

Usefulness of recombinant γ -gliadin 1 for identifying patients with celiac disease and monitoring adherence to a gluten-free diet

Bharani Srinivasan, PhD,^a Margarete Focke-Tejkl, PhD,^a Milena Weber, MSc,^a Sandra Pahr, PhD,^a Alexandra Baar, PhD,^a Raja Atreya, MD,^b Markus F. Neurath, MD,^b Harald Vogelsang, MD,^c Wolf-Dietrich Huber, MD,^d and Rudolf Valenta, MD^a *Vienna, Austria, and Erlangen, Germany*

Background: Celiac disease (CD) is an inflammatory disease of the small intestine caused by an immunologic hypersensitivity reaction to dietary wheat gluten.

Objectives: We sought to clone, express, and perform IgA epitope mapping of a CD-specific wheat antigen and to study its usefulness for identifying patients with CD and monitoring adherence to a gluten-free diet.

Methods: A synthetic gene coding for γ -gliadin 1 (GG1) was expressed in *Escherichia coli*. Recombinant γ -gliadin 1 (rGG1) was purified and characterized biochemically, structurally, and immunologically by using sera from patients with CD and control subjects. Overlapping GG1 peptides were synthesized for IgA and IgG epitope mapping. GG1 and peptide-specific antibodies were raised for tracing GG1 in cereals and dietary wheat products and to study its resistance to digestion.

Results: rGG1 was expressed and purified. rGG1-based IgA ELISAs performed in populations of patients with CD and control subjects showed a specificity of 92.9%, which was higher than that of gliadin extract (e). Furthermore, it allowed monitoring of adherence to a gluten-free diet in patients.

A 26-amino-acid peptide from the proline-glutamine-rich

repetitive N-terminal region was identified as the immunodominant IgA epitope. GG1-related antigens were found in rye, barley, and spelt but not in oat, rice, or maize. GG1 was detected in dietary wheat products after baking, and in particular, the major IgA epitope-containing region was resistant against digestion.

Conclusions: rGG1 and its epitope might be useful for identifying patients with CD, monitoring treatment, and studying the pathomechanisms of CD and development of preventive and therapeutic strategies. (*J Allergy Clin Immunol* 2015;■■■■:■■■-■■■.)

Key words: Celiac disease, cloning, recombinant gamma gliadin 1, epitope

Celiac disease (CD) is an inflammatory disease of the small intestine caused by a hypersensitive immune response to dietary gluten proteins that affects 0.7% to 2% of the European and American population.^{1,2} CD is characterized by small intestinal villous atrophy, crypt hyperplasia, and infiltration of gliadin-specific lymphocytes in the lamina propria.³ On gluten exposure, patients usually secrete high titers of anti-gliadin antibodies (AGAs) and antibodies against endomysial proteins (EMA) proteins.⁴ Tissue transglutaminase 2 (tTG2) was identified as one of the endomysial autoantigens.⁵ Interestingly, deamidation of gliadin peptides by tTG2 enhances activation and IFN- γ secretion by gliadin-specific T cells, which is thought to lead to mucosal damage.⁶ Assays measuring levels of anti-tTG2 and anti-deamidated gliadin peptide (DGP) antibodies are being used for the diagnosis of CD. Although these tests are very useful for diagnosis, they are not ideally suited for the monitoring of a patient's adherence to a gluten-free diet (GFD).⁷ Measurement of AGA levels for diagnosis is less frequently performed because of their lower specificity and sensitivity.⁸

In fact, AGA tests are based on an alcohol-soluble fraction of wheat, termed the gliadin fraction, which is a complex mixture of water-insoluble seed storage proteins. Within the gliadin fraction, many proteins can be found, among them cysteine-rich α/β -gliadins, γ -gliadins, and cysteine-poor ω -gliadins, which occur in numerous isoforms and heterogeneities, all of which might not be equally toxic to patients with CD.⁹⁻¹¹

We have recently developed a subfractionation technique for the gliadin fraction to enrich highly CD-specific IgA-reactive antigens. Analysis of the subfractions for IgA reactivity with sera from patients with CD and control subjects revealed that the subfraction containing α/β -gliadins had lower specificity than a subfraction that was rich in γ -gliadins. Within this CD-specific IgA-reactive fraction, we identified peptide sequences derived from γ -gliadins as possible CD-specific IgA-reactive wheat antigens based on mass spectrometry and protein sequencing.¹²

From ^athe Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, ^cthe Department of Gastroenterology and Hepatology, and ^dthe Department of Pediatrics and Adolescent Medicine, Medical University of Vienna; and ^bMedical Clinic 1, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen.

Supported by the FWF-funded PhD program IAI DK W1212 and the Medical University of Vienna and in part by a research grant from Phadia/Thermo Fisher Scientific, Uppsala, Sweden.

Disclosure of potential conflict of interest: R. Atreya has consultant arrangements with AbbVie, Takeda, InDex, and MSD Sharp & Dohme and has received research support from the German Research Council. M. F. Neurath has consultancy arrangements with MSD, Giuliani, Pentax, InDex Pharmaceuticals, Shire, and Tillotts Pharma; has received research support from the German Research Council and German Cancer Aid; has received payment for lectures from AbbVie, Boehringer Ingelheim, Celgene Corporation, Falk Foundation, Ferring, MSD, Janssen, and Takeda; has a patent for anti-IL-12 therapy in patients with Crohn disease; and receives royalties from Thieme and VG Wort. H. Vogelsang has received research support from the Austrian Science Fund. R. Valenta has received research support and a consulting fee or honorarium from Thermo Fisher Scientific, has consultancy arrangements from Biomay AG and Fresenius Medical Care, and has received research support from Biomay AG. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication June 11, 2014; revised April 22, 2015; accepted for publication April 23, 2015.

Corresponding author: Rudolf Valenta, MD, Division of Immunopathology, Department of Pathophysiology and Allergy Research, Centre for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria. E-mail: rudolf.valenta@meduniwien.ac.at.

0091-6749

© 2014 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

<http://dx.doi.org/10.1016/j.jaci.2015.04.040>

Abbreviations used

AGA:	Anti-gliadin antibodies
AUC:	Area under the curve
CD:	Celiac disease
DGP:	Deamidated gliadin peptide
EMA:	Endomysial proteins
GFD:	Gluten-free diet
GG1:	γ -Gliadin 1
ID:	Intestinal disorder
MALDI-TOF:	Matrix-assisted laser desorption/ionization time of flight
NCBI:	National Center for Biotechnology Information
PBST:	PBS with 0.05% Tween 20
rGG1:	Recombinant γ -gliadin 1
tTG2:	Tissue transglutaminase 2

The aim of this study was the molecular characterization, cloning, and epitope mapping of a CD-specific wheat antigen. We also sought to study its usefulness for identifying patients with CD and monitoring adherence to a GFD. Based on the previously obtained γ -gliadin peptide sequences, we have now constructed a synthetic gene coding for a γ -gliadin, which we designated γ -gliadin 1 (GG1), expressed the recombinant protein in *Escherichia coli*, purified it to homogeneity and performed a biochemical and structural analysis of the recombinant protein. The recombinant protein was then evaluated for specific IgA reactivity with sera from patients with CD and various control populations. Furthermore, we studied the usefulness of IgA anti-recombinant γ -gliadin 1 (rGG1) antibodies for monitoring adherence to GFD. IgA epitopes were mapped by testing a series of overlapping synthetic peptides. Rabbit antisera were raised against the recombinant protein and its major IgA-reactive peptide to identify related antigens in other cereals and to investigate its resistance to baking and digestion.

METHODS**Identification of GG1 based on amino acid sequence data, cloning, and expression of rGG1**

Using a combined biochemical and immunologic approach, we have identified a subfraction of wheat gliadin that reacted specifically to IgA antibodies from patients with CD.¹² Within this subfraction, 2 peptide sequences (ie, N-terminus: NIQVDPGSGQ; internal peptide, APFASIVA-GIGGQ) belonging to γ -gliadins were obtained from the approximately 35-kDa protein band. When we searched the SwissProt database for wheat sequences coding for proteins of the corresponding molecular weight containing both peptides, we identified GG1 from *Triticum aestivum* (SwissProt accession no. P08453.1) as a candidate antigen. A National Center for Biotechnology Information (NCBI) Protein blast (<http://blast.ncbi.nlm.nih.gov>) of the amino acid sequence of GG1 with sequences from *Taestivum* (taxid:4565) deposited in the NCBI nonredundant protein sequences (nr) database yielded 25 γ -gliadin isoforms of similar molecular weights containing both peptides, which differed only in a few amino acids.

A synthetic gene containing the sequence coding for the mature protein of the GG1 gene (GenBank: M16064, SwissProt ID: P08453.1) along with a C-terminal 6x Histidine tag and methionine as a start codon, which was codon optimized for expression in *E coli*, was obtained from GenScript (Piscataway Township, NJ) and cloned into pET-27b(+) (Novagen, Darmstadt, Germany).

