study was to evaluate the appearance and relationship of DGP antibodies to TGAA in a select group of children known to have celiac autoimmunity. We use the term ''celiac autoimmunity'' to refer only to individuals with TGAA positivity, whether or not biopsy has been performed. This term implies that an autoimmune process is present, as evidenced by autoantibodies, whether the celiac process is latent or overt. Only those children with biopsies confirming histological abnormalities were actually considered to have celiac disease, even though all of them had persistent TGAA positivity. Antibodies to DGP are specific, and they parallel the fluctuations of TGAA in children with celiac autoimmunity. In this study, we were able to observe the natural history of the appearance of these antibodies and compare it with TGAA, and follow their course after a GFD was begun.

We were struck by how well DGP antibodies parallel TGAA, especially considering that 1 antibody is formed by exposure to exogenous proteins and the other is an

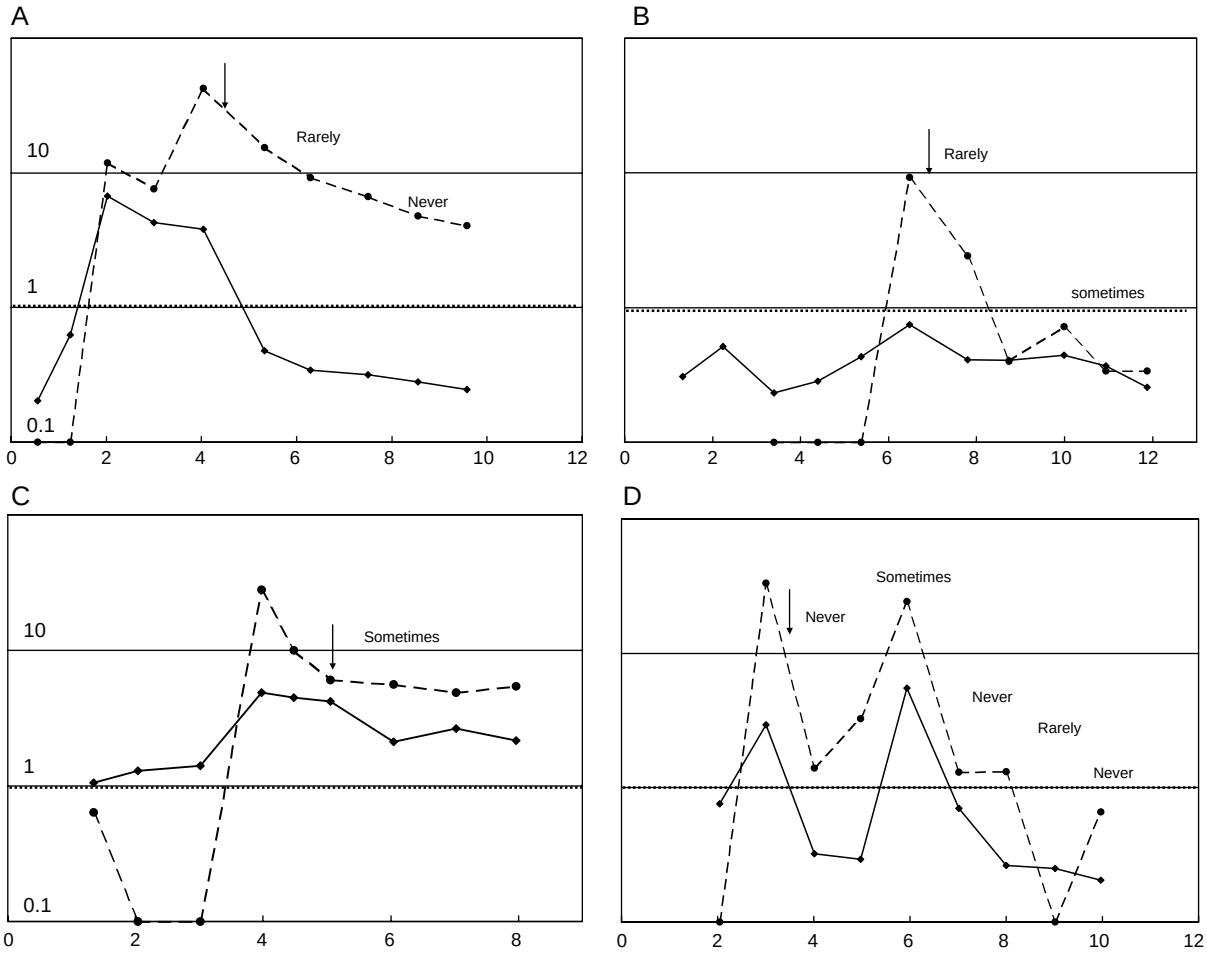

FIG. 5. Representative graphs of children treated with GFD over time. Arrow indicates time of biopsy with confirmed celiac disease, and institution of GFD. "Sometimes," "rarely," and "never" indicate patient-reported frequency of dietary indiscretion. A, Example of persistently positive TGAA with resolution of DGP antibodies. B, Example of transiently positive TGAA while DGP remained negative at all time points. C, Example of persistently positive TGAA and DGP antibodies. D, Example of complete resolution of both TGAA and DGP antibodies.

autoantibody. The specific nature of the antibodies and the appearance of DGP antibodies in individuals with celiac disease emphasize the importance of transglutaminase deamidation of selective gliadin peptides in the pathogenic process. The parallel nature of DGP antibodies and TGAA also underlines the intimate relationship between gliadin and transglutaminase as part of this process, particularly at the onset of celiac autoimmunity. It is intriguing