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# Intestinal T Cell Responses to Gluten Peptides Are Largely Heterogeneous: Implications for a Peptide-Based Therapy in Celiac Disease<sup>1</sup>

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The identification of gluten peptides eliciting intestinal T cell responses is crucial for the design of a peptide-based immunotherapy in celiac disease (CD). To date, several gluten peptides have been identified to be active in CD. In the present study, we investigated the recognition profile of gluten immunogenic peptides in adult HLA-DQ2<sup>+</sup> celiac patients. Polyclonal, gliadin-reactive T cell lines were generated from jejunal mucosa and assayed for both proliferation and IFN- $\gamma$  production in response to 21 peptides from wheat glutenins and  $\alpha$ -,  $\gamma$ -, and  $\omega$ -gliadins. A magnitude analysis of the IFN- $\gamma$  responses was performed to assess the hierarchy of peptide potency. Remarkably, 12 of the 14 patients recognized a different array of peptides. All  $\alpha$ -gliadin stimulatory peptides mapped the 57–89 N-terminal region, thus confirming the relevance of the known polypeptide 33-mer, although it was recognized by only 50% of the patients. By contrast,  $\gamma$ -gliadin peptides were collectively recognized by the great majority (11 of 14, 78%) of CD volunteers. A 17-mer variant of 33-mer, QLQFPQPQLPYQPQP, containing only one copy of DQ2- $\alpha$ -I and DQ2- $\alpha$ -II epitopes, was as potent as 33-mer in stimulating intestinal T cell responses. A peptide from  $\omega$ -gliadin, QPQQPFPQPQPFPWQP, although structurally related to the  $\alpha$ -gliadin 17-mer, is a distinct epitope and was active in 5 out of 14 patients. In conclusion, these results showed that there is a substantial heterogeneity in intestinal T cell responses to gluten and highlighted the relevance of  $\gamma$ - and  $\omega$ -gliadin peptides for CD pathogenesis. Our findings indicated that  $\alpha$ -gliadin (57–73),  $\gamma$ -gliadin (139–153), and  $\omega$ -gliadin (102–118) are the most active gluten peptides in DQ2<sup>+</sup> celiac patients. *The Journal of Immunology*, 2009, 182: 4158–4166.

Celiac disease (CD)<sup>3</sup> is a common food-sensitive enteropathy in humans. In CD patients, the ingestion of wheat gluten, and of homologous proteins of barley and rye, induces pronounced T cell-mediated inflammatory reactions, mainly in the small intestine (1). The marked genetic association with HLA class II genes and the isolation of gluten-reactive, HLA-DQ2/DQ8-restricted CD4<sup>+</sup> T cells from the intestinal mucosa of celiac patients have highlighted the key role of the adaptive T cell response in the CD lesion (2–6).

Despite the efforts of several laboratories to define relevant gluten epitopes (2, 3, 5, 6), the characterization of the complete repertoire of peptides involved in the pathogenesis of CD remains a daunting task because of the great heterogeneity of gluten proteins (7). So far, several T cell stimulatory peptides from  $\alpha$ -gliadin,

$\gamma$ -gliadin, and glutenins have been identified using mass spectrometry analysis or by screening large peptide libraries (5, 6, 8–10). Furthermore, only in recent years has the key role of tissue transglutaminase (TG2) been elucidated in deamidating gluten peptides to facilitate their binding to HLA-DQ2 and -DQ8 molecules (11, 12). These findings led to an important step forward in the knowledge of CD pathogenesis, and also to the identification of immunogenic gluten peptides on the basis of their susceptibility to be deamidated by TG2 (Ref. 13 and G. Mamone, A. Camarca, O. Fierro, F. Addeo, G. Mazzarella, S. Auricchio, R. Troncone, and C. Gianfrani, manuscript in preparation).

Despite the large number of immunogenic gluten peptides so far identified, Arentz-Hansen et al. showed that intestinal T cell responses in HLA-DQ2<sup>+</sup> Norwegian CD patients are mainly focused on two overlapping peptides spanning the 57–68 and 62–75 region of  $\alpha$ -gliadin (8). Remarkably, Khosla and coworkers demonstrated that a single 33-mer peptide encompassing the 57–89 N-terminal region of certain  $\alpha$ -gliadin proteins displayed the optimal T cell stimulatory capacity in a cohort of adult Scandinavian subjects (14). This 33-mer peptide, encompassing six copies of three reported T cell epitopes (DQ2- $\alpha$ -I, DQ2- $\alpha$ -II, and DQ2- $\alpha$ -III), is resistant to gastrointestinal proteolysis, does not require intracellular processing for presentation, and binds efficiently to HLA-DQ2. Collectively, these chemical and immunological properties add further weight to the contention that this 33-mer peptide is the immunodominant T cell stimulatory gluten peptide in HLA-DQ2<sup>+</sup> CD patients (15, 16). Parallel studies have shown that following consumption of wheat-containing food, gluten-reactive T cells secreting IFN- $\gamma$  transiently circulate in peripheral blood of previously “gluten-free” celiac patients (17). By this innovative approach, the screening of a peptide library spanning the entire

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<sup>3</sup> Abbreviations used in this paper: CD, celiac disease; B-LCL, EBV-lymphoblastoid cell line; iTCL, intestinal T cell line; PT, peptic-tryptic; TCC, T cell clone; TG2, tissue transglutaminase.

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Table I. Peptides analyzed in this study

Peptide	Position	Sequence <sup>a</sup>	Reference
<b><math>\alpha</math>-Gliadins</b>			
33-mer	Glia- $\alpha$ 2 (57–89)	LQLQFFPQPQLPYQPQLPYQPQLPYQPQPF	Shan et al. (14)
25-mer	Glia- $\alpha$ 2 (64–89)	PQLPQFLQPYPQPQLPYQPQPF	Mamone et al. (13)
18-mer	Glia- $\alpha$ 2 (71–89)	QPQYPQPQLPYQPQPF	Mamone et al. (13)
17-mer	$\alpha$ -Glia (57–73)	QLQFFPQPQLPYQPQPF	Anderson et al. (17)
AG11	$\alpha$ -Glia (78–95)	PQPQFFLPQLPYQPQPS	This study
AG12	$\alpha$ -Glia (82–98)	PQPQFFPQLPYQPQPS	This study
T65	$\alpha$ -Glia (57–73)	QLQFFPQPQLPYQPQPS	This study
13-mer	Glia- $\alpha$ 9 (56–68)	LQLQFFPQPQLPY	Arentz-Hansen et al. (8)
Glia-20	Glia- $\alpha$ 20 (93–106)	FFRPQQPYPQPQPY	Vader et al. (18)
p(31–49)	$\alpha$ -Glia (31–49)	LGQQQFFPQPYPQPQPF	Mamone et al. (13) and Maiuri et al. (33)
<b><math>\gamma</math>-Gliadins</b>			
DQ2- $\gamma$ -I	$\gamma$ -Glia (139–153)	PQQPQQSFPQQQPA	Sjöström et al. (12)
DQ2- $\gamma$ -II	$\gamma$ -Glia (222–236)	GQGI IQPQQPAQLIR	Vader et al. (18)
14-mer-1	$\gamma$ -Glia (105–118)	PQQQTLQPQPAQL	Mamone et al. (13)
DQ2- $\gamma$ -III	$\gamma$ -Glia (83–97)	FFPQQPQQPYPQQPQ	Arentz-Hansen et al. (9)
DQ2- $\gamma$ -IV	$\gamma$ -Glia (117–132)	QQFSQPQQFPQPQQ	Arentz-Hansen et al. (9)
DQ2- $\gamma$ -V	$\gamma$ -Glia (78–97)	LQPQQFFPQPQQPYPQQPQ	Arentz-Hansen et al. (9)
14-mer-2	$\gamma$ -Glia (173–186)	PQQPFFPSQQQQLI	Mamone et al. (13)
<b><math>\omega</math>-Gliadins</b>			
DQ2- $\omega$ -1	$\omega$ -Glia (102–118)	QPQQFFPQPQQFFPQWP	This study <sup>b</sup>
<b>Glutenins</b>			
Glt-156	Glt (42–56)	QQPFFSQQQSPFSQ	Vader et al. (18)
Glt(19–39)	Glt (19–39)	SHIPGLERPSQQQLPQQTL	Mamone et al. (13)
<b>Gluten</b>			
Glu-5	Unknown	QIPQQQQIPQQPQQF	Arentz-Hansen et al. (9)

<sup>a</sup> Underline Q indicates a TG2-deamidated amino acid residue.