The pET-GG1 construct was transformed into *E coli* BL21 (DE3; Invitrogen, Carlsbad, Calif), and rGG1 was expressed in liquid cultures and purified, as previously described.¹³

Multiple sequence alignment of GG1 with homologous proteins from other cereals

An NCBI Protein blast (<http://blast.ncbi.nlm.nih.gov>) search of GG1 (SwissProt ID: P08453.1) against spelt (*Triticum spelta*), rye (*Secale cereale*), barley (*Hordeum vulgare L*), maize (*Zea mays*), rice (*Oryza sativa*), and oat (*Avena sativa*) was performed. A protein sequence showing maximum identity with GG1 from each of the cereals was chosen for multiple sequence alignment by using Clustal Omega software from the EMBL-EBI Web site (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).¹⁴

Protein extracts and SDS-PAGE

Gliadin extract (e) from *Taestivum* was prepared according to the Osborne protocol.¹⁵ Seed extracts from wheat, rye, spelt, barley, maize, oat, and rice and protein extracts of crust and crumbs of 3 types of commonly consumed breads were prepared for immunoblotting, as previously described.¹³

Migration patterns of extracts and rGG1 under reducing and nonreducing conditions were compared by mixing 10 μ g of rGG1 with Laemmli sample buffer containing β -mercaptoethanol (reducing) or without β -mercaptoethanol (nonreducing) by using 12% SDS-PAGE and staining of gels with Coomassie Brilliant Blue R-250.¹²

Matrix-assisted laser desorption and ionization-time-of-flight (MALDI-TOF) mass spectrometry of rGG1 was performed in a positive linear mode by using MALDI-TOF with the Compact MALDI II instrument (Kratos, Manchester, United Kingdom; piCHEM, R&D, Graz, Austria).

Circular dichroism analysis of purified rGG1

The rGG1 protein was dialyzed in 40 mmol/L acetic acid, and the circular dichroism spectra of rGG1 were analyzed by using a Jasco J-810 Spectropolarimeter (JASCO, Tokyo, Japan). The far-UV circular dichroism spectra from 190 to 260 nm were recorded in a 2-mm path length quartz cuvette (Hellma, Mullheim, Baden, Germany) with a resolution of 1 nm, a scan speed of 50 nm/min, and a protein concentration of 0.10 mg/mL, and an average of 3 scans were obtained. Measurements were taken at 21°C. Results are expressed as mean residue ellipticity (degrees per square centimeter per decimole) $\times 10^3$ at a given wavelength. Secondary structure analysis was performed with the CDSSTR algorithm from DICHROWEB.

Sera from patients with CD and control subjects

Sera were taken from 63 untreated patients with CD (median age, 26 years; range, 0.4-65 years; male/female sex, 16/49) who received a diagnosis according to European Society for Paediatric Gastroenterology, Hepatology and Nutrition guidelines.¹⁶ All the patients had positive test results regarding EMA, for IgA reactivity to tTG2 QUANTA Lite ELISA diagnostic kits (Inova Diagnostics, San Diego, Calif) and for the presence of either IgA, IgG, or both against deamidated gliadin (DGP) QUANTA Lite ELISA diagnostic kits (Inova Diagnostics). Additional serum samples were obtained from 6 patients (median age, 8 years; range, 1-10 years; male/female sex, 0/6) before and after GFD (GFD: 6 months to 81 months) from 18 patients with CD who had received GFD for a period of 4 months up to 7 years (median age, 8 years; range, 1-69 years; male/female sex, 4/14). The AGA⁺/EMA⁻ subjects (n = 13; median age, 1 year; range, 0.5-14 years; male/female sex, 9/4) had negative results regarding EMA. In this group 13 of 13 subjects also had negative test results for IgA against tTG2, 11 of 13 had negative results for IgA against DGP, and 9 of 13 had negative results for IgG against DGP. For control purposes, sera from 55 patients with intestinal disorders (IDs; eg, Crohn disease [n = 26; median age, 38 years; range, 0.4-75 years; male/female sex, 10/16] and ulcerative colitis [n = 22; median age, 32.5 years; range, 21-76 years; male/female sex, 14/8]) or intestinal adenocarcinoma, colon cancer, or polyps (n = 7; median age, 59 years; range, 47-77 years; male/female sex, 4/3); sera from 32 allergic subjects (wheat food allergy: n = 7; median age, 39 years; range, 36-49 years; male/female sex, 2/3; information on 2 patients was not available), patients with baker's asthma (n = 6; median age, 37 years; range, 12-45 years; male/female sex, 5/1), patients with milk allergy (n = 13; median age, 24 years; range, 1-64 years; male/female sex, 4/9), or patients

with respiratory allergy ($n = 6$; median age, 35 years; range, 23–54 years; male/female sex, 5/1); and sera from 41 healthy volunteers were tested. Subjects from the control groups had negative test results for CD by using the diagnostic test kits mentioned above. Sera were obtained in the course of routine diagnostic procedures, and residual serum samples were tested in an anonymized and retrospective manner with permission from the Ethical Committee of the Medical University of Vienna.

rGG1-derived synthetic peptides

For IgA and IgG epitope mapping, 19 overlapping peptides with lengths of between 18 to 26 amino acids spanning the length of rGG1 were synthesized and purified, as previously described.¹⁷

IgA reactivity to rGG1 and gliadin extract (e) and epitope mapping using synthetic overlapping peptides by means of ELISA

ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were either coated with gliadin extract (e) or rGG1. For peptide mapping, plates were coated with rGG1 or rGG1-derived synthetic peptides. The antigens were coated at a concentration of 5 $\mu\text{g/mL}$ diluted in bicarbonate buffer (pH 9.0) overnight at 4°C and washed twice with PBS with 0.05% Tween 20 (PBST). The plates were then blocked with 1% BSA in PBST for 2 hours at room temperature and incubated overnight at 4°C with human sera diluted 1:100 and 1:1000 for IgA and IgG binding, respectively, or with buffer alone. The plates were washed with PBST and incubated with an anti-human IgA₁/IgA₂ mAb (BD Biosciences, San Jose, Calif) at 1:1000 dilution or a monoclonal anti-human IgG (BD Biosciences) at 1:1000 for 5 hours at 4°C. After washing, the bound antibodies were detected by incubating the plates with 1:1000 diluted sheep anti-mouse horseradish peroxidase-conjugated antibodies (GE Healthcare, Buckinghamshire, United Kingdom). Results represent means of duplicate determinations with less than 2% variation.

Production of rabbit antibodies specific for rGG1 and peptides 4 and 18

Antibodies specific for rGG1 were obtained by means of immunization of rabbits, as previously described.¹² Antibodies to rGG1-derived peptide 4 and peptide 18 were obtained by coupling the peptides to keyhole limpet hemocyanin by using Imject EDC mKHLH Spin Kit (Thermo Fisher Scientific, Rockford, Ill) before immunization.

In vitro digestion of rGG1, simulating gastric and duodenal digestion

In vitro digestion of rGG1 was performed by using 2 enzymes: first digestion with pepsin and then chymotrypsin.^{18,19} Briefly, rGG1 solubilized in 50 mmol/L acetic acid was digested first with 1:100 wt/wt pepsin. The peptic digestion was carried out at 37°C, and aliquots of digested samples were collected at 15, 30, 60, 90, and 120 minutes. The reaction was stopped by increasing the pH of the samples up to 8.0 by addition of 1 mol/L NaOH. The peptic digest was then further digested with 1:100 wt/wt chymotrypsin in a solution containing 100 mmol/L Tris and 10 mmol/L CaCl₂ (pH 8.3). The reaction mixture was incubated at 37°C, and aliquots were collected at 15, 30, 60, 90, 120, and 240 minutes and 20 hours. The reaction was stopped by heating the samples to 95°C for 5 minutes.

According to pilot SDS-PAGE stained with Coomassie blue, comparable amounts of cereal extracts, bread crumbs, and crust extracts were separated by means of SDS-PAGE²⁰ and blotted onto nitrocellulose.²¹ GG1 moieties were detected with specific rabbit antibodies.¹²

Statistics

Data were analyzed by using GraphPad Prism statistical software (GraphPad Software, San Diego, Calif). The receiver operating characteristic

curve was plotted, and the AUC and Youden index (J)²² were calculated and used to determine the best cutoff value for anti-rGG1 and anti-gliadin extract (e) antibodies. Statistical significance between 2 groups was analyzed by using the Mann-Whitney U test and Wilcoxon matched-pairs test.

