

# Gliadin-specific, HLA-DQ( $\alpha 1^*0501, \beta 1^*0201$ ) Restricted T Cells Isolated from the Small Intestinal Mucosa of Celiac Disease Patients

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## Summary

Celiac disease (CD) is most probably an immunological disease, precipitated in susceptible individuals by ingestion of wheat gliadin and related proteins from other cereals. The disease shows a strong human HLA association predominantly to the *cis* or *trans* encoded HLA-DQ( $\alpha 1^*0501, \beta 1^*0201$ ) (DQ2) heterodimer. T cell recognition of gliadin presented by this DQ heterodimer may thus be of immunopathogenic importance in CD. We therefore challenged small intestinal biopsies from adult CD patients on a gluten-free diet *in vitro* with gluten (containing both gliadin and other wheat proteins), and isolated activated CD25<sup>+</sup> T cells. Polyclonal T cell lines and a panel of T cell clones recognizing gluten were established. They recognized the gliadin moiety of gluten, but not proteins from other cereals. Inhibition studies with anti-HLA antibodies demonstrated predominant antigen presentation by HLA-DQ molecules. The main antigen-presenting molecule was established to be the CD-associated DQ( $\alpha 1^*0501, \beta 1^*0201$ ) heterodimer. The gluten-reactive T cell clones were CD4<sup>+</sup>, CD8<sup>-</sup>, and carried diverse combinations of T cell receptor (TCR) V $\alpha$  and V $\beta$  chains. The findings suggest preferential mucosal presentation of gluten-derived peptides by HLA-DQ( $\alpha 1^*0501, \beta 1^*0201$ ) in CD, which may explain the HLA association.

Celiac disease (CD)<sup>1</sup> is a disorder of the small intestine, characterized by crypt-cell hyperplasia and villous atrophy (1, 2). As a result, mild to severe malabsorption and diarrhea are often found. The disease is precipitated in susceptible individuals by ingestion of cereal proteins. In particular, wheat gluten, but also rye, and to a lesser extent, barley and oat, may cause disease. Once these nutrients are withdrawn from the diet, the intestinal morphology will normalize.

CD is most probably an immunological disease (1–3), and shows a well-defined HLA association. We previously found that 93 of 94 Norwegian CD patients carried the DQA1<sup>\*0501</sup> and DQB1<sup>\*0201</sup> genes in *cis* or *trans* position (4), which encode the DQ( $\alpha 1^*0501, \beta 1^*0201$ ) heterodimer (5). This strong association is found in most patient populations (6, 7). However, the link between the immunopathology and the HLA association has not been elucidated, since gluten-reactive T

cells from the mucosa of CD patients have previously not been reported (8).

Challenge of treated CD patients (i.e., on a gluten-free diet) with gluten induces a systemic and mucosal immune activation (9–12). When small intestinal biopsies from CD patients on a gluten-free diet are challenged *ex vivo* with gluten, CD4<sup>+</sup> T cells in the lamina propria are activated and express CD25 (IL-2 receptor L chain) (13). Similar findings are not made in biopsies from healthy individuals. Here we show that such activated T cells mainly recognize gluten when presented by the HLA-DQ( $\alpha 1^*0501, \beta 1^*0201$ ) heterodimer. The results suggest that an important feature of celiac disease may be presentation, at the site of the disease lesion, of gluten-derived peptides by the CD-associated HLA-DQ heterodimer itself.

## Materials and Methods

**Patients.** Biopsies were taken from eight CD patients, two with untreated disease and six on a gluten-free diet. The diagnosis was based on typical mucosal lesions with crypt hypertrophy, villous atrophy, and an increased number of intraepithelial lymphocytes.

<sup>1</sup> Abbreviations used in this paper: B-LCL, B lymphoblastoid cell line; CD, celiac disease; IHWS, International Histocompatibility Workshop; TCC, T cell clone.

The patients were all typed serologically to be HLA-DR3,DQ2, i.e., they carried the DQ( $\alpha$ 1\*0501, $\beta$ 1\*0201) heterodimer. All treated CD patients improved clinically on a gluten-free diet. By the time biopsies were taken for the present study, formalin-fixed biopsies were H-E stained and evaluated by light microscopy. Some but not all patients had shown complete normalization on a presumptive gluten-free diet. Patients controls ( $n = 5$ ) had clinical symptoms partly suggestive of CD, but their small intestinal mucosa was histologically normal.