<sup>b</sup> Also, S. A. Tye-Din, J. A. Stewart, J. A. Dromey, T. Beissbarth, D. A. Van Heel, A. Tatham, K. Henderson, S. Mannering, C. Gianfrani, D. Jewell, A. V. S. Hill, and R. P. Anderson, manuscript in preparation.

sequence of A-gliadin identified a 17-mer peptide (a truncated variant of the 33-mer peptide mapping to the residues 57–73) active in the great majority of CD volunteers. In contrast with these findings, Koning and coworkers showed that only 50% of CD intestinal T cell lines obtained mainly from children and a few adult celiac donors were reactive to two overlapping peptides spanning the region 57–75 of  $\alpha$ -gliadins (18). Interestingly, the authors in that study identified several immunostimulatory  $\gamma$ -gliadin-derived peptides (18).

The present study analyzes the repertoire of intestinal T cell responses in CD patients to determine whether T cells in CD recognize multiple and heterogeneous gluten peptides or that the T cell response is focused on a single immunodominant peptide. The reactivity of 14 adult HLA-DQ2<sup>+</sup> celiac patients was analyzed toward a large panel of immunogenic gliadin and glutenin peptides. Furthermore, we also assessed the hierarchy of bioactive gluten peptides on the basis of the intensity of IFN- $\gamma$  responses. Polyclonal, gliadin-reactive intestinal T cell lines (iTCLs) were generated from intestinal mucosa and assayed for recognition of 21 peptides, including known epitopes from  $\alpha$ -gliadin: 33-mer (57–89) and its truncated forms, 25-mer (64–89), 18-mer (71–89), 17-mer (57–73), 13-mer (56–68), and glia-20; from  $\gamma$ -gliadin, as DQ2- $\gamma$ -I, DQ2- $\gamma$ -II, DQ2- $\gamma$ -III, DQ2- $\gamma$ -IV, and DQ2- $\gamma$ -V, and the two recently identified peptides 14-mer-1 (105–118) and 14-mer-2 (173–186); two glutenin peptides, Glt-19–39 and glt-156 (42–56); and glu-5 of undefined origin. Furthermore, three polymorphisms of  $\alpha$ -gliadin 17-mer and an  $\omega$ -gliadin-derived peptide (DQ2- $\omega$ -1) were also analyzed for recognition, as well as the innate immunity eliciting  $\alpha$ -gliadin 31–49 peptide (Table I).

Since immunomodulatory strategies based on the administration of the T cell stimulatory peptides are under clinical investigation for several diseases, including food allergies and autoimmune disorders (19, 20), the defining of the repertoire and hierarchy of gluten peptides that stimulate intestinal T cells in the great major-

ity of CD patients could have important implications in the design of a peptide-based immunotherapy for CD (21).

## Materials and Methods

### Patients

Jejunal biopsies were obtained from 14 adult HLA-DQ2<sup>+</sup> CD patients (Table II). Six patients (mean age, 25.6 years; range, 18–34 years) were consuming a normal diet and their mucosa was atrophic. Eight patients (mean age, 35.7 years; range, 18–49 years) had followed a gluten-free diet for at least 2 years. Patients were typed for *DQA1*, *DQB1*, and *DRB1* genotypes using commercial HLA typing kits (Dynal Biotech). Patients from southern Italy were randomly recruited from those referred to the Gastroenterology Unit of a local hospital, and they gave their full informed consent to the study.

### Gliadin digestion and peptide synthesis

Peptic-tryptic digestion of gliadin (PT-gliadin) extracted from Strampelli grain was performed as previously described (13). Peptides were synthesized by automated continuous-flow solid-phase following the Fmoc/tBu strategy on a PerSeptive Biosystems Pioneer peptide synthesis system GEN600611, according to the manufacturer's protocol. The identity of synthesized peptides was confirmed by MALDI-TOF mass spectrometry (Voyager DE-Pro; PE Biosystems). Peptide purity (92–98%) was assessed by reversed phase HPLC on a Vydac C18 column using a Kontron HPLC system (Promega). PT-gliadin and peptides were incubated at 37°C for 4 h with TG2 (1:1 enzyme/substrate ratio) in 5 mM Tris-HCl buffer (pH 6.8) containing 5 mM CaCl<sub>2</sub>, 10 mM NaCl, and 10 mM DTT.

### Generation of gliadin-specific T cell lines and T cell clones

Gliadin-reactive iTCLs were generated as described previously (22). Briefly, mucosal explants were digested with collagenase A and cells were suspended at 2–3  $\times$  10<sup>5</sup>/ml in complete medium (X-Vivo15 medium supplemented with 5% AB-pooled human serum and antibiotics, all provided from BioWhittaker). Cells were stimulated with 1.5  $\times$  10<sup>6</sup> irradiated PBMC and TG2-treated (deamidated) PT-gliadin (50  $\mu$ g/ml). IL-15 (R&D Systems) was added after 48 h at 10 ng/ml. On days 7 and 21 following cell stimulation with Ag, iTCLs were restimulated with irradiated autologous PBMC and deamidated PT-gliadin. T cell clones (TCCs)

Table II. Patients enrolled in the study

Patient	Age (Years)/Sex	Disease State	DQA1	DQB1	DRB1	DQ2 Phenotype
CD230204	18/M	In remission	0505, 0201	02*, 03*	*07/*05	DQ2.5
CD220201	40/F	In remission	05*, 03*	0201, 0301	03/11	DQ2.5
CD210205	21/M	In remission	0505, 0201	02*, 03*	*07/*05	DQ2.5
CD280900	27/M	In remission	0501, 05*	0201, 0301	03/11	DQ2.5
CD090401	28/F	In remission	0501, other	0201, other	03/14	DQ2.5
CD140102	18/F	In remission	0505, 0201	02*, 03*	*07/*13	DQ2.5
CD041051	36/F	In remission	0505, 0201	02*, 03*	*07/*05	DQ2.5
CD041052	49/F	In remission	0505, 0201	02*, 03*	03/07	DQ2.5
CD061204	34/F	Atrophic	0501, 0201	02*, 02*	03/07	DQ2.5+2.2
CD171204	24/F	Atrophic	0501, other	02*, other	*03/*13	DQ2.5
CD310504	32/F	Atrophic	0501, other	02*, other	*01/*03	DQ2.5
CD202006	18/F	Atrophic	0501, 0201	02*, 02*	03/07	DQ2.5+2.2
CD130406	21/F	Atrophic	0501, 0201	02*, 02*	03/07	DQ2.5+2.2
CD290306	25/F	Atrophic	0505, 0201	02*, 03*	*07/*05	DQ2.5

were derived from iTCL of patient CD230204 by limiting dilution (one cell per well) in the presence of  $5 \times 10^4$  irradiated allogeneic PBMC,  $5 \times 10^3$  EBV-lymphoblastoid cell line (B-LCL), and 0.5  $\mu\text{g/ml}$  PHA, as previously described (23).