RESULTS

Domain architecture of wheat GG1, which was produced as recombinant antigen

We have previously identified 2 peptide sequences of wheat γ -gliadin in a subfraction of wheat gliadin that showed highly specific IgA reactivity in sera from patients with CD.¹² Based on sequence comparisons, we identified a corresponding wheat GG1 protein in the database (SwissProt accession no. P08453.1, see Fig E1 in this article's Online Repository at www.jacionline.org). This protein has also been described to contain a T-cell epitope (ie, 134–153: QQLPQPQQPQQSFPQQQRPF) recognized by a T-cell clone isolated from the gut of a patient with CD (Fig 1, light blue box).^{23,24} The architecture of the corresponding GG1 precursor protein is shown in Fig E1, A. The precursor protein comprises 327 amino acids, of which the 19 N-terminal amino acids are cleaved to yield the mature protein containing 308 amino acids. GG1 contains high amounts of Gln (35.4%) and Pro (17.5%), as is typical for γ -gliadins.²⁵ GG1 can be grouped into 5 domains (see Fig E1, A).²⁶ Domain I is a short N-terminal nonrepetitive region, whereas domain II represents the Pro- and Gln-rich repetitive region. In closely related γ -gliadins from wheat, the characteristic PQQPFPQ repeat occurs 8 times and the PQQPFPQQPQQPFPQTQQ sequence occurs 3 times in this domain, and the domain is usually devoid of negatively charged amino acids. GG1 domain III is a cysteine-rich region with 5 cysteine residues. Domain IV is rich in glutamine, and domain V is a C-terminal nonrepetitive region, which, in the case of GG1, contains 2 cysteine residues.

Wheat GG1 has highest sequence homology with spelt, rye, and barley prolamins

A comparison of the amino acid sequence of the GG1 precursor protein with the sequences deposited in the sequence databases revealed that it shared significant sequence homology (>49% sequence identity) with cereal prolamins from spelt, rye, and barley, whereas sequence homology with prolamins from oat and rice was much lower (<42% sequence identity, Fig 1). The spelt protein (GenBank Accession no. AAD30440.1) showed a sequence identity of 79.2%, the rye protein (ADP95484.1) showed a sequence identity of 68%, and the barley protein (ABH01262.1) showed a sequence identity of 49.6%. Prolamins from oat (CCH22519.1) and rice (ABU89796.1) showed sequence identities of 42.2% and 32.6%, respectively. No proteins with relevant sequence identity were identified for maize in the databases searched. Sequence identities were low within the Pro- and Gln-rich domain, whereas homology was higher in domains III and IV. Furthermore, there was a considerable variation in length and composition in domains II and IV among the compared proteins. Domains III and V showed similar length among the various prolamins. The 7 cysteine residues of GG1 located in domains III and V (Fig 1, boxed in red) were highly conserved among

FIG 1. Multiple sequence alignment of the GG1 amino acid sequence with amino acid sequences of prolamins from closely related cereals (spelt, rye, and barley) and distantly related rice and oat. Identical amino acids are represented by *dots*, and gaps are represented by *dashes*. The coloring of the amino acids is based on the domain architecture, as described in Fig E1, A. The *open green* and *dark blue rectangles* represent peptides 4 and 18, respectively. The T-cell epitope described by Molberg et al²⁴ has been *boxed in light blue*. The conserved cysteines are represented by *open red rectangles*. Percentages of sequence identities between GG1 and respective prolamins are indicated in bold italics in the *bottom right corner*.

Analysis of the secondary structure of rGG1 by means of circular dichroism (Fig 2, B) and subsequent calculation of the contents of secondary structure with CDSSTR software showed that the protein was largely unordered, with an alpha-helix content of less than 25%. No difference was observed when the circular dichroism spectra were recorded with rGG1 in 70% ethanol or in 10^{-5} mol/L HCl solutions (data not shown).

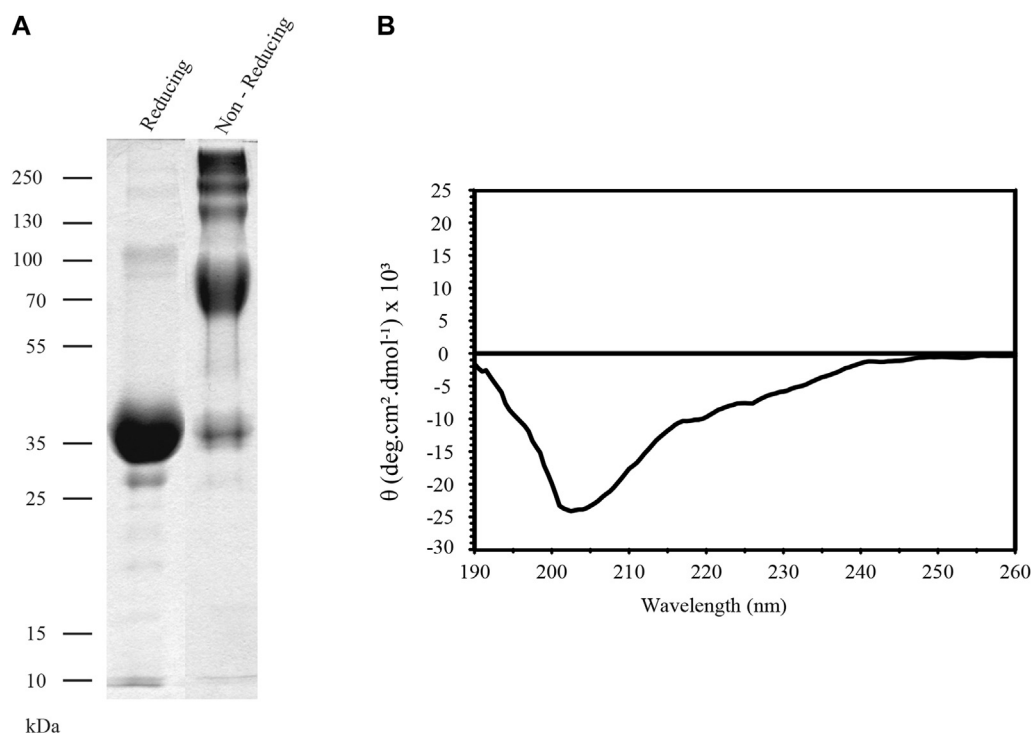


FIG 2. Biochemical and structural characterization of rGG1. **A**, Coomassie-stained SDS-PAGE containing rGG1 separated under reducing (*left lane*) and nonreducing conditions (*right lane*). Molecular weight markers (in kilodaltons) are indicated on the *left margin*. **B**, Circular dichroism analysis of purified rGG1. The spectra are expressed as mean residue ellipticities (*y-axis*) at given wavelengths (*x-axis*).

rGG1 is a major CD-specific, IgA-reactive antigen

To investigate whether native gliadin-based AGA tests can be improved by using rGG1, we tested serum IgA reactivity of patients with active CD, patients with IDs, healthy subjects, patients with IgE-associated allergies (including food allergy), and AGA⁺/EMA⁻ subjects to rGG1 and gliadin extract (e) (Fig 3, A). When IgA reactivity to rGG1 was analyzed, 46 of 63 patients with CD, 1 of 55 patients with IDs, 4 of 41 healthy subjects, 2 of 32 allergic patients, and 3 of 13 AGA⁺/EMA⁻ subjects had positive test results (Fig 3, A). Testing IgA reactivity to gliadin extract (e) showed that 56 of 63 patients with CD, 5 of 55 patients with IDs, 7 of 41 healthy subjects, 9 of 32 allergic patients, and 13 of 13 AGA⁺/EMA⁻ subjects had positive results (Fig 3, A). In the CD group the number of patients with positive IgA reactivity to gliadin extract (e) was higher than the number of patients with positive IgA reactivity to the rGG1 protein, but in each of the control groups, the number of patients showing false-positive IgA reactivity to rGG1 was lower than the number of patients showing false-positive IgA reactivity to gliadin extract (e). The difference in IgA reactivity of patients with CD to rGG1 and gliadin extract (e) was not significant ($P = .1078$; 95.05% CI, -0.0290 to 0.2570), whereas the differences in IgA reactivity of patients with IDs, healthy subjects, patients with IgE-associated allergies, and AGA⁺/EMA⁻ subjects to rGG1 and gliadin extract (e) were significant ($P = .0002$ [95.05% CI, 0.0200 - 0.0500], $P = .0193$ [95.02% CI, 0.0100 - 0.0800], $P = .0002$ [95.15% CI, 0.0400 - 0.1300], and $P < .0001$ [95.01% CI, 0.3400 - 1.410], respectively). Importantly, the receiver operating characteristic AUC for rGG1 (AUC, 0.9314; 95% CI, 0.8740-0.9529; SE, 0.02; $P < .0001$) was larger than that for gliadin extract (e) (AUC, 0.8828; 95%

CI, 0.8375-0.9281; SE, 0.02; $P < .0001$; Fig 3, B). The use of rGG1 in an ELISA-based serum IgA pilot test thus provided a sensitivity of 73.01% (95% CI, 0.60-0.83) and a specificity of 92.9% (95% CI, 0.87-0.96), whereas gliadin extract (e) had a sensitivity of 88.8% (95% CI, 0.78-0.95) and a specificity of 75.8% (95% CI, 0.67-0.82). The positive predictive values for rGG1 and gliadin extract (e) were 82.14% and 62.2%, respectively, and the negative predictive values for rGG1 and gliadin extract (e) were 88.5% and 93.8%, respectively.