**Antigens and Peptides.** Cereal protein antigens were prepared based on their solubility in dilute acids and alcohol (14). Flour was defatted and water-soluble proteins extracted by washing twice in water-saturated *n*-butanol. Wheat gluten (containing both gliadins and glutenins) and corresponding proteins from other cereals were extracted with 0.01 M acetic acid, whereas gliadin was extracted with 70% (vol/vol) ethanol. Trade gluten (ICN Nutritional Biochemicals, Cleveland, OH) or gliadin (Sigma Chemical Co., St. Louis, MO) was dissolved in water or in 0.01 M acetic acid. The cereal protein was partially digested with pepsin and trypsin (Sigma Chemical Co.) largely according to Frazer et al. (15). Flours used were from the wheat cultivars Kolibri, Yamhill, Scout 66, Cheyenne, Folke, and Tjalve; the blended wheat preparations Canadian Western Red Spring, Dark Northern Spring, Northern Spring, Canadian Hard Winter; or trade (in Norway)-blended wheat, rye, barley, and oat. As control antigens, not related to cereals, we used purified protein derivative of *Mycobacterium tuberculosis* (provided by the National Institute of Public Health, Oslo, Norway), sonicated *M. tuberculosis* (strain H37Ra, provided by Dr. Oftung, The Norwegian Radium Hospital, Oslo, Norway), or streptokinase (Behringwerke, Marburg, Germany). Seven overlapping synthetic peptides covering the NH<sub>2</sub>-terminal region (residue 1-58) of  $\alpha$ -gliadin (16-18) were synthesized as described in detail elsewhere (Gjertsen H. A., K. E. A. Lundin, L. M. Sollid, J. A. Eriksen, and E. Thorsby, manuscript submitted for publication). The peptide sequences were (one letter code) p14: VRVPVPLQPNPSQ-QQPQ; p15: QNPSQQPQEQVPLVQQQ; p16: QVPLVQQQ-FLGQQQPFPPQ; p19: LGQQQPFPPQYPQPQPF; p209: FPGQQQPFPPQYPQPQPF; p211: FPGQQQPFPPQYPQPQPF; and p62: QPYPQPFPFSQQPYLQL.

**Ex Vivo Gluten Stimulation of Biopsies.** Four to six biopsy specimens were taken by upper gastrointestinal endoscopy obtaining biopsies from the distal duodenum. Biopsies from treated CD patients on a gluten-free diet were challenged for ~18 h with 5 mg/ml of a peptic/tryptic digest of commercial gluten (ICN Nutritional Biochemicals) in an organ culture chamber at 37°C with 5% CO<sub>2</sub> and 95% O<sub>2</sub> at 1 bar pressure in RPMI 1640 (Gibco, Paisley, Scotland) containing 20% inactivated, pooled human serum and antibiotics (13). This gluten challenge step was not done for biopsies from individuals on a gluten-containing diet. The biopsies were cut in pieces and digested enzymatically with 1 mg/ml collagenase A (Boehringer Mannheim, Mannheim, Germany) at 37°C. Cells liberated from the biopsy fragments were harvested after 30 min, 60 min, and in one case, 120 min by filtration through a nylon filter (mesh width, 200  $\mu$ m) and named according to the patient code and duration of collagenase treatment (i.e., CD281 30', etc.).

**T Cell Lines and T Cell Clones (TCC).** Activated T cells were immunomagnetically isolated using Dynabeads (M-450; Dynal, Oslo, Norway) coated with an antibody to CD25 (Amersham International, Amersham, Bucks, UK), as described elsewhere (19, 20). To the cell suspension from three to five biopsies, 100  $\mu$ l beads of a 0.1 mg/ml solution was used. Positively selected cells were resuspended immediately in 200  $\mu$ l of a suspension of  $2 \times 10^5$  au-