### T cell assays

iTCLs or TCCs were assayed for responses to deamidated PT-gliadin and PT-gluten peptides by the detection of both IFN- $\gamma$  by ELISA and proliferation, as previously described (22). Autologous or allogeneic HLA-matched B-LCLs were used as APCs. PT-gliadin (50  $\mu\text{g/ml}$ ) or gluten peptides (at the indicated concentration) were added to APCs ( $1 \times 10^5$  or  $5 \times 10^4$  for TCL and TCC assays, respectively) concomitantly with responder T cells ( $3 \times 10^4$  and  $1 \times 10^4$  for TCLs and TCCs, respectively). In the experiments with blocking mAbs, B-LCLs were preincubated for 10 min with anti-human HLA-DR (10  $\mu\text{g/ml}$ , clone L243; BD Pharmingen) or anti-human HLA-DQ (10  $\mu\text{g/ml}$ , clone SPV-L3; Biodesign International) before the addition of indicated peptides. In dose-response assays, irradiated APCs were incubated overnight at 37°C with escalating concentrations of indicated peptides. Before addition of T cells, APCs were washed to remove unbound Ag. In experiments with fixed APCs, cells were incubated with 0.05% glutaraldehyde in RPMI 1640 for 90 s at room temperature before 0.2 M glycine was added for an additional 60 s. APCs were washed and cocubated overnight at 37°C in U-bottom 96-well plates with peptides (1  $\mu\text{M}$ ) and then incubated with T cells. Cell supernatants (50  $\mu\text{l}$ ) were collected after 48 h for determination of IFN- $\gamma$ . To detect cell proliferation, [ $^3\text{H}$ ]thymidine (Amersham Biosciences) was added (0.5  $\mu\text{Ci/well}$ ) to cultures for an additional 16 h. [ $^3\text{H}$ ]thymidine incorporation was measured by liquid scintillation counting (TopCount; Packard Instruments). Each peptide was assayed in duplicate and in at least three independent experiments. IFN- $\gamma$  ELISA was performed as previously described (23) and had a sensitivity of 62 pg/ml.

### Bioinformatics analysis

The hierarchy of peptide responses was evaluated in IFN- $\gamma$  ELISA experiments by calculating the ratio between the optical densities (OD) in response to peptide and the OD observed with medium alone. The resulting IFN- $\gamma$  intensity, expressed as numerical value, was analyzed by using Cluster 3.0 (24). Data were normalized according to the following procedure: all values were transformed in  $\log_2$  using the log transform data tool and were centered for both columns (peptides) and rows (patients), so that the median value of each column/row was 0. Then, all values were normalized by a scale factor such that the sum of the squares of the values in each row/column was 1. A clustering algorithm was applied by similarity metrics based on a Pearson correlation that builds a hierarchical structure among objects and shows a correlation. The TreeView program was used for visualizing and browsing the clustered data. The alignment of peptides was made by the ClustalW program (25), and the sequence identity percentages between the peptides were evaluated using the FASMA (format and analyze sequences in multiple alignments) tool developed by our group (26).

### HLA-DQ2 binding assay

HLA-DQ2 molecules were purified from homozygous HLA-DR3<sup>+</sup> B-LCLs by affinity chromatography, as previously described (27). Peptide binding assays were performed by incubating purified HLA-DQ2 mole-

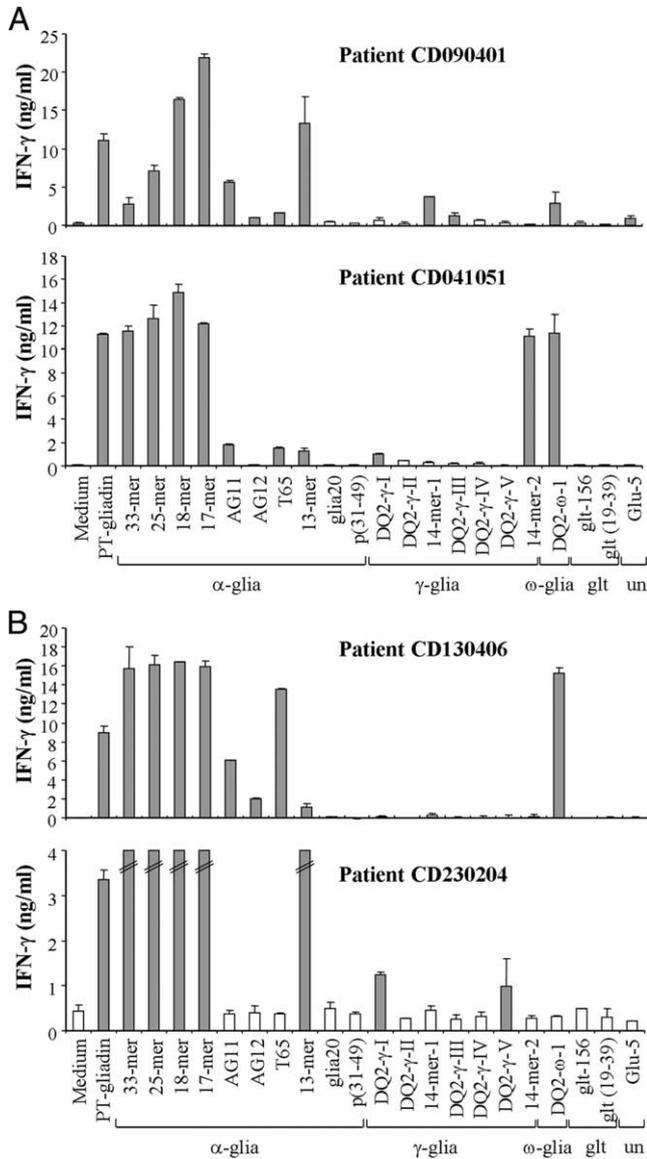
cules (5–500 nM) with various concentrations of unlabeled peptide inhibitors and 0.1–1 nM  $^{125}\text{I}$ -radiolabeled probe peptide for 48 h in PBS containing 0.05–0.15% Nonidet P-40 in the presence of a protease inhibitor cocktail (28, 29). MHC binding of the radiolabeled peptide was determined by capturing MHC/peptide complexes on SPV-L3 (anti-HLA-DQA) or HB180 (anti-HLA-DRA/DQA) Ab-coated Lumitrac 600 plates (Greiner Bio-One) and measuring bound radioactivity using the TopCount microscintillation counter (Packard Instruments). The concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide ( $\text{IC}_{50}$ ) was then calculated. Peptides were typically tested at six different concentrations covering a 100,000-fold dose range, and in three or more independent assays.

## Results

### Recognition patterns of gluten peptides by polyclonal intestinal T cell lines

We selected a large panel of 21 gluten peptides, including known HLA-DQ2-restricted epitopes, and selected candidate epitopes on the basis of high sequence similarity with known immunogenic peptides (Table II). All peptides, except for DQ2- $\gamma$ -V, were tested in their deamidated form. To obtain comparable and unbiased results, we have screened polyclonal, gliadin-reactive T cell lines established by repeated stimulation of intestinal mucosal cells with deamidated PT-gliadin extracted from the same wheat flour. Furthermore, only adult HLA-DQ2<sup>+</sup> CD patients were enrolled (Table I). We considered responsive those iTCLs in which the IFN- $\gamma$  production to gliadin/peptide was at least twice the value of cells cultured with medium alone. Collectively, we found four different profiles of peptide recognition. Results of peptide responsiveness are illustrated in Figs. 1, 2 (A and B), and 3.