to consider how antibodies to DGP could be specific to celiac disease. Two possible explanations have been offered as to why antibodies to deamidated gliadin epitopes exist in celiac patients, as cited by Aleanzi et al (16). Given that T cell reactivity is enhanced by deamidated (as opposed to native) gliadin sequences, B cell activation may be more robust to previously deamidated peptides. In addition, such deamidated peptides may be in higher concentration in people with celiac disease than in healthy individuals because of increased transglutaminase activity in the mucosa. It has been hypothesized that at the time of diagnosis, a broad humoral immune response is directed against gliadin, TG, and TG–gliadin complexes that becomes restricted to only TG after the elimination of gluten from the diet (24). Our results are consistent with this hypothesis. We can also speculate that DGP antibodies become undetectable sooner than TGAA either because the autoimmune process takes longer to resolve than the humoral process against an exogenous antigen or possibly because the TGAA assay is simply more sensitive. In our series, 2 of 3 of children remained TGAA positive even while receiving a GFD after an average of 3 years, and this similar frequency of persistent positivity has been observed in 1 independent series in Italy (24) but was higher in another that

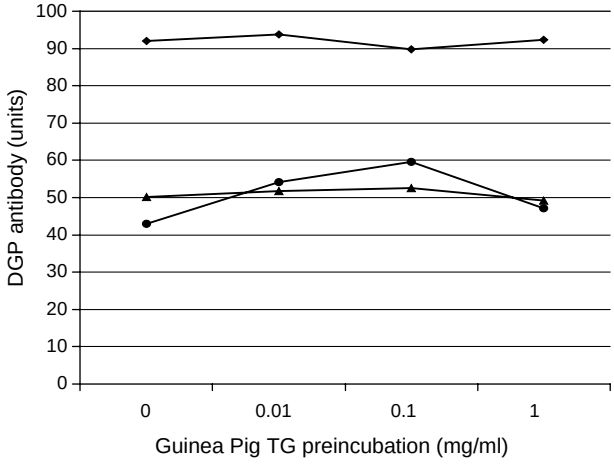

FIG. 6. DGP antibody levels following increased fluid-phase competition with guinea pig TG. Sera from 3 children positive for both TGAA and DGP antibodies were tested for antibody cross-reactivity between TG and DGP (sera 1–3). Sera were preincubated with guinea pig TG at increasing concentrations before testing with the DGP ELISA. TG was unable to absorb DGP antibodies, as indicated by a stable DGP antibody signal despite increasing TG competition.

monitored only children (1 of 3 remained positive) (25). It is important to note that both studies also used the TGAA radioimmunoassay, and although TGAA was persistent, it did decrease in overall titer.