tologous, irradiated PBMC in complete medium (CM: RPMI 1640 with 15% pooled, inactivated human serum and penicillin-streptomycin) and 5 U/ml rIL-2 (Amersham International). This cell suspension was transferred to one well of a flat-bottomed 96-well plate and grown for 1 wk. Cultures were thereafter restimulated weekly with autologous, irradiated PBMC, 1  $\mu$ g/ml PHA (Wellcome, Dartford, UK) and 5 U/ml IL-2. T cell lines isolated from biopsies from individuals on a gluten-containing diet were split into two subcultures, one which received gluten and one which received PHA. In both cases, autologous, irradiated PBMC were also here used as APC in the presence of 5 U/ml IL-2. For some of the T cell lines, TCC were established by limiting dilution (0.3-0.5 cells/well) in 20- $\mu$ l wells (Terasaki; Nunc, Roskilde, Denmark), using  $10^4$  irradiated PBMC, 1  $\mu$ g/ml PHA, and 5 U/ml IL-2. Growing TCC were transferred to 96-well plates and later to 24-well plates (Costar Corp., Cambridge, MA), also in these cases with weekly restimulation with PHA, PBMC, and IL-2. TCC were screened for reactivity and cryopreserved in the vapor phase of liquid N<sub>2</sub>.

**Phenotyping of T Cells.** Phenotyping of T cells for the markers CD2, CD3, CD4, CD8, and TCR- $\alpha/\beta$  was done by FACS<sup>®</sup> analysis (FACScan<sup>®</sup>, Becton Dickinson & Co., Palo Alto, CA) or by a rosette-forming assay using antibody-coated Dynabeads M-450 (21).

**Proliferative Assays and Inhibition Studies.** Proliferative assays were performed in 0.2 ml CM on 96-well round-bottomed plates using  $2 \times 10^4$  T cells stimulated in triplicates with either  $5 \times 10^4$  EBV-transformed B lymphoblastoid cell lines (B-LCL; irradiated 10,000 rad) or  $5-10 \times 10^4$  PBMC (3,000 rad) in the presence or absence of antigen. Peptic-tryptic digests of cereal proteins were used at 1-2 mg/ml, synthetic peptides at 50  $\mu$ M. APC were usually preincubated overnight with antigen, as this was shown to enhance the proliferative response of the T cells (data not shown). Cultures were pulsed with [<sup>3</sup>H]thymidine after ~48 h, and harvested 18 h thereafter. APC used were either HLA homozygous B-LCL distributed during the 10th and 11th International Histocompatibility Workshops (IHWS) (22, 23), or PBMC or B-LCL from local CD patients or healthy controls (4, 5). In inhibition assays with anti-HLA mAb, APC were preincubated with gluten and mAb before addition of the T cells. mAb used for inhibition studies were W6/32, anti-HLA class I monomorphic (American Type Culture Collection [ATCC], Rockville, MD); L243, anti-HLA-DR monomorphic (ATCC); B8.11, anti-HLA-DR monomorphic (a gift from B. Malissen, Centre d'Immunologie, Marseille, France); GSP4.1: anti-HLA-DR monomorphic (a gift from J. Hansen, Fred Hutchinson Cancer Research Center, Seattle, WA); SPV-L3, anti-HLA-DQ monomorphic (a gift from H. Spits, DNAX Research Institute, Palo Alto, CA); FN81, anti-HLA-DQ monomorphic (a gift from S. Funderud, The Norwegian Radium Hospital, Oslo, Norway); 2.12.E.11, anti-HLA-DQ2 (a gift from H. Viken, our institute); XIII 358.4, anti-HLA-DQ2, (a gift from C. Mazzilli, University of Rome, Rome, Italy); and B7/21, anti-HLA-DP monomorphic (a gift from F. Bach, University of Minnesota Hospital, Minneapolis, MN).

**HLA-DQB Transfected B Cells.** Transfection with HLA-DQB1\*0201 cDNA was performed essentially as described elsewhere (24, and Paulsen, G. et al., manuscript in preparation). In short, DQB1\*0201 cDNA (a gift from Janet Lee, Sloan-Kettering Institute for Cancer Research, New York) from the DR7DQ2<sup>+</sup> cell line BH (10th IHWS, No. 9046) was transfected by a retroviral method into the DR5DQ7<sup>+</sup> cell line Sweig007 (10th IHWS, No. 9037), which carries DQA1\*0501 and DQB1\*0301. Transfected cells were propagated in selective medium for several cycles to ensure stable

expression of the transferred gene product. Actual expression of DQ( $\beta$ 1\*0201) was shown by FACS analysis<sup>®</sup> (Becton Dickinson & Co.) using the mAb XIII 358.4.