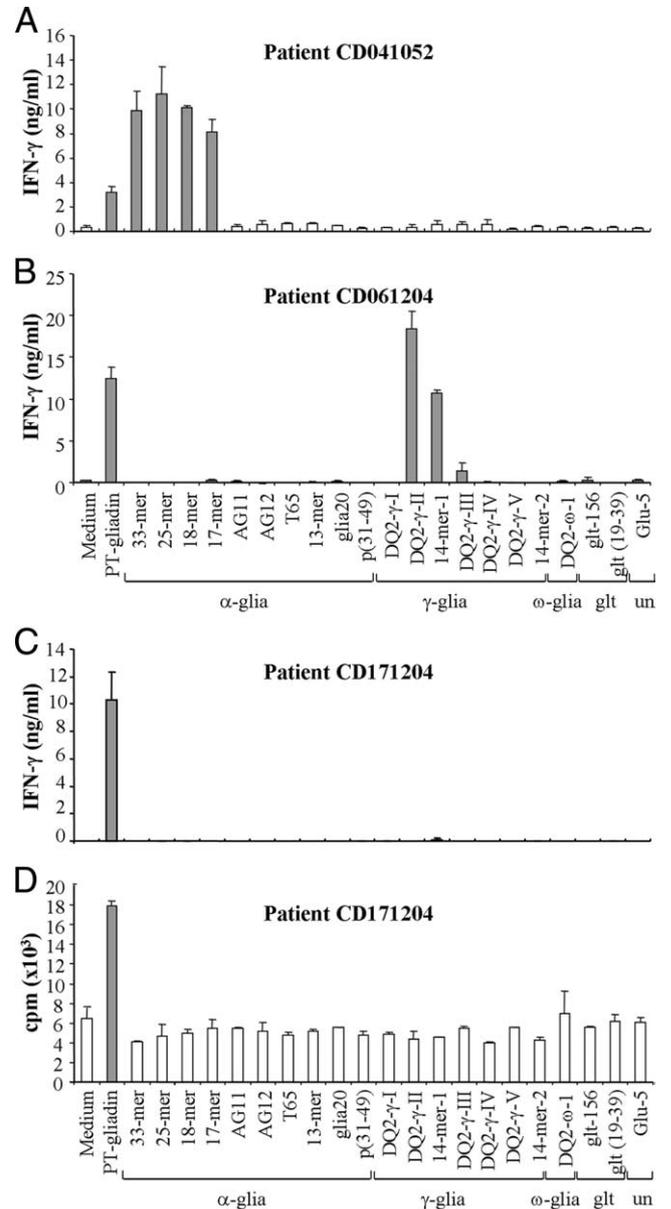
**Recognition of peptides from three gliadin families.** Four of the 14 (28.5%) patients (CD090401, CD041051, CD210205, CD140102) produced IFN- $\gamma$  in response to peptides from either  $\alpha$ -,  $\gamma$ -, or  $\omega$ -gliadins, although with a variable intensity. Patient CD090401 produced very high levels of IFN- $\gamma$  in response to some truncations of 33-mer (18-mer, 17-mer, and 13-mer) and intermediate IFN- $\gamma$  levels to the 33-mer, to the  $\gamma$ -gliadin 14-mer-1, and to the DQ2- $\omega$ -1 peptide. Interestingly, this patient recognized also two polymorphisms of the known  $\alpha$ -gliadin 17-mer, the AG12 and T65, as well as the DQ2- $\gamma$ -III peptide. A weak IFN- $\gamma$  response to glu-5 peptide was also observed. Similarly, patient CD041051 showed high response to 33-mer peptide and to its truncated sequences with the exception of the 13-mer peptide, which induced weak IFN- $\gamma$  production; in contrast, IFN- $\gamma$  responses to  $\gamma$ -gliadin peptide 14-mer-2 and to DQ2- $\omega$ -1 were very high (Fig. 1A). CD210205 responded to 33-mer and to all of its truncated peptides except the 13-mer (IFN- $\gamma$  values ranging from  $42.1 \pm 0.8$  to  $28.0 \pm 2.2$  ng/ml; medium,  $0.24 \pm 0.05$  ng/ml), and this patient



**FIGURE 1.** Recognition profile of peptides from multiple gliadin families. IFN- $\gamma$  responses of intestinal T cell lines to 21 gluten peptides and to PT-gliadin are illustrated. *A*, IFN- $\gamma$  responses of CD patients recognizing peptides derived from all three  $\alpha$ -,  $\beta$ -, and  $\gamma$ -gliadins and (*B*) from two gliadin families ( $\alpha$ - and  $\omega$ - or  $\alpha$ - and  $\gamma$ -gliadins) are illustrated. T cells ( $3 \times 10^4$ ) were stimulated with autologous EBV-B cells ( $6 \times 10^4$ ) and with TG2-deamidated peptides (30  $\mu$ g/ml, corresponding to a range of concentration of 8–19  $\mu$ M) or PT-gliadin (50  $\mu$ g/ml). IFN- $\gamma$  production was evaluated by ELISA after 48 h of incubation. Results are shown as means  $\pm$  SD of duplicate wells. Levels of IFN- $\gamma$  (ng/ml) are referred to  $10^6$  T cells/ml. Responses were considered positive (indicated with filled bars) when the peptide-specific IFN- $\gamma$  level was 2-fold or more than the level of IFN- $\gamma$  obtained in the absence of peptide. Representative results of three separate experiments performed for each iTCL are illustrated.

reacted to the 17-mer polymorphism, AG11 ( $4.7 \pm 0.7$  ng/ml). Positive responses were observed also to the DQ2- $\omega$ -1 peptide ( $20.0 \pm 10$  ng/ml) and to the DQ2- $\gamma$ -IV epitope ( $2.7 \pm 0.7$  ng/ml). CD140102 responded well to the DQ2- $\gamma$ -IV and to the DQ2- $\omega$ -1 ( $86.0 \pm 3.1$  and  $13.7 \pm 2.90$  ng/ml, respectively; medium,  $1.8 \pm 0.3$  ng/ml); surprisingly, among the  $\alpha$ -gliadin peptides only AG11 was very actively recognized by this patient ( $119.5 \pm 9.0$  ng/ml) (Fig. 3).

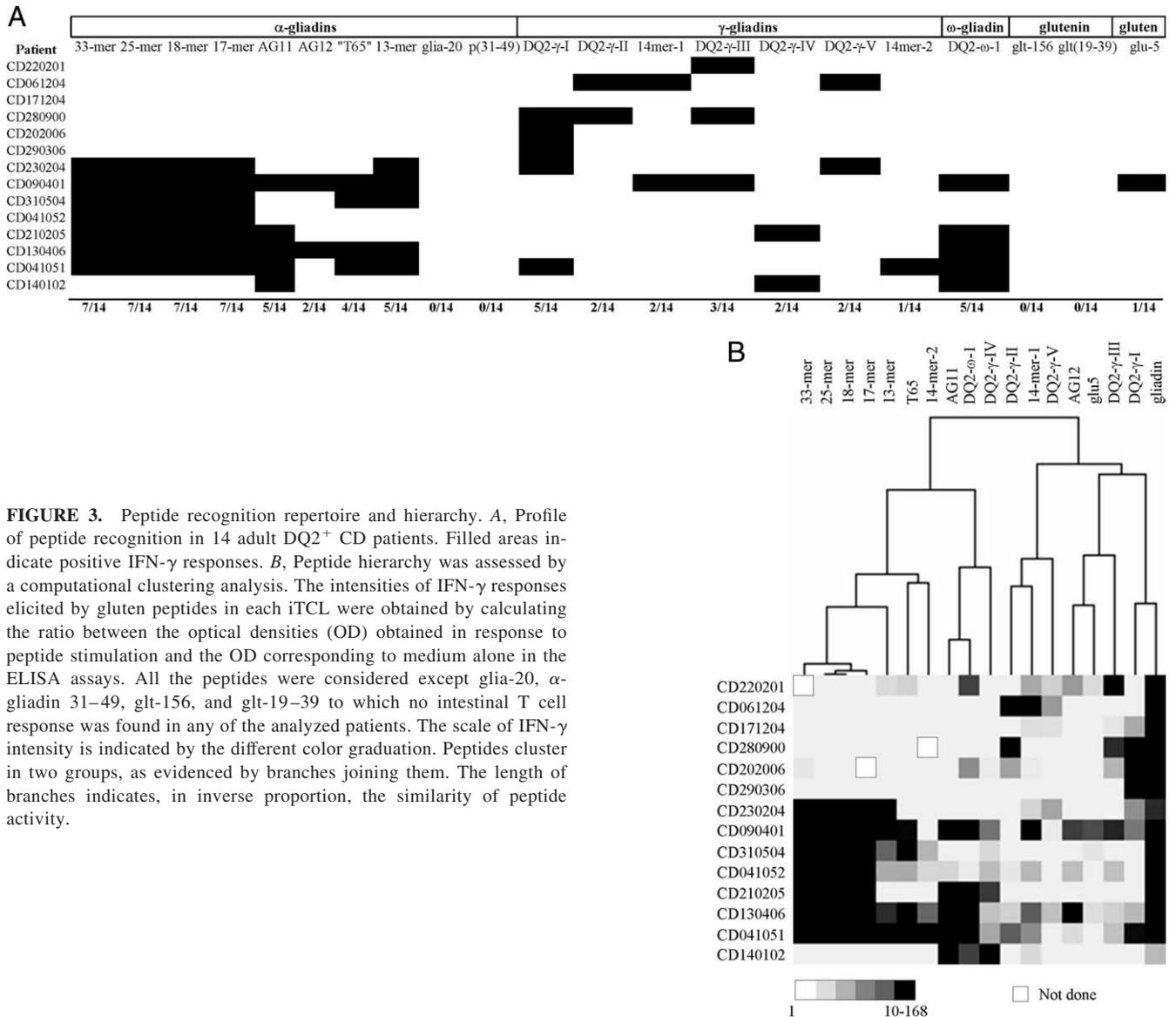
**Recognition of peptides from two gliadin families.** Two patients (14.2%) reacted to peptides from two gliadin families (Fig. 1*B*).