IgA against rGG1 for monitoring adherence to a GFD

In another pilot study we compared antibody levels against rGG1, tTG2, and DGP in sera obtained from patients with CD before and after a GFD using longitudinal testing. Serum samples taken before a GFD were positive in each of the tests, showing comparable sensitivity of the tests in the tested patients (Fig 4, A). After a GFD, antibody titers decreased significantly against the tested proteins (rGG1: $P = .0313$ [96.8% CI, 0.2830-1.189]; tTG2 IgA: $P = .0313$ [96.8% CI, 31.45-315.5]; DGP IgA: $P = .0312$ [96.8% CI, 45.40-123.4]; and DGP IgG: $P = .0313$ [96.8% CI, 3.710-85.00]), but according to the manufacturer's recommended cutoffs, 5 of 6 serum samples were still positive for IgA against tTG2, 2 of 6 still showed anti-DGP IgA, and 4 of 6 continued to mount anti-DGP IgG (Fig 4, A). By contrast, each of the 6 patients lost IgA reactivity to rGG1 after a GFD (Fig 4, A).

Using sera from 18 patients with CD on a GFD for cross-sectional testing (Fig 4, B), we found that each of the 18 patients had negative results for rGG1-specific IgA, whereas 2 showed IgA

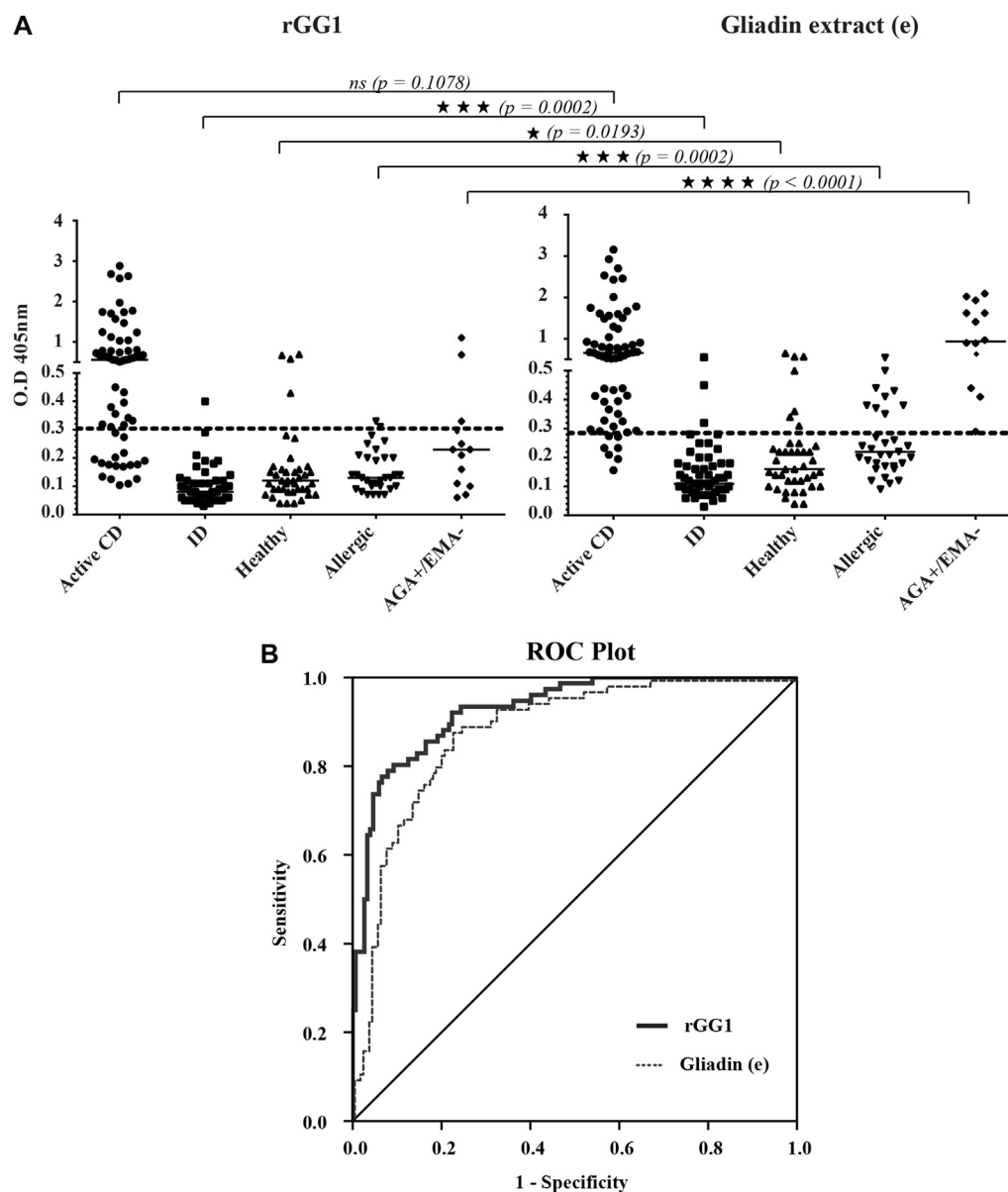


FIG 3. IgA reactivity to rGG1 and gliadin extract (e). **A**, Sera from patients with CD, patients with IDs, healthy persons, patients with IgE-associated allergies, and AGA⁺/EMA⁻ subjects were tested for IgA reactivity to rGG1 and gliadin extract (e) by means of ELISA. The results are shown as a scatter plot, and OD values corresponding to the amounts of bound antibodies are displayed on the y-axes. The horizontal lines within groups indicate the median, and the dotted horizontal lines indicate the cutoff values based on the Youden index (P values between 2 groups are indicated to denote significance). **B**, Receiver operating characteristic (ROC) curves comparing IgA reactivity with rGG1 and gliadin extract (e) as CD-specific markers.

reactivity to gliadin extract (e), and the difference in IgA reactivity was significant ($P = .0461$; 95.32% CI, 0.00200-0.0790). Eight of 18 patients still had positive test results for tTG2-specific IgA, 3 of 18 had positive results for anti-DGP IgA, and 8 of 18 had positive results for DGP-specific IgG (Fig 4, B).

The major IgA-reactive epitope of GG1 is a peptide in the N-terminal proline- and glutamine-rich domain II

Peptides 4, 5, 6, 7, and 9 belonging to domain II showed strong IgA reactivity to serum from patients with active CD. Peptide 4

from domain II (Fig 1, boxed in green) was the most strongly and frequently recognized peptide (Fig 5, A). The sequence of peptide 4 was highly conserved in the GG1 homologue from spelt but less in barley, whereas the peptide 4-homologous region was either missing or not conserved in the prolamins from rye, rice, and oat (Fig 1). In addition to peptide 4, we found IgA reactivity to peptides 2 and 3 of domain II, peptide 13 of domain III, and peptide 18 of domain V, but these peptides showed much weaker reactivity (Fig 5, A). None of the other peptides was recognized by IgA from patients with CD, and none of the healthy subjects had peptide-specific or rGG1-specific IgA reactivity. rGG1 exhibited specific IgA reactivity for all patients with CD (Fig 5, A).