**TCR Sequencing.** TCR  $\alpha$  and  $\beta$  gene sequencing was performed using modified PCR techniques as described elsewhere (25). At least three M13 plaques for each TCR gene were sequenced, except that, because of a PCR slippage problem, we were only able to find one M13 plaque which included the CDR3 $\alpha$  of the V $\alpha$ 22<sup>+</sup> transcript of TCC 1.27.

## Results

**Growth and Gluten Reactivity of T Cells from Small Intestinal Biopsies.** Activated, CD25<sup>+</sup> T cells were positively selected from small intestinal biopsies from 13 individuals. T cell growth was observed in 11 of the 13 cases, and the T cell lines were tested after 2–3 wk. T cell lines could be established from biopsies from four of the six patients with treated CD, where positive selection of activated, CD25<sup>+</sup> T cells was done with ex vivo challenge with gluten. Of these four, gluten-specific responses were observed in T cell lines from three individuals, two showed strong responses and one showed a weak response which was not investigated further (data not shown). The small intestinal morphology and the subsequent gluten reactivity of biopsy-derived T cell lines are depicted in Table 1. A weak gluten-specific proliferative response was observed for one T cell line from one untreated CD patient, but not from the other untreated CD patient or the five patient controls (Table 2, and data not shown).

**Polyclonal T Cell Recognition of Gluten and Preferential Restriction by the CD-associated DQ Heterodimer.** Three T cell lines (termed CD281 30', CD280 60', and CD280 120') showing reactivity towards gluten were studied in mAb inhibition experiments. CD280 is HLA-DR3,6, DQ1,2 and CD281 is HLA-DR3, DQ1,2. mAb inhibition studies showed a complete inhibition of the CD281 30' and CD280 120' lines by anti-DQ mAb, whereas the response of the CD280 60' line was partly inhibited by anti-DR, partly by anti-DQ mAb (Fig. 1).

We next stimulated the lines from CD280 and CD281 with gluten, using cells from a panel of allogeneic donors as APC.

**Table 2.** Proliferative Responses of T Cell Lines Directly Isolated from Untreated Patients and Controls

	CD294* DR3,6 DQ1,2 CD pt.	CD289 DR3,6 DQ1,2 CD pt.	CD297 DR3,7 DQ2 Contr.	CD290 DR2,4 DQ1,3 Contr.
T cells alone	0.1	0.1	0.7	0.1
APC alone	1.6	0.2	1.5	0.4
T + APC	1.6	0.2	1.8	0.4
T + APC + gluten	3.1	0.2	1.2	0.3
T + APC + PHA + IL-2	<b>51.6</b>	<b>19.0</b>	<b>33.6</b>	<b>40.4</b>

Values show mean cpm ( $\times 10^{-3}$ ) of triplicate cultures. SD was usually <10%. Positive values are given in boldface.