**FIGURE 2.** Recognition profile of peptides from one or none gliadin family. Recognition profile of intestinal T cell lines to 21 gluten peptides and to PT-gliadin are illustrated. *A* and *B*, IFN- $\gamma$  responses of two iTCLs recognizing peptides derived from only one gliadin family are illustrated. *C*, IFN- $\gamma$  production and (*D*) cell proliferation of the iTCLs from patient CD171204 are shown. T cells were stimulated as indicated in Fig. 1. Levels of IFN- $\gamma$  (ng/ml) are referred to  $10^6$  T cells/ml. Positive responses are indicated by the filled bars. Representative results of three separate experiments performed for each iTCL are illustrated.

CD130406 responded to all  $\alpha$ -gliadin peptides except for glia-20 and glia-31–49, and to DQ2- $\omega$ -1; CD230204 reacted very well to all peptides mapping the 57–89 region of  $\alpha$ -gliadin and weakly to the  $\gamma$ -gliadin peptides DQ2- $\gamma$ -I and DQ2- $\gamma$ -V (Fig. 1*B*).

**Recognition of peptides from one gliadin family.** This profile of response was found in half the patients. CD041052 reacted to all 33-mer truncated analogs except the 13-mer (Fig. 2*A*). CD310504 strongly recognized 33-mer, 25-mer, 18-mer, and 17-mer and its polymorphism T65 (values of IFN- $\gamma$  in the range of 20–25 ng/ml; Fig. 3*A*). The remaining five patients showed a significant IFN- $\gamma$  production toward one or more  $\gamma$ -gliadin peptides. The profile of responses from one representative patient, CD061204, recognizing



**FIGURE 3.** Peptide recognition repertoire and hierarchy. *A*, Profile of peptide recognition in 14 adult DQ2<sup>+</sup> CD patients. Filled areas indicate positive IFN- $\gamma$  responses. *B*, Peptide hierarchy was assessed by a computational clustering analysis. The intensities of IFN- $\gamma$  responses elicited by gluten peptides in each iTCL were obtained by calculating the ratio between the optical densities (OD) obtained in response to peptide stimulation and the OD corresponding to medium alone in the ELISA assays. All the peptides were considered except glia-20,  $\alpha$ -gliadin 31–49, glt-156, and glt-19–39 to which no intestinal T cell response was found in any of the analyzed patients. The scale of IFN- $\gamma$  intensity is indicated by the different color graduation. Peptides cluster in two groups, as evidenced by branches joining them. The length of branches indicates, in inverse proportion, the similarity of peptide activity.

DQ2- $\gamma$ -II, DQ2- $\gamma$ -III, and 14-mer-1 is illustrated in Fig. 2*B*. Responses from the other patients are illustrated in Fig. 3.

*No peptide recognition.* Patient CD171204, although very reactive to gliadin, did not respond to any peptide tested (Fig. 2, *C* and *D*), thus suggesting the existence of other gluten immunogenic peptides active in our cohort of patients.

#### Recognition frequency and hierarchy of gliadin immunogenic peptides

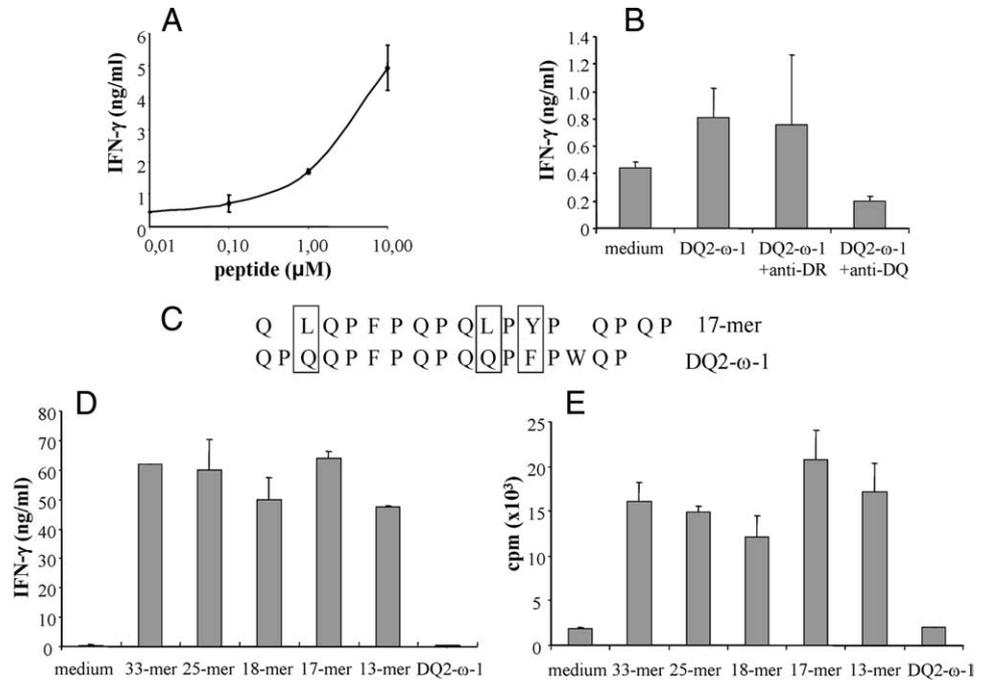
Quantitative assessment of T cell stimulatory activity as well as the consistency of peptide recognition are crucial for determining immunodominance (30, 31). As expected by its known stimulatory properties, the 33-mer induced very intense T cell activation in the great majority of responders (8, 14, 32). However, 33-mer was recognized by only half (7 of 14) of the patients (Fig. 3*A*). Remarkably, all patients responding to 33-mer reacted similarly to the 25-mer, 18-mer, and 17-mer, and only five of them responded to the shortest truncation 13-mer (Fig. 3*A*). Furthermore, five of these seven patients recognized at least one polymorphism of 17-mer (Figs. 1 and 2*A*). Reactivity toward the  $\gamma$ -gliadin-derived peptides

was more heterogeneous than observed for  $\alpha$ -gliadin peptides. Each  $\gamma$ -gliadin peptide was recognized by between 1 and 5 patients (for DQ2- $\gamma$ -I) (Fig. 3*A*), and, overall,  $\gamma$ -gliadin peptides were recognized by 11 (78%) patients (Figs. 1 and 2*B*). Collectively, these findings suggest that  $\gamma$ -gliadin-derived peptides are relevant to CD pathogenesis, consistent with previous studies (16, 18).

Interestingly, DQ2- $\omega$ -1 peptide displayed strong stimulatory capacity and was recognized by five (35%) patients. Only one patient (CD090401) produced IFN- $\gamma$ , but at very low level, in response to glu-5 (Fig. 3*A* and data not shown). Finally, no responses were observed toward the glia-20 or the 31–49 peptide of  $\alpha$ -gliadin (33, 34).