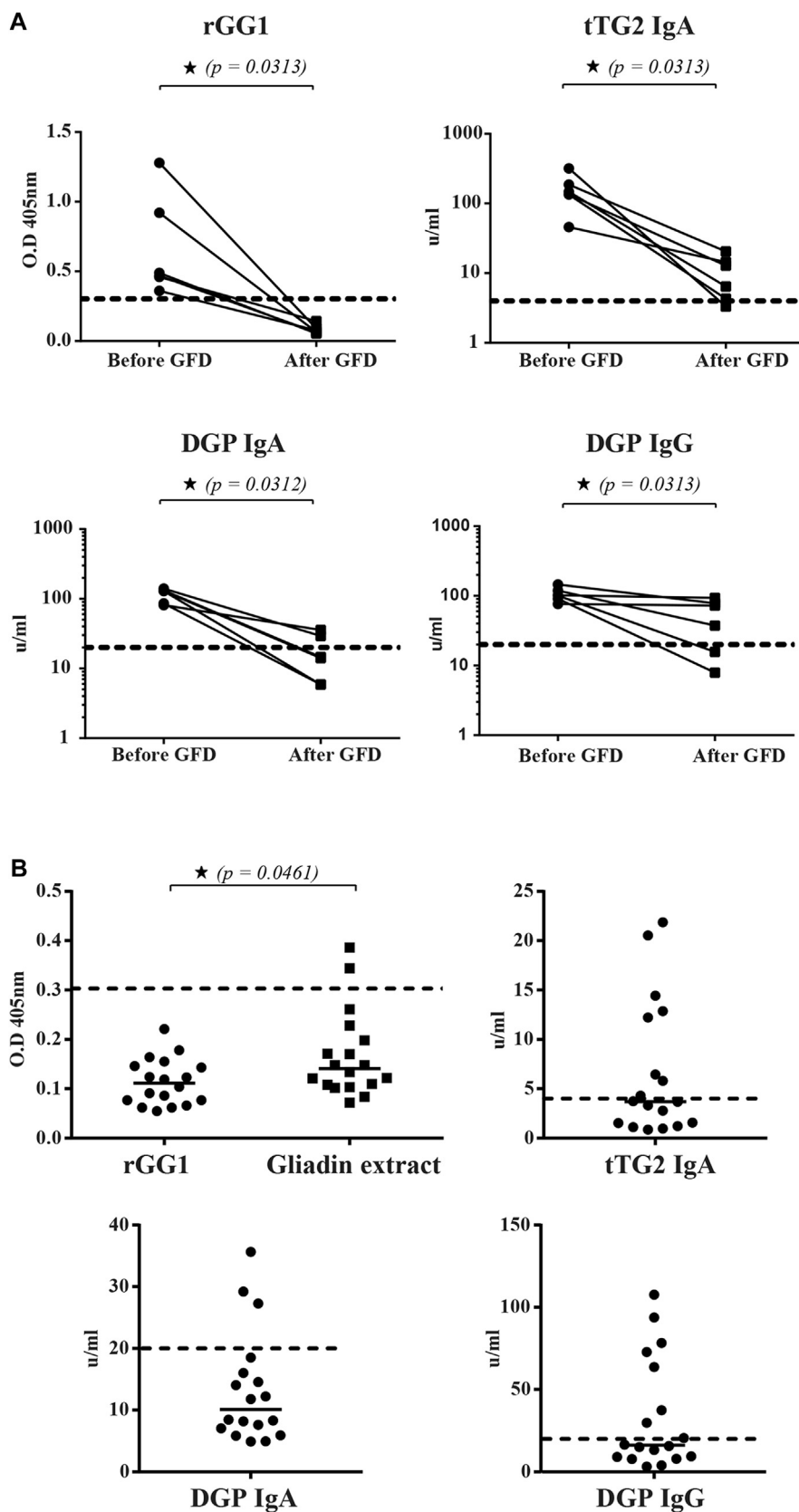


FIG 4. Antibody reactivities of patients with CD on a GFD. Sera obtained from 6 patients with CD “before and after” a GFD (**A**) and from 18 patients with CD on a GFD (**B**) were tested for IgA reactivity to rGG1, tTG2, and DGP, as well as for IgG against DGP. OD values and units per milliliter corresponding to bound antibody levels are shown on the y-axes. Cutoff values for each assay are indicated by horizontal dashed lines (P values between 2 groups are indicated to denote significance).

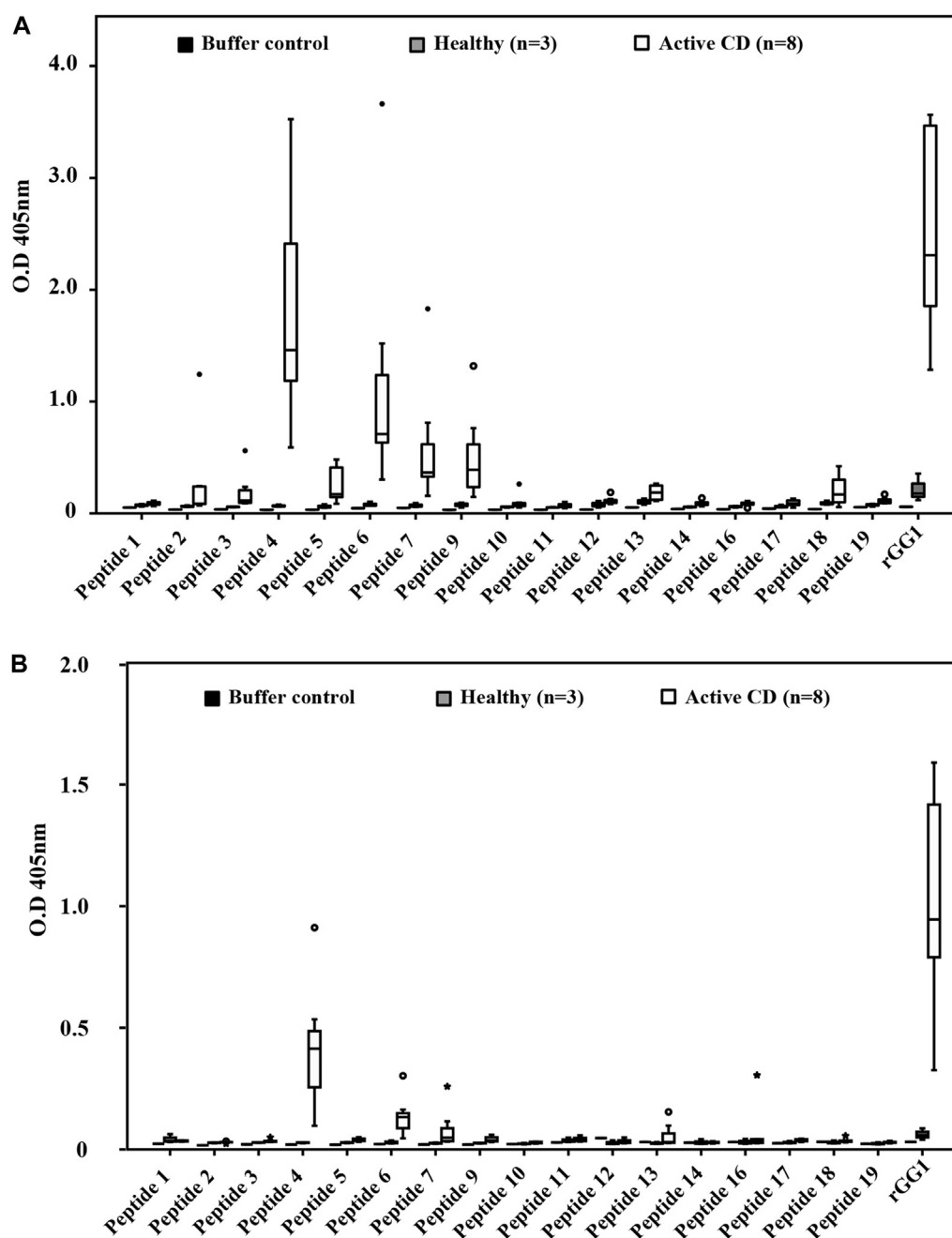


FIG 5. Epitope mapping of GG1 using synthetic overlapping peptides. IgA (A) and IgG (B) reactivity of patients with active CD (n = 8) and healthy persons (n = 3) to GG1-derived peptides (Fig 1) or to rGG1 (x-axes) by using ELISA. The results are shown as box plots of OD values for each group, where 50% of the values are within the boxes, lines within the boxes indicate median values, and open circles and stars indicate outliers and extremes, respectively.

For IgG, we found a similar recognition profile, but at the given serum dilution, the reactions were much weaker than for IgA. Again, peptide 4 was the most strongly recognized peptide, and peptides 6, 7, and 13 were also reactive, although to a lower extent. All patients with CD had IgG reactivity to rGG1, and none of the healthy subjects had relevant IgG reactivity to any of the peptides or rGG1 (Fig 5, B).

Testing of the peptides with rabbit anti-rGG1 antibodies showed that they recognized most of the peptides, although at varying intensities (see Fig E3 in this article's Online Repository

at www.jacionline.org). Rabbit anti-peptide 4 and anti-peptide 18 antibodies showed high specificity with the immunogens. The preimmune sera showed no reactivity (see Fig E1).

rGG1-like proteins can be detected in certain cereals and are resistant to baking

Using rabbit anti-rGG1 antibodies, we searched for cross-reactive proteins in several cereals by means of immunoblotting, and Fig 6, A, shows that proteins of comparable molecular weight