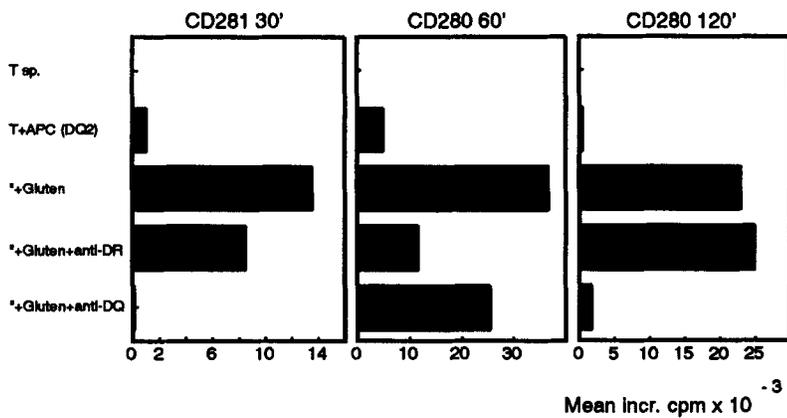
Since only weak responses were seen with PBMC as APC (possibly because of the low expression of HLA-DQ on such cells) we used B-LCL. Some results are given in Fig. 2. In some cases, weak or moderate alloresponses to the APC in the absence of gluten was observed, but strong gluten-specific proliferative responses were always observed using B-LCL from donors being DR3,DQ2, i.e., carrying the DQA1\*0501 and DQB1\*0201 genes in *cis* position. When instead APC were used from a donor being DR5,DQ7/DR7,DQ2, i.e., carrying the DQA1\*0501 and DQB1\*0201 genes in *trans* position, a weak gluten-specific response of the CD280 120' fraction was seen. However, for the two other polyclonal T cell lines, weak gluten-specific responses were also seen using APC from some donors not being DR3,DQ2. From the results of the antibody inhibition experiments and the panel studies we conclude that a preponderance of the small intestinal-derived gluten-reactive T cells tested were restricted by the CD-associated DQ heterodimer.

The antigen specificity of the T cell lines was then investigated (Table 3, and data not shown). They recognized gluten from a variety of wheat formulations, but not rye and not

**Table 1.** Histology of the Small Intestinal Biopsies from Treated Patients

	Morphology	Cell density	Gluten reactivity
CD280	Partial villous atrophy	Increase of IEL and LPL	Yes
CD281	Partial villous atrophy	Increase of IEL and LPL	Yes
CD286	Minor villous changes	Normal IEL and LPL	No
CD287	No pathological changes	Normal IEL and LPL	No T cell growth
CD288	Partial villous atrophy	Normal IEL, slight increase of LPL	No T cell growth
CD295	Partial villous atrophy	Increase of IEL and LPL	Yes (weak)

The cell density of intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) was judged by light microscopy on H-E stained sections. Also indicated is gluten reactivity of T cell lines derived from the biopsies.



**Figure 1.** mAb inhibition of gluten-specific responses of polyclonal T cell lines from the small intestinal mucosa suggested preferential HLA-DQ restriction. T cell lines shown are the 30' fraction from patient CD281, and the 60' and 120' fractions from patient CD280. Gluten was presented by allogeneic DR3,DQ2<sup>+</sup> B-LCL to the T cell lines. The mAb L243 (anti-DR) or SPV-L3 (anti-DQ) were used as 1:500 dilutions of ascites. Bars show mean cpm of triplicate cultures. (*T sp.*) T cells alone.

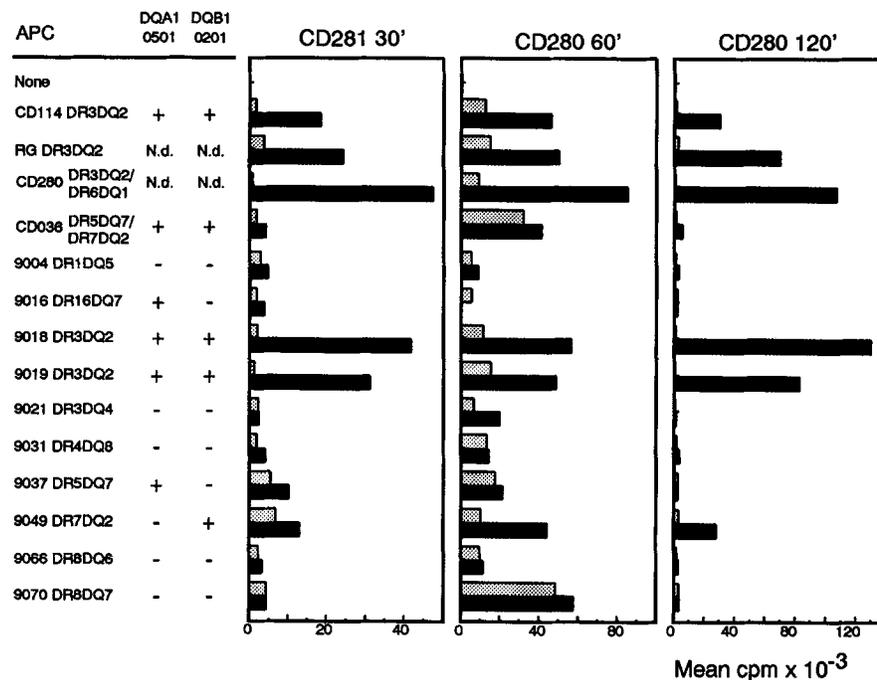
pepsin-trypsin, which had also been present during the primary ex vivo stimulation of the biopsies. The T cell lines all recognized the gliadin moiety of gluten. No responses to control antigens not related to cereals were observed.