Hierarchical clustering analysis of active peptides, performed by the specific algorithm (see Refs. 24–26 and *Materials and Methods* for details) and based on the intensity of IFN- $\gamma$ -responses elicited in the 14 individuals, showed that peptides clustered into two main groups (Fig. 3*B*). The first one included the majority of  $\alpha$ -gliadin sequences that elicited the strongest and more consistent responses: 33-mer, 25-mer, 18-mer 17-mer, 13-mer, AG11, T65, but not AG12, as well as the DQ2- $\omega$ -1 peptide (with each peptide

**FIGURE 4.** Characterization of an immunogenic  $\omega$ -gliadin-derived peptide. **A**, iTCLs from patient CD041051 are assayed for IFN- $\gamma$  production in response to 10-fold increased concentrations of DQ2- $\omega$ -1 peptide. **B**, Cells from the same patient are stimulated with the DQ2- $\omega$ -1 (15  $\mu$ M) in the presence or absence of anti-HLA-DR and anti-HLA-DQ blocking mAbs (10  $\mu$ g/ml). **C**, Alignment of the  $\omega$ -gliadin peptide DQ2- $\omega$ -1 with the  $\alpha$ -gliadin 17-mer peptide is illustrated; differences of amino acid residues are delimited by a boxed area. **D** and **E**, Analysis of DQ2- $\omega$ -1 cross-reactivity on DQ2- $\alpha$ -I/III-specific T cell clones from patient CD230204 is shown. T cells were stimulated with N-terminal  $\alpha$ -gliadin peptides and DQ2- $\omega$ -1 (each peptide was assayed at 30  $\mu$ g/ml corresponding to a range of concentrations of 8–19  $\mu$ M) and assayed as indicated in Fig. 1. Representative IFN- $\gamma$  and proliferative responses of three separate experiments performed are illustrated.



being active in at least four and up to seven patients). Surprisingly, this group also included the infrequently recognized  $\gamma$ -gliadin 14-mer-2 and DQ2- $\gamma$ -IV (Fig. 3A) that revealed a stimulatory capacity comparable to those of  $\alpha$ -gliadin epitopes (Figs. 1A and 3B and data not shown). The second group, including the remaining  $\gamma$ -gliadin epitopes (DQ2- $\gamma$ -I, DQ2- $\gamma$ -II, DQ2- $\gamma$ -III, DQ2- $\gamma$ -V, and 14-mer-1) and the  $\alpha$ -gliadin AG12, was characterized by a more heterogeneous pattern of recognition and low intensity of IFN- $\gamma$  responses. Clustering results among the active peptides were in agreement with their degree of sequence similarity. The 33-mer, 25-mer, 18-mer, and 17-mer peptides had sequence identity ranging from 80% to 100%, and in the clustering diagram, these epitopes are joined by very short branches, meaning that they induced very similar production of IFN- $\gamma$ . The second group included the majority of the  $\gamma$ -gliadin peptides with more diverse sequences. In the clustering diagram, the  $\gamma$ -gliadin peptides are linked by long branches, indicating variability in their stimulatory activity.

Interestingly, four of six patients nonresponsive to  $\alpha$ -gliadin peptides have atrophic mucosa. When we next compared the hierarchical clustering results with the intestinal mucosal damage and HLA genotypes of responders, no correlation with the IFN- $\gamma$  responses was found (data not shown), thus suggesting that a larger number of patients is needed to reach a significant correlation between the profile of responses to gluten peptides and disease status.

In conclusion, clustering analysis confirmed a more focused and intense responses to peptides of the N-terminal region of  $\alpha$ -gliadin and to DQ2- $\omega$ -1, and more diffuse responses to  $\gamma$ -gliadin peptides.

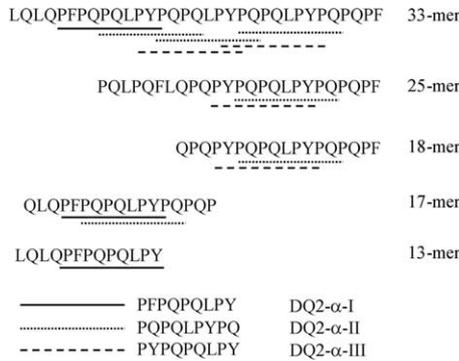
#### DQ2- $\omega$ -1 is an epitope different from the related $\alpha$ -gliadin 17-mer

The DQ2- $\omega$ -1 peptide has been previously shown to stimulate PBMC of HLA-DQ2<sup>+</sup> CD patients after a short oral gluten challenge (S. A. Tye-Din, J. A. Stewart, J. A. Dromey, T. Beissbarth, D. A. Van Heel, A. Tatham, K. Henderson, S. Mannering, C. Gianfrani, D. Jewell, A. V. S. Hill, and R. P. Anderson, manuscript in preparation). In the present study we have further characterized the immunogenicity of this  $\omega$ -gliadin peptide on intestinal mucosal T cells. DQ2- $\omega$ -1 was active in five patients (Figs. 1A and 3A).

Using T cells from the responder patient CD041051, we found that DQ2- $\omega$ -1 retained stimulatory activity even at low concentrations (Fig. 4A), and that its recognition was restricted by HLA-DQ2 (Fig. 4B). Interestingly, alignment analysis revealed that DQ2- $\omega$ -1 has 70% of sequence identity with the known  $\alpha$ -gliadin 17-mer (Fig. 4C). Furthermore, the  $\omega$ -gliadin peptide contains the 9-mer PFPQPQQPF that shares 78% sequence identity with the DQ2- $\alpha$ -I epitope PFPQPQLPY. This similarity could explain why four of five patients reactive to DQ2- $\omega$ -1 also recognized the 17-mer peptide (Fig. 3A). To further dissect whether the DQ2- $\omega$ -1 peptide is a functional analog of the 17-mer  $\alpha$ -peptide or a distinct epitope, we performed cross-reactivity experiments using three different 17-mer-specific intestinal T cell clones. As shown in Fig. 4, D and E, TCCs obtained from patient CD230204 produced high levels of IFN- $\gamma$  and proliferated in response to 17-mer but not to DQ2- $\omega$ -1, indicating that no cross-reactivity occurred between the two peptides. Furthermore, TCCs vigorously reacted to 33-mer and to all its truncated variants, thus strengthening the finding that DQ2- $\omega$ -1 is distinct from 17-mer and from other peptides mapping the N-terminal region of  $\alpha$ -gliadins.

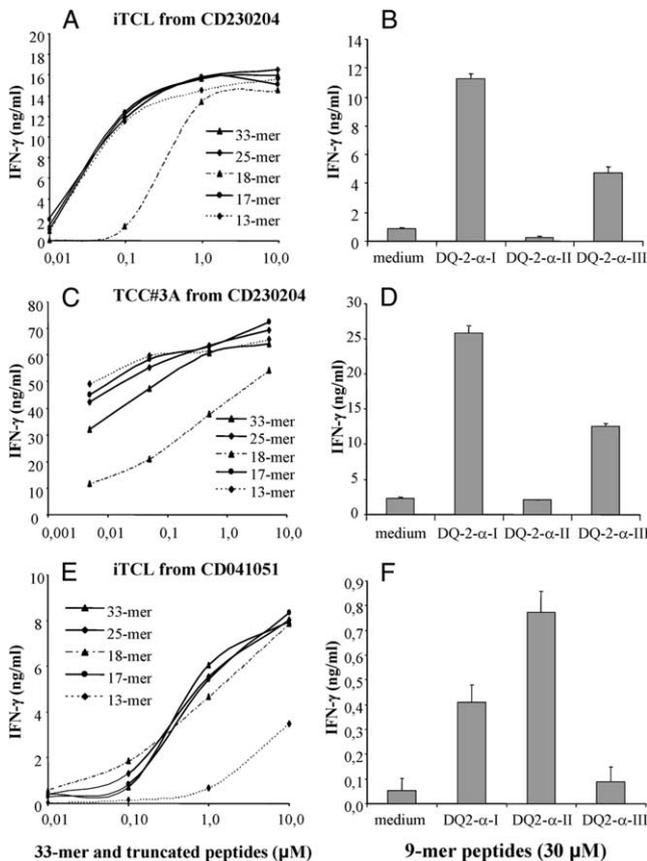
#### Dominance of 33-mer and of its truncated peptides: role of 9-mer epitopes