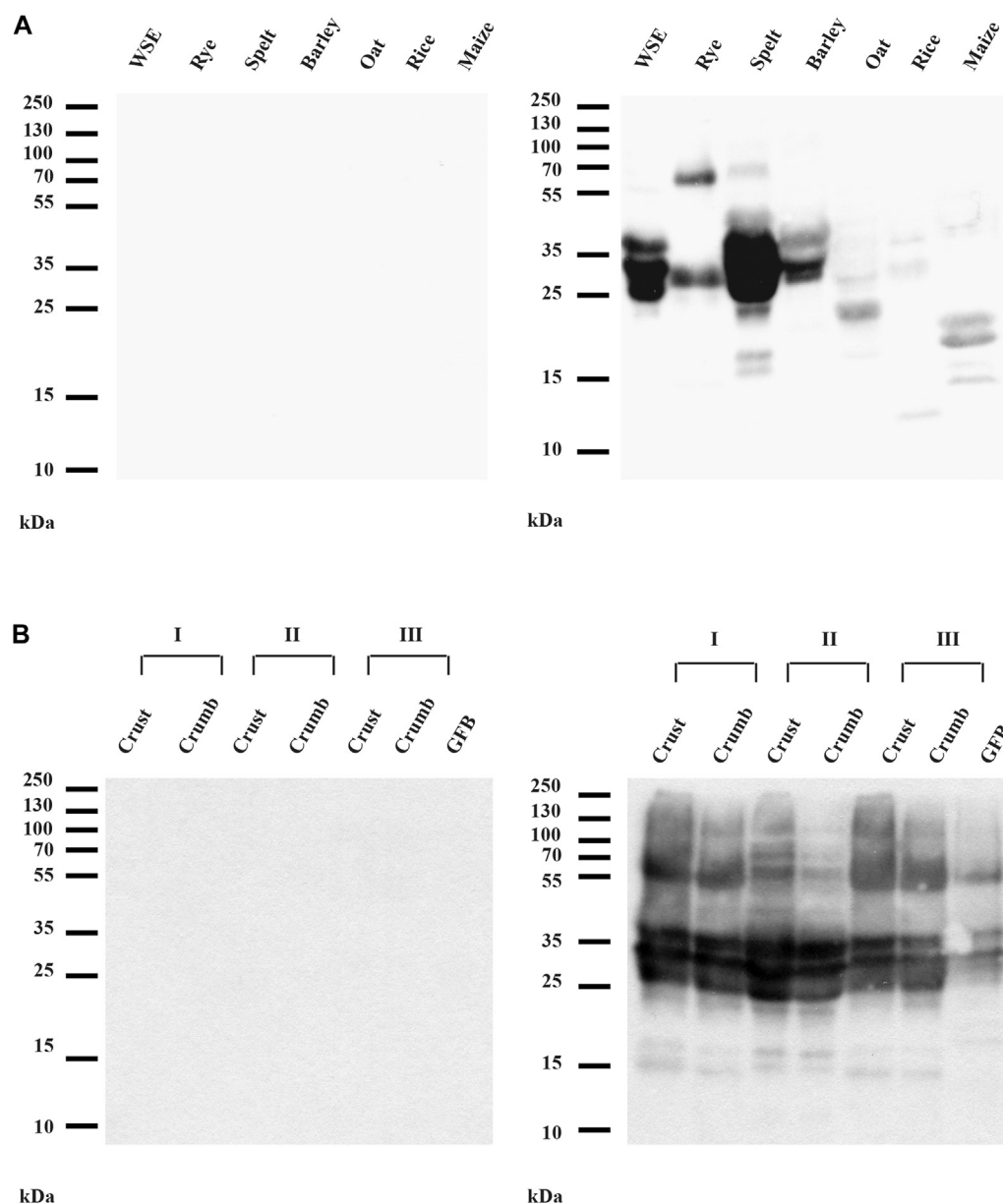


FIG 6. Detection of GG1-like proteins in cereal and bread extracts. Protein extracts from wheat, rye, spelt, barley, oat, rice, and maize (**A**) and extracts from the crumb and crust of (I) common bread (II), rolls (III), rye bread (III), and gluten-free bread (GFB; **B**) were blotted onto nitrocellulose membrane and probed with preimmune serum (*left panels*) or rabbit anti-rGG1 antibodies (*right panels*). Molecular weights in kilodaltons are shown on the *left margins*.

(ie, approximately 30-35 kDa) are detected in wheat and spelt and, to a lesser degree, in rye and barley. Only weak reactivity was observed to protein bands of different molecular weights in oat, rice, and maize, which is also in agreement with the results from multiple sequence alignment (Fig 1) and clinical experience that patients with CD can eat products derived from oat, rice, and maize.^{27,28} No reactivity was observed with the corresponding preimmune serum (Fig 6, A, left panel).

It has been reported that high temperatures induce changes in the gliadin structure through formation of new hydrophobic and disulfide bonds^{29,30} and that such changes cause aggregation of gliadins and decrease their digestibility.³¹ Therefore we studied whether GG1 can be found in processed food and after baking. For this purpose, we analyzed the presence of GG1 protein in

breadcrumbs and crust protein extracts from 3 different types of commonly consumed breads (Fig 6, B). Immunoblot analysis with rabbit anti-rGG1 antibodies indeed shows that an approximately 35-kDa protein band is present in both the crumb and crust in each of the tested products but only in trace amounts in gluten-free bread. Again, no reactivity of the preimmune serum was observed (Fig 6, B, left panel).

rGG1 and its major IgA-reactive epitope are resistant to gastric digestion

GG1 was first digested with pepsin for 2 hours, and aliquots were taken at 30, 60, and 120 minutes. After 2 hours of peptic digestion, the rest of the samples were further digested with

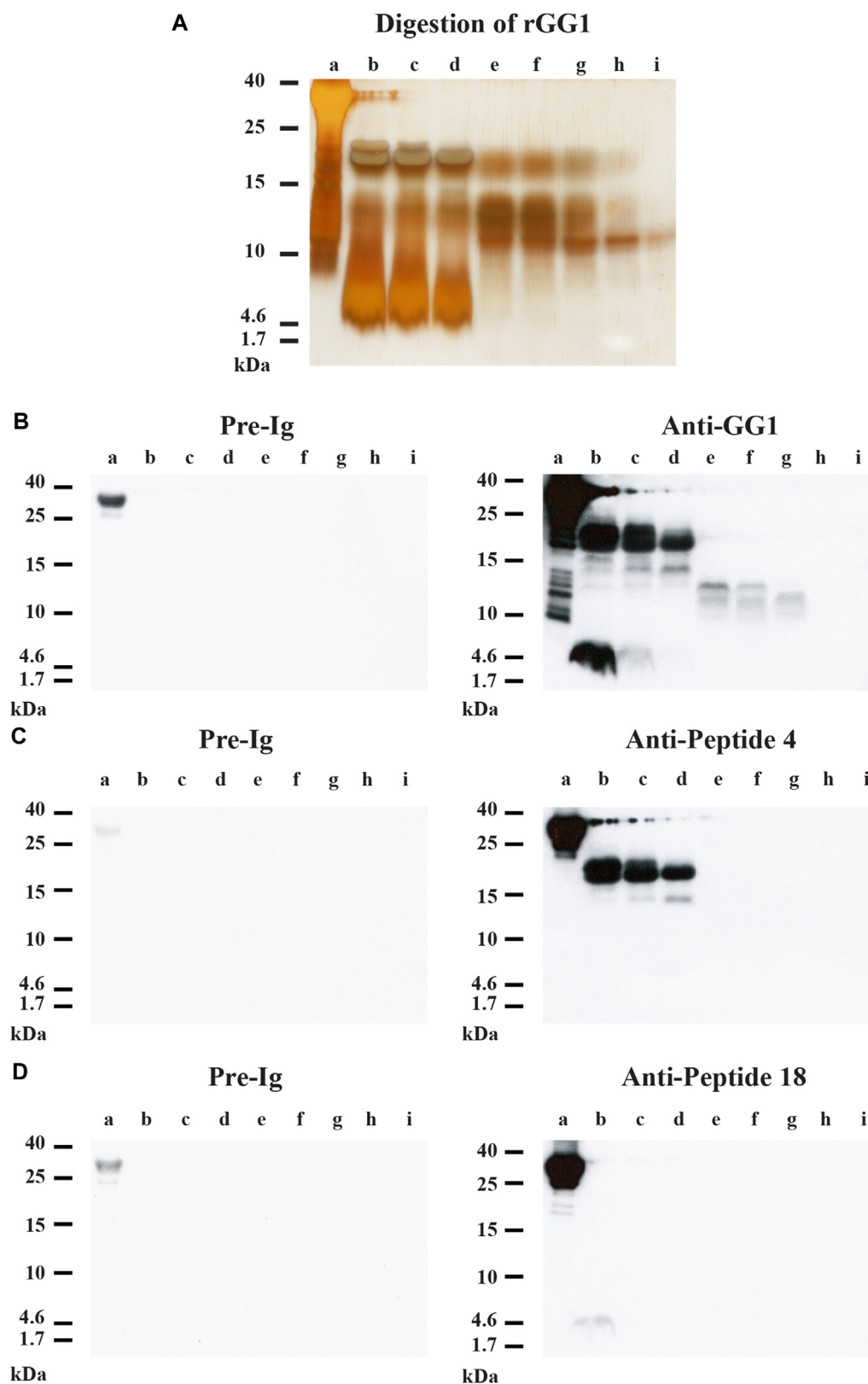


FIG 7. Immunoblot detection of GG1 after peptic and chymotryptic digestion. **A**, Silver-stained SDS-PAGE gels of undigested rGG1 (lane a) and rGG1 digested with pepsin for 30 (lane b), 60 (lane c), and 120 (lane d) minutes. Lanes e to i contain rGG1 samples, which after 120 minutes of peptic digestion were further digested with chymotrypsin for 30 (e), 60 (f), 120 (g), 240 (h), or 1200 (i) minutes. Molecular weights are indicated as kilodaltons. **B-D**, Identically prepared gels were blotted onto nitrocellulose and then probed with rabbit anti-rGG1 antibodies (right panel) or the corresponding preimmune serum (Pre-Ig; left panel) in Fig 7, B; with rabbit anti-peptide 4 antibodies (right panel) or the preimmune serum (left panel) in Fig 7, C; or with rabbit anti-peptide 18 antibodies (right panel) or the preimmune serum (left panel) in Fig 7, D.