**Isolation of Gluten-specific TCC.** To investigate the fine specificity of individual T cells, we raised TCC. All TCC were screened for reactivity towards gluten presented by B-LCL being DR3,DQ2, and tested in mAb inhibition assays using anti-DR and anti-DQ mAb. A varying proportion of TCC from each cell line showed gluten reactivity. 7 of 80 TCC from the CD281 30' fraction (e.g., TCC 1.27, 1.50, and 1.63) and 4 of 80 TCC (e.g., TCC 5.14 and 5.48) from the CD280 60' fraction were strictly gluten reactive. Other TCC showed a complex pattern, partly alloreactive, partly gluten reactive, and were uniformly inhibited by anti-DR, but not by anti-DQ mAb. Gluten specificity was observed for 76 of 83 TCC

from the CD280 120' fraction (e.g., TCC 4.19). However, most or all these TCC appeared to be sister clones (based on sequencing of the TCR and/or reactivity staphylococcal exotoxins, data not shown). Importantly, all strict gluten-reactive TCC were HLA-DQ restricted, with one exception.

**Gluten-specific TCC Were Restricted by the CD-associated DQ Heterodimer.** Some TCC with strict gluten-specific responses were further analyzed, all carrying the CD2<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup>, TCR- $\alpha/\beta$ <sup>+</sup> phenotype. Three different kinds of experiments were performed.

First, we performed inhibition studies using a panel of anti-HLA mAb to inhibit the response to gluten in the presence of APC being DR3,DQ2. For all TCC except one, mAb against HLA class I, HLA-DR, or HLA-DP did not inhibit the gluten-specific responses (data not shown). In contrast, a range of HLA-DQ2-reactive mAb inhibited the reactivity



**Figure 2.** APC from donors being DR3, DQ2 preferentially induced a gluten-specific response of the T cell lines tested (same as in Fig. 1). The APC were either local B-LCL (CD114, RG, CD280, CD036) or homozygous B-LCL distributed during the 10th or 11th IHWS. (*Hatched bars*) T cell responses (mean of triplicate cultures) in the presence of APC alone. (*Solid bars*) T cell responses to gluten plus APC. (+, -) Presence or absence of the CD-associated DQA1\*0501 and DQB1\*0201 genes. (*N.d.*) Not determined by genomic typing.

**Table 3.** Antigen Specificity of T Cell Lines and TCC

	CD281 30'	CD280 120'	4.19	5.14	5.48	1.27
T cells alone	0,1	0,0	0,0	0,0	0,0	0,0
APC alone	1,0	1,0	0,6	0,7	0,7	0,7
T + APC	1,8	1,4	0,8	0,7	0,8	0,7
T + APC + ICN	<b>24,6</b>	<b>37,2</b>	<b>43,2</b>	<b>26,8</b>	<b>13,3</b>	<b>18,0</b>
T + APC + Sigma	<b>21,7</b>	<b>12,4</b>	<b>23,5</b>	<b>15,8</b>	<b>13,5</b>	<b>15,5</b>
T + APC + CWRS	<b>17,4</b>	<b>29,2</b>	<b>27,1</b>	<b>7,7</b>	<b>12,5</b>	<b>10,7</b>
T + APC + NS	ND	ND	7,7	8,8	9,6	4,6
T + APC + CHW	ND	ND	5,1	7,2	7,4	3,7
T + APC + Kolibri	<b>17,8</b>	<b>23,8</b>	<b>15,9</b>	<b>3,5</b>	<b>9,1</b>	<b>8,1</b>
T + APC + Rye	4,2	1,4	0,6	0,7	1,5	0,7
T + APC + Barley	ND	1,7	0,9	0,7	1,9	0,7
T + APC + Oat	ND	ND	0,7	0,7	0,8	0,7
T + APC + p14	1,8	1,1	0,7	0,7	0,9	0,6
T + APC + p15	1,6	1,3	0,8	0,7	0,9	0,6
T + APC + p16	ND	1,3	0,6	0,7	0,8	0,6
T + APC + p19	1,6	1,1	0,6	0,7	1,0	0,6
T + APC + p209	1,7	1,2	0,6	0,6	0,9	0,6
T + APC + p211	1,8	1,2	0,6	0,6	1,0	0,7
T + APC + p62	1,6	1,3	0,8	0,7	1,0	0,7