The polypeptide 33-mer is a very potent peptide (14, 15). We confirmed the prominent stimulatory capacity of this peptide, a finding that was further corroborated by computational clustering analysis (Fig. 3B). Interestingly, truncated peptides 17-mer, 25-mer, 18-mer, and 13-mer (Fig. 5) although containing a reduced number of the known 9-mer epitopes, were as potent as 33-mer in eliciting T cell responses either on polyclonal iTCLs (Figs. 1, 2A, and 3B) or on peptide-specific TCCs (Fig. 4, D and E). To further dissect the contributions of distinct 9-mer epitopes to the immunodominance of the N-terminal  $\alpha$ -gliadin peptides, dose-response curves were performed using DQ2- $\alpha$ -I/III-specific iTCLs and TCCs (from CD230204; Fig. 6, A and B), and also DQ2- $\alpha$ -II-specific iTCLs from CD041051 (Fig. 6, E and F). IFN- $\gamma$  production and cellular proliferation of DQ2- $\alpha$ -I/III-specific T cells induced by the 33-mer were equivalent to those induced by the shorter variants of this peptide (Fig. 6A–D and data not shown).

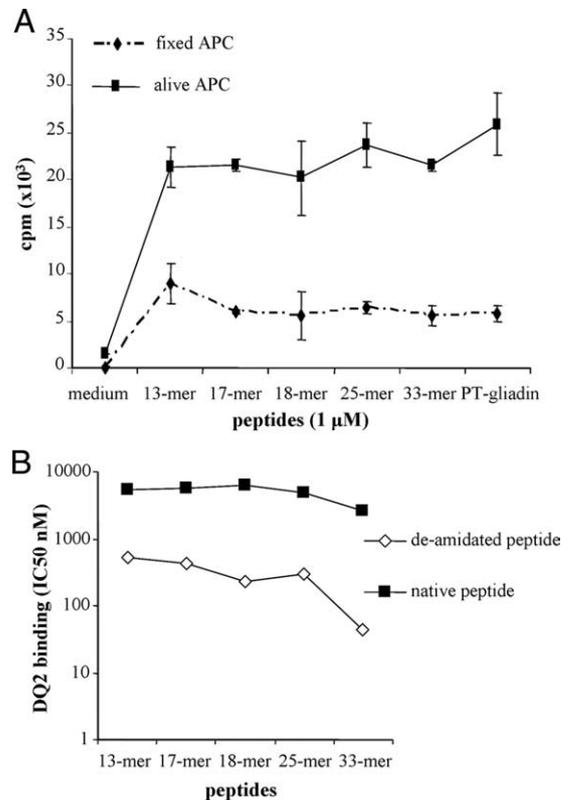


**FIGURE 5.** Sequence alignment of 33-mer with its truncated peptides.  $\alpha$ -Gliadin peptides were aligned on the basis of their position at the N-terminal region. All 9-mer DQ2- $\alpha$ -I, DQ2- $\alpha$ -II, and DQ2- $\alpha$ -III epitopes contained in each peptide are underlined.

Remarkably, the 33-mer, 25-mer, 17-mer, and 18-mer, which all contained at least one copy of DQ2- $\alpha$ -II, possessed similar potency in activating DQ2- $\alpha$ -II-specific iTCLs (Fig. 6, *E* and *F*). In con-



**FIGURE 6.** Correlation between the stimulatory capacity of N-terminal  $\alpha$ -gliadin peptides and the known 9-mer epitopes. Dose-response curves of peptides encompassing the 57–89 region of  $\alpha$ -gliadin were performed on both iTCLs and TCCs. Irradiated APCs (autologous B-LCL) were incubated overnight with 10-fold increasing doses of TG2-deamidated peptides before DQ2- $\alpha$ -I/III- or DQ2- $\alpha$ -II-specific T cells were added ( $3 \times 10^4$  for T cells or  $1 \times 10^4$  for TCCs). T cell lines and clones were also analyzed in response to DQ2- $\alpha$ -I, -II, and -III 9-mer epitopes (30  $\mu$ M). *A* and *B*, IFN- $\gamma$  production of DQ2- $\alpha$ -I/III-responsive iTCLs from patient CD230204 is illustrated. *C* and *D*, From the same patient, DQ2- $\alpha$ -I/III-specific TCCs were generated and tested for peptide recognition. *E* and *F*, Cells from patient CD041051 reacting to DQ2- $\alpha$ -II epitope were stimulated with increasing peptide doses. Values (mean of duplicates) are representative of three separate experiments.



**FIGURE 7.** The stimulatory activity of 33-mer and of its truncated peptides is processing-independent. *A*, The proliferative response of CD230204 iTCLs (DQ2- $\alpha$ -I-specific) to 33-mer, 25-mer, 18-mer, 17-mer, and 13-mer peptides of  $\alpha$ -gliadins was assessed using both alive or fixed APCs pulsed overnight with peptides (1  $\mu$ M) or PT-gliadin (50  $\mu$ g/ml). After a thorough washing, Ag-loaded APCs were incubated with T cells for an additional 48 h. *B*, Binding to purified DQ2 heterodimers was performed by a competitive inhibitory assay. IC<sub>50</sub> indicates the concentration of each analyzed peptide that induced the 50% of inhibition of a probe peptide binding.

trast, the 13-mer that included only DQ2- $\alpha$ -I had weak stimulatory capacity and only at very high concentration (10  $\mu$ M). Collectively, these results demonstrated that the most active  $\alpha$ -gliadin peptides need to contain only one copy of DQ2- $\alpha$ -I and of DQ2- $\alpha$ -II epitopes. These findings have important implications for the definition of the minimal  $\alpha$ -gliadin immunodominant peptide relevant for a peptide-based therapeutic vaccine.

#### Cellular processing and binding to HLA-DQ2 by N-terminal $\alpha$ -gliadin peptides

We next investigated whether processing by APCs and binding affinity for purified HLA-DQ2 might influence the stimulatory activity of peptides encompassing the 57–89 region. To address the role of cellular processing, we performed proliferation assays using both irradiated (“live”) and glutaraldehyde-fixed APCs with the iTCLs from patient CD230204 (DQ2- $\alpha$ -I/III specific) as responder cells (Fig. 7*A*). At a peptide concentration that stimulated maximal T cell response (1  $\mu$ M; Fig. 6*A*), no substantial differences were observed between the proliferation elicited by 33-mer and its shorter variants using live or fixed APCs. Interestingly, peptide-pulsed, live APCs displayed a more efficient stimulatory capacity for T cells than fixed cells, most likely due to more efficient peptide uptake and processing, or to better preserved co-stimulatory molecules. It is noteworthy that the 33-mer was efficiently presented by fixed cells and, consistent with previous reports, does not require cellular processing (18). Binding to purified HLA-DQ2 molecules showed that the 33-mer possessed the

highest affinity ( $IC_{50}$  of 44.0 nM). Nevertheless, all of the other truncated peptides were efficient binders to HLA-DQ2, with an  $IC_{50}$  ranging from 232 nM for 18-mer to 536 nM for 13-mer (Fig. 7B). In accordance with previous findings (16, 35–38), the native peptides bound weakly to HLA-DQ2 with an  $IC_{50}$  ranging from 10- to 58-fold lower than corresponding deamidated peptides. The data demonstrated that each of the truncated  $\alpha$ -gliadin peptides bound efficiently to HLA-DQ2 without requiring further cellular processing. From these overall results we concluded that the 17-mer that contains only one copy of DQ2- $\alpha$ -I and of DQ2- $\alpha$ -II is the minimal immunodominant  $\alpha$ -gliadin peptide with optimal T cell stimulatory capacity.