The appearance of DGP antibodies even before TGAA in 9 children suggests that measurement of these antibodies may be useful for earlier screening in celiac disease. More important, the more rapid resolution of DGP antibodies in children treated with GFD does suggest to us that measurement of this antibody may be useful in monitoring dietary adherence, and it may be superior to the measurement of TGAA in this regard. The 5 children who became DGP negative while receiving a regular diet either had low titers of antibodies for a relatively brief time (4 children) or had never been DGP positive and had only recently become TGAA positive (1 child). Therefore, this may be more of a reflection of a less sensitive assay rather than spontaneous DGP antibody seroconversion to negative. By contrast, our series shows that of those children who are DGP positive, DGP remains detectable in only 18% after GFD. Serum to examine the natural history of new antibody assays such as DGP can be obtained only through ongoing long-term prospective studies of at-risk individuals, such as this study, and others in Germany (26) and Finland (27), which can provide valuable insight into the behavior of celiac disease autoimmunity. Future studies involving this new DPG antibody assay include the investigation of assay sensitivity, ability to monitor strict dietary control including gluten challenge, and multiple antibody panels for the diagnosis and prediction of celiac disease.

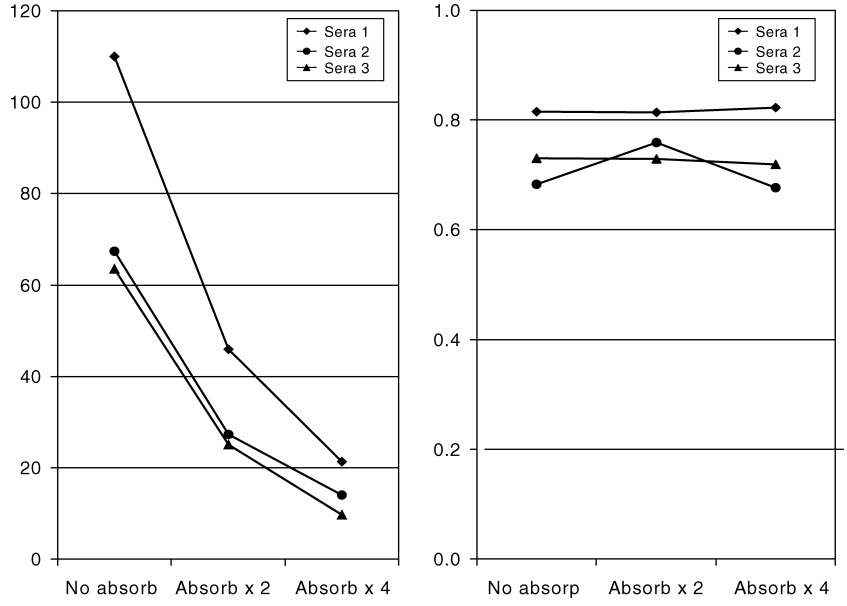

FIG. 7. To further confirm that antibodies to TG and to DGP were not cross-reactive, sera from 3 children positive for both TGAA and DGP antibodies were first passed through plate-bound DGP up to 4 times to absorb DGP antibodies. Sera were then tested for DGP antibodies (left) and TGAA (right). A decrease in DGP signal indicates preabsorption by DGP resulting in depletion of the antibody. A lack of a decrease in TGAA signal indicates that TGAA is unable to be absorbed by DGP.

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