Values show mean cpm ( $\times 10^{-3}$ ) of triplicate cultures. SD was usually <10%. Positive values are given in boldface, antigens used were proteins from various cereals, or synthetic peptides covering residues 1-58 from  $\alpha$ -gliadin. ICN, Sigma, CWRS, NS, CHW, and Kolibri denote various wheat varieties, as outlined in Materials and Methods.

of these TCC. Only one gluten-specific TCC (5.26) was inhibited by an anti-DR mAb, but its poor expansion potential made full characterization impossible.

Second, we performed panel studies using a large panel of HLA homozygous 10th and 11th IHWS B-LCL as APC. Some results for four of the TCC appear in Fig. 3. Five of five B-LCL homozygous for the HLA-DR3DQ2 haplotype (carrying DQA1\*0501 and DQB1\*0201 genes in *cis* position) induced strong gluten-specific responses, whereas by and large, this was not observed for B-LCL not carrying this haplotype. No APC being DR5,DQ7 (carrying DQA1\*0501 but not DQB1\*0201) induced a gluten-specific response, whereas APC being DR7,DQ2 (carrying DQB1\*0201 but not DQA1\*0501) induced a weak gluten-specific response of TCC 4.19, but not the other TCC. We also used more than 25 different B-LCL from healthy individuals and CD patients as APC (Fig. 3, Table 4, and data not shown). Also in this case, B-LCL from all donors being DR3,DQ2 induced a gluten-specific response of the TCC.

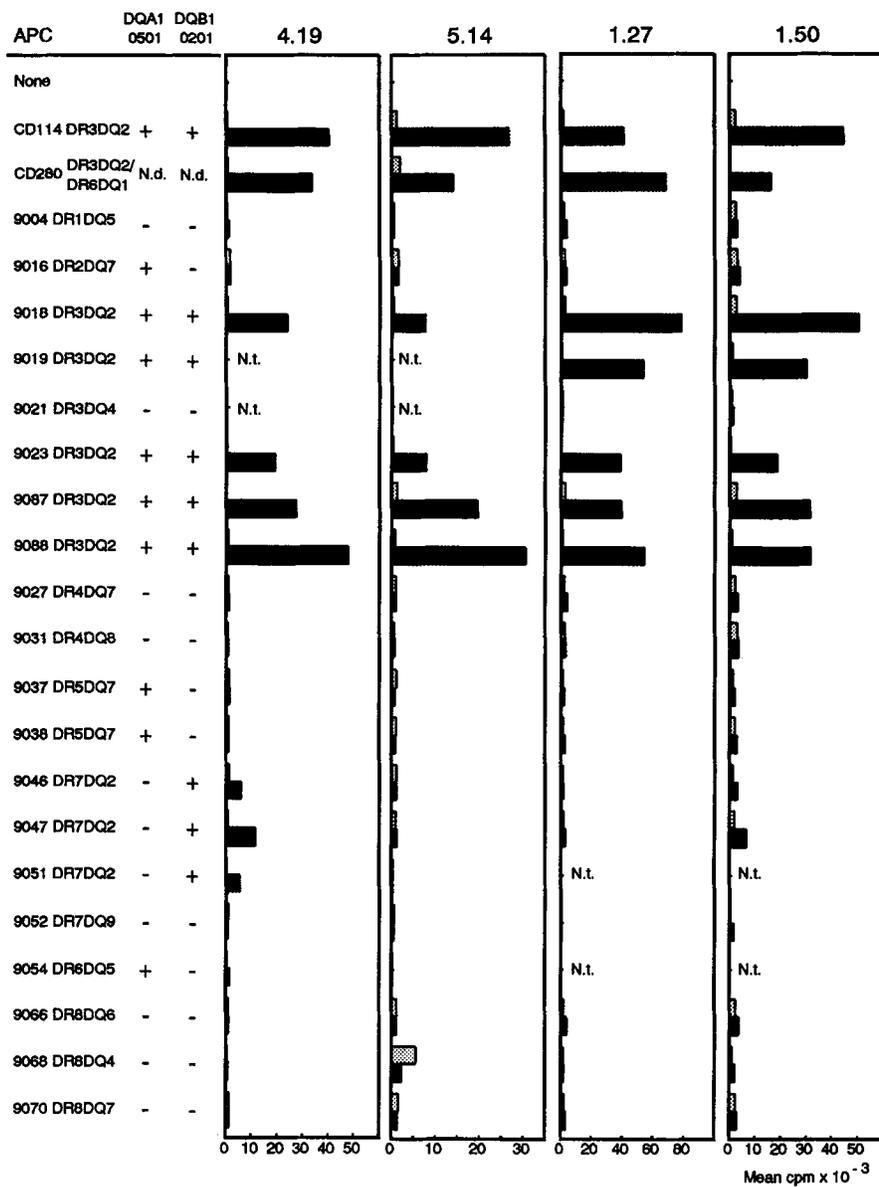
B-LCL from a family involving a DR5DQ7/DR7DQ2 heterozygous CD patient (5) who thus carry the DQA1\*0501 and DQB1\*0201 genes in *trans* position, were then investigated. Three of the TCC (5.14, 5.48, and 1.63) recognized gluten presented by B-LCL from the CD patient, but not