## Discussion

There is a general consensus that HLA-DQ-restricted CD4<sup>+</sup> T cells are pivotal in CD pathogenesis (1). At present, many gluten peptides with stimulatory capacity for intestinal CD4<sup>+</sup> T cells have been identified by several groups in various cohorts of CD patients (8–10, 13–15, 16). In the present study we screened intestinal T cells isolated from 14 adult Italian CD patients for recognition of 21 peptides derived from wheat glutenin and  $\alpha$ -,  $\gamma$ -, and  $\omega$ -gliadins. Consistent with previous studies (18), our results demonstrate that HLA-DQ2<sup>+</sup> CD patients respond to a wide and heterogeneous array of peptides; in some cases many peptides from multiple or single gliadin families are recognized, while in others only one of the peptides tested was active. The great majority of patients reacted to a particular set of peptides, thus confirming that a large number of gluten epitopes might be implicated in CD development. Collectively, we found 17 of 21 peptides, derived from  $\alpha$ -,  $\gamma$ -, and  $\omega$ -gliadins, to be recognized by T cell lines raised against deamidated PT-gliadin. The failure of the gliadin-specific T cell line from patient CD171204 to recognize any of the 21 peptides tested suggests that other gliadin peptides are relevant in some HLA-DQ2<sup>+</sup> CD patients.

T cell responses of adult CD patients toward the overall 10  $\alpha$ -gliadin-derived peptides assayed in this study indicated that they are mainly focused on the N-terminal 57–89 region of  $\alpha$ -gliadins. In fact, the 33-mer and its shorter forms (25-mer, 18-mer, 17-mer) were the most frequently recognized among the peptides assessed, and they stimulated the greatest IFN- $\gamma$  production and proliferation by intestinal T cells. In contrast, responses elicited by  $\gamma$ -gliadin-derived peptides were more less focused than those induced by  $\alpha$ -gliadin-derived peptides, most likely reflecting their more diverse sequences. Furthermore, the great majority of patients reacted to at least one  $\gamma$ -gliadin peptide, and an overall half recognized DQ2- $\gamma$ -I. This frequent recognition of  $\gamma$ -gliadin peptides by intestinal T cells from CD individuals suggests that their contribution to CD pathogenesis may be greater than is currently appreciated. We also found that intestinal T cell lines were frequently and strongly stimulated by the  $\omega$ -gliadin-derived peptide, DQ2- $\omega$ -1 (QPQQPFPQPQQPFPWQP). DQ2- $\omega$ -1 is recognized by peripheral blood cells of HLA-DQ2<sup>+</sup> CD patients following in vivo challenge with wheat (S. A. Tye-Din, J. A. Stewart, J. A. Dromey, T. Beissbarth, D. A. Van Heel, A. Tatham, K. Henderson, S. Mannering, C. Gianfrani, D. Jewell, A. V. S. Hill, and R. P. Anderson, manuscript in preparation). Interestingly, although DQ2- $\omega$ -1 shares substantial amino acid sequence similarity with the 17-mer  $\alpha$ -gliadin (57–73) peptide, we demonstrated that it is a distinct T cell epitope.

Despite self and foreign Ags harboring many potential epitopes, T cell responses are primed by only a selected few and others are recognized by T cells only as the immune response evolves with progression of disease (39). Understanding the hierarchy and consistency of epitopes is important, as recent studies have shown that

immunodominant epitopes can have therapeutic application to tolerize the adverse immune reaction in several T cell-mediated diseases (19, 20, 40). In the case of immunodominance of known gluten peptides, conflicting data have been reported. Here we found that the 33-mer was recognized by T cell lines from 50% of our cohort of CD patients. This finding was consistent with that of Vader et al., who also showed that 50% of T cell lines derived from Dutch children and adults were reactive to peptides 57–68 and 62–75 (18). In contrast, there was universal recognition of the 33-mer by HLA-DQ2<sup>+</sup> Norwegian CD patients (9, 14). Interestingly, a recent study demonstrated that following a brief oral gluten challenge, peripheral blood HLA-DQ2-restricted CD4<sup>+</sup> T cells expressing the gut-homing integrin  $\alpha_4\beta_7$  from a large number of treated HLA-DQ2<sup>+</sup> CD patients of English and Australian origin reacted to an  $\alpha$ -gliadin 17-mer (57–73) peptide, QLQPFQQLPYPQPQS, that is a shorter version of the known immunodominant 33-mer (17).

Many reasons could explain the observed discrepancies between studies that address gluten peptide recognition by T cells in CD. For example, the origin of CD individuals, the particular wheat cultivar composition of their diet, as well as the disease status may be important. However, the most reasonable explanation is the difference in methodology used to generate and expand T cell lines from intestine. In common with the group from the Netherlands, we have generated TCLs following stimulation of intestinal cells with PT-gliadin-pulsed PBMC, while the Norwegian group has established TCLs by stimulation after incubating the entire mucosal explant with chymotrypsin-digested gliadin, thus using the tissue resident cells as APCs.

Mucosal resident APCs, particularly dendritic cells, could have an important role in shaping the intestinal immune response to gluten peptides and to food proteins in general. To date, the few studies that have investigated the phenotype and function of dendritic cells resident in the small intestinal mucosa of celiac patients have had discrepant results (41, 42). It may be that the different profile of gluten peptides we found active in our cohort of patients, compared with Norwegians, could be due to the different type or maturation of APCs used to generate gliadin-specific T cell lines. Interestingly, the increased intensities of responses to gluten peptides observed when viable irradiated APCs are used in T cell specificity assays compared with fixed cells strengthen the hypothesis of a pivotal role of APCs in determining the different profile of active gluten peptides.

When we compared the stimulatory potency of peptides encompassing the sequence of 33-mer, the 25-mer, 18-mer, 17-mer, and 13-mer, we observed that the magnitude of elicited responses was not strictly dependent on the copy number of 9-mer epitopes contained in each peptide. Furthermore, consistent with a previous report, experiments performed with fixed APCs revealed a very similar stimulatory activity of 33-mer and also of its shorter forms (15). We found that these related  $\alpha$ -gliadin peptides had high binding affinity for HLA-DQ2 molecules. Taken together, these findings indicate that this family of related  $\alpha$ -gliadin peptides can efficiently bind to HLA-DQ2 molecules on the cell surface without requiring processing.

From the overall results, we conclude that the 17-mer peptide with one copy each of the DQ2- $\alpha$ -I and of DQ2- $\alpha$ -II epitopes is as potent as the polypeptide 33-mer in stimulating CD4<sup>+</sup> T cell responses in CD patients. Interestingly, data bank research revealed that  $\alpha$ -gliadins contain several polymorphisms of the 17-mer immunodominant epitope. In this study, we screened three related 17-mers (AG11, AG12, and T65). We showed that 6 of 14 individuals reacted to at least one of these peptides. Surprisingly, four TCLs reacted with variable intensity to peptide T65, containing a threonine in position 65. These results suggested that polymorphisms of known epitopes can be

efficiently recognized by CD patients, and for this reason should be considered in the design of immunotherapy.

In conclusion, our findings point out the great heterogeneity of immunogenic gluten peptides and the relevance of both  $\gamma$ - and  $\omega$ -gliadins epitopes in CD pathogenesis. Furthermore, we found peptides derived from the 57–89 region of  $\alpha$ -gliadins, the DQ2- $\gamma$ -I and the DQ2- $\omega$ -1, to be highly active. We also defined the shortest truncation of 33-mer, the 17-mer, as the minimal  $\alpha$ -gliadin peptide with optimal immunogenicity. The present study has relevance for the design of peptide-based immunotherapy for CD.

## Acknowledgments

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## Disclosures

R. P. Anderson is an inventor who is involved in commercialization of patents relating to diagnostics, therapeutics, and nontoxic glutes based on knowledge of peptides recognized by T cells in celiac disease. R. P. Anderson is also Chief Executive, Director and a substantial shareholder in Nexpep and Nexgrain.

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