chymotrypsin for 20 hours, and aliquots were taken at 30, 60, 120, 240, and 1200 minutes. Silver-stained SDS-PAGE (Fig 7, A) shows that digestion with pepsin generated fragments of approximately 23 to approximately 2 kDa (Fig 7, A, lanes b-d). Subsequent digestion with chymotrypsin further digested the 20-kDa band, generating additional smaller fragments to approximately 11 kDa (Fig 7, A, lanes e-i). Immunoblot analysis with rabbit anti-rGG1 antibodies showed that after 2 hours of digestion with pepsin and 2 hours of digestion with chymotrypsin, certain immune-reactive rGG1 fragments remained intact (Fig 7, B, right panel, lanes b-g). Probing the samples with the anti-peptide 4 antibodies revealed that the region containing peptide 4 was very resistant to pepsin digestion (Fig 7, C, right panel, lanes b-d). By contrast, the region containing peptide 18, which was not recognized by IgA antibodies from patients with CD, was digested almost completely (Fig 7, D, right panel, lanes b-d). Treatment of pepsin-digested samples with chymotrypsin resulted in generation of protein fragments, which were still reactive with anti-rGG1 antibodies (Fig 7, B, lanes e-g), and the reactivity was lost during very prolonged digestion. Chymotrypsin-digested samples did not show reactivity with anti-peptide 4 and anti-peptide 18 antibodies (Fig 7, C and D, right panel, lanes e-i).

DISCUSSION

We cloned, expressed, and purified rGG1, which was specifically recognized by IgA antibodies from 73.01% of patients with CD, demonstrating that it is a major IgA-reactive wheat antigen. In pilot tests sensitivity of the rGG1-based IgA testing was lower when compared with gliadin extract (e)-based testing, but by using rGG1, a higher specificity of 92.9% was obtained. This specificity was higher when compared with that of native gliadin extract (e), which had a specificity of only 75.8%. Thus our results indicate that rGG1 is a major CD-specific antigen.

Because the only treatment option available for patients with CD is to be on a lifelong GFD, monitoring the patient's adherence to this diet is important. Although in our pilot study we could test only a limited number of patients before and after a GFD and a second set of sera after a GFD, our results suggest that IgA reactivity to rGG1 might be better suited for monitoring GFD adherence when compared with measuring antibody levels against tTG2 or DGP. Thus IgA reactivity to rGG1 can be used as a marker for monitoring GFD adherence in patients with CD.

We next determined the IgA epitopes of GG1 using a set of synthetic overlapping peptides spanning the GG1 sequence. IgA antibodies from patients with CD identified mainly peptides (ie, peptides 4, 5, 6, 7, and 9) in the proline- and glutamine-rich repetitive region at the N-terminus, of which one peptide (ie, peptide 4) was most strongly and frequently recognized. Interestingly, IgG antibodies, although to a weaker extent, were directed against these peptides, indicating a coevolution of the antibody response in the IgA and IgG class in patients with CD. Of note, the sequence motif QPQQPF, which has previously been identified by means of random phage cloning with sera from patients with CD, occurs in peptides 4, 5, 6, and 7, and a similar motif (QPQQPQ) can be found in peptide 9.³²

Our assumption that GG1 could be an important CD-specific antigen is also supported by the fact that it contains a T-cell epitope, which has been previously mapped with a T-cell clone that was isolated from the gut of a patient with CD.^{23,24} This T-cell

epitope overlaps with the IgA-reactive peptide 9 (Figs 1 and 2). The sequence alignment of GG1 with the corresponding prolamins from other cereals showed that the formerly described T-cell epitope is well conserved only in spelt but not in rye and barley, which might explain the selective T-cell recognition reported earlier.²³ By contrast, the rabbit anti-rGG1 antibodies showed cross-reactivity with those cereals (ie, rye, barley, and spelt) that are also not well tolerated by patients with CD, whereas little or no reactivity was observed with oat, rice, and maize, which can be part of the diet of patients with CD.^{27,28} These data suggest that it might be possible to use the rabbit anti-rGG1 antibodies for screening of various types of processed food to detect GG1- and GG1-related proteins and for subsequent food labeling. This assumption is further supported by the finding that anti-rGG1 antibodies detected GG1 strongly in various breads, whereas only weak signals were obtained with gluten-free bread. Using anti-rGG1 antibodies, we also could demonstrate that GG1 is resistant to baking. This finding might explain why it can serve as a major CD antigen in baked and processed foods. Perhaps even more important was the finding that rGG1 was resistant to pepsin and partly to chymotrypsin digestion because peptide fragments reactive to anti-rGG1 were still detected after full pepsin digestion and a subsequent 4 hours of chymotrypsin digestion. Interestingly, the major IgA-reactive region of rGG1 was particularly resistant because fragments reactive to the anti-peptide 4 antibodies but not to anti-peptide 18 antibodies were observed after peptic digestion.

In summary, we have cloned and expressed a major CD-specific wheat antigen. Based on the GG1 sequence and epitope mapping, it might be possible to develop new forms of prevention and treatment for CD.

Clinical implications: rGG1 and a synthetic peptide containing a major IgA-reactive epitope thereof can be used as markers for CD.

REFERENCES

1. Rewers M. Epidemiology of celiac disease: what are the prevalence, incidence, and progression of celiac disease? *Gastroenterology* 2005;128:S47-51.
2. Rubio-Tapia A, Ludvigsson JF, Brantner TL, Murray JA, Everhart JE. The prevalence of celiac disease in the United States. *Am J Gastroenterol* 2012;107:1538-44, quiz 7, 45.
3. Schuppan D, Junker Y, Barisani D. Celiac disease: from pathogenesis to novel therapies. *Gastroenterology* 2009;137:1912-33.
4. Di Sabatino A, Corazza GR. Coeliac disease. *Lancet* 2009;373:1480-93.
5. Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO, et al. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* 1997;3:797-801.
6. Meresse B, Ripoche J, Heyman M, Cerf-Bensussan N. Celiac disease: from oral tolerance to intestinal inflammation, autoimmunity and lymphomagenesis. *Mucosal Immunol* 2009;2:8-23.
7. Leffler DA, Edwards George JB, Dennis M, Cook EF, Schuppan D, Kelly CP. A prospective comparative study of five measures of gluten-free diet adherence in adults with coeliac disease. *Aliment Pharmacol Ther* 2007;26:1227-35.
8. Rostom A, Dube C, Cranney A, Saloojee N, Sy R, Garrity C, et al. The diagnostic accuracy of serologic tests for celiac disease: a systematic review. *Gastroenterology* 2005;128:S38-46.
9. Wieser H. Chemistry of gluten proteins. *Food Microbiol* 2007;24:115-9.
10. Okita TW, Cheesbrough V, Reeves CD. Evolution and heterogeneity of the alpha-type beta-type and gamma-type gliadin DNA-sequences. *J Biol Chem* 1985;260:8203-13.
11. Spaenij-Dekking L, Kooy-Winkelaar Y, van Veelen P, Drijfhout JW, Jonker H, van Soest L, et al. Natural variation in toxicity of wheat: potential for selection of nontoxic varieties for celiac disease patients. *Gastroenterology* 2005;129:797-806.
12. Srinivasan B, Focke-Tejkl M, Swoboda I, Constantin C, Mittermann I, Pahr S, et al. A combined biochemical, biophysical and immunological approach towards the

- identification of celiac disease-specific wheat antigens. *Amino acids* 2013;45:889-900.
13. Baar A, Pahr S, Constantin C, Scheibelhofer S, Thalhamer J, Giavi S, et al. Molecular and immunological characterization of Tri a 36, a low molecular weight glutenin, as a novel major wheat food allergen. *J Immunol* 2012;189:3018-25.
 14. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li WZ, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 2011;7:539.
 15. Weiss W, Vogelmeier C, Gorg A. Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma. *Electrophoresis* 1993;14:805-16.
 16. Husby S, Koletzko S, Korponay-Szabo IR, Mearin ML, Phillips A, Shamir R, et al. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J Pediatr Gastroenterol Nutr* 2012;54:136-60.
 17. Focke M, Mahler V, Ball T, Sperr WR, Majlesi Y, Valent P, et al. Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. *FASEB J* 2001;15:2042-4.
 18. Gass J, Bethune MT, Siegel M, Spencer A, Khosla C. Combination enzyme therapy for gastric digestion of dietary gluten in patients with celiac sprue. *Gastroenterology* 2007;133:472-80.
 19. Ehren J, Moron B, Martin E, Bethune MT, Gray GM, Khosla C. A food-grade enzyme preparation with modest gluten detoxification properties. *PLoS One* 2009;4:e6313.
 20. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
 21. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;76:4350-4.
 22. Shapiro DE. The interpretation of diagnostic tests. *Stat Methods Med Res* 1999;8:113-34.
 23. Lundin KE, Scott H, Hansen T, Paulsen G, Halstensen TS, Fausa O, et al. Gliadin-specific, HLA-DQ(alpha 1*0501,beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J Exp Med* 1993;178:187-96.
 24. Molberg O, McAdam SN, Korner R, Quarsten H, Kristiansen C, Madsen L, et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat Med* 1998;4:713-7.
 25. Sugiyama T, Rafalski A, Soll D. The nucleotide-sequence of a wheat gamma-gliadin genomic clone. *Plant Sci* 1986;44:205-9.
 26. Qi PF, Wei YM, Ouellet T, Chen Q, Tan X, Zheng YL. The gamma-gliadin multigene family in common wheat (*Triticum aestivum*) and its closely related species. *BMC Genomics* 2009;10:168.
 27. Koskinen O, Villanen M, Korponay-Szabo I, Lindfors K, Maki M, Kaukinen K. Oats do not induce systemic or mucosal autoantibody response in children with coeliac disease. *J Pediatr Gastroenterol Nutr* 2009;48:559-65.
 28. Kagnoff MF. Overview and pathogenesis of celiac disease. *Gastroenterology* 2005;128:S10-8.
 29. Menkovska M, Lookhart GL, Pomeranz Y. Changes in the gliadin fraction(S) during breadmaking—isolation and characterization by high-performance liquid-chromatography and polyacrylamide-gel electrophoresis. *Cereal Chem* 1987;64:311-4.
 30. Lagrain B, Rombouts I, Brijs K, Delcour JA. Kinetics of heat-induced polymerization of gliadin. *J Agric Food Chem* 2011;59:2034-9.
 31. Simonato B, Pasini G, Giannattasio M, Peruffo AD, De Lazzari F, Curioni A. Food allergy to wheat products: the effect of bread baking and in vitro digestion on wheat allergenic proteins. A study with bread dough, crumb, and crust. *J Agric Food Chem* 2001;49:5668-73.
 32. Osman AA, Gunnel T, Dietl A, Uhlig HH, Amin M, Fleckenstein B, et al. B cell epitopes of gliadin. *Clin Exp Immunol* 2000;121:248-54.

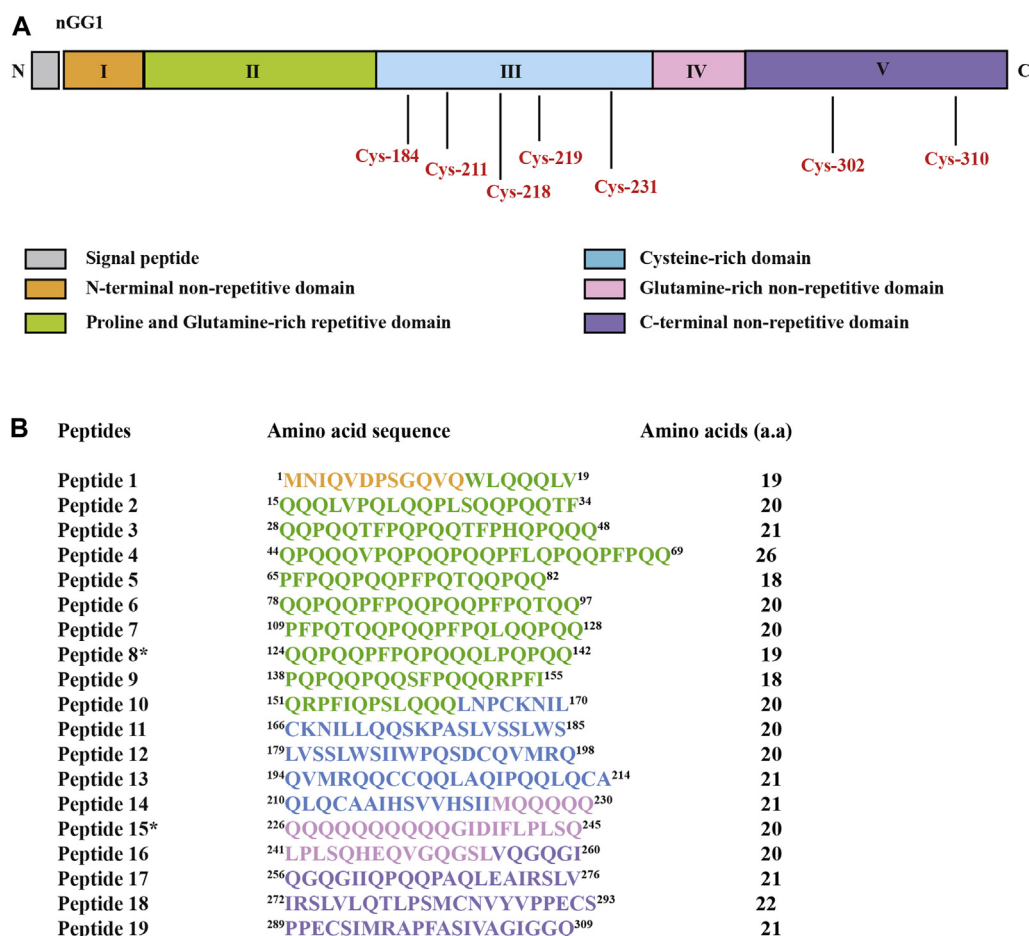


FIG E1. Schematic representation of GG1. **A**, Depiction of the domain architecture of GG1. The signal peptide is followed by a short N-terminal nonrepetitive domain (I), a highly variable Pro- and Gln-rich repetitive domain (II), a cysteine-rich nonrepetitive domain (III), a Gln-rich region (IV), and the C-terminal nonrepetitive domain containing 2 conserved cysteine residues (V). **B**, Sequence of synthetic GG1 peptides. Displayed are peptide numbers, amino acid sequences, positions of the peptides in the full length GG1, and their lengths. Asterisks indicate peptides that have not been tested. The coloring of the letters is based on the domain architecture, as described in Fig E1, A.

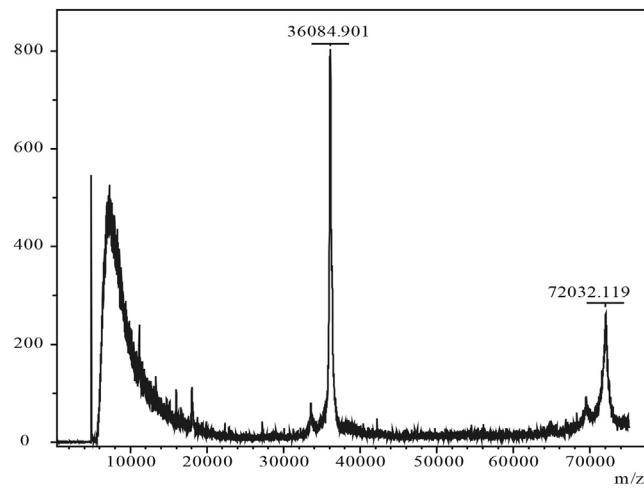


FIG E2. MALDI-TOF analysis of purified rGG1. The m/z ratio is provided on the x-axis, and the signal intensity (in arbitrary units) is shown on the y-axis.

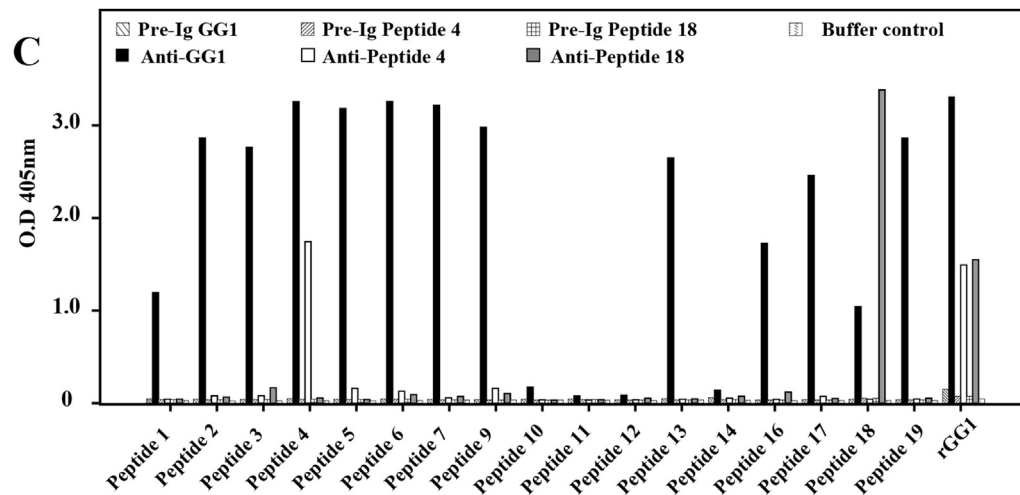


FIG E3. IgG reactivity of preimmune sera (*Pre-Ig*) and immune sera from rabbits immunized with rGG1, peptide 4, or peptide 18 of GG1-derived peptides (Fig E1) with GG1-derived peptides or rGG1 (*x-axes*) as tested by using ELISA. OD values (*y-axis*) correspond to the amount of bound antibodies.