from the father (DR4DQ7/DR5DQ7) or the mother (DR7DQ2/DR8DQ4) (Table 4, and data not shown). This demonstrates that these TCC were able to recognize gluten when presented by the DQ( $\alpha$ 1\*0501, $\beta$ 1\*0201) heterodimer encoded by genes in the *trans* position.

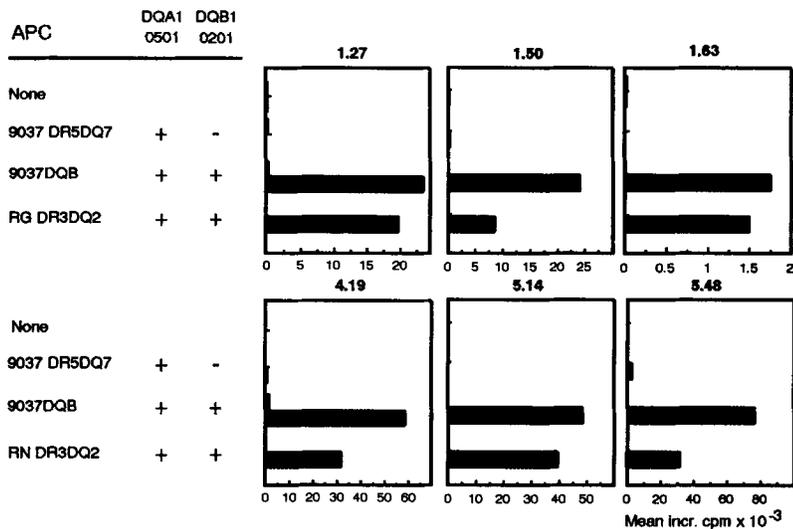
Third, B-LCL being DR5,DQ7 (carrying DQA1\*0501 and DQB1\*0301) which had been transfected with a DQB1\*0201 gene, was used as APC. This transfectant expressed the endogenous DQ( $\alpha$ 1\*0501, $\beta$ 1\*0301) as well as a DQ( $\alpha$ 1\*0501, $\beta$ 1\*0201) heterodimer. All TCC responded to gluten presented by the transfected B-LCL, but not when presented by the original untransfected B-LCL (Fig. 4).

*TCC Recognized Gluten from a Variety of Wheat Cultivars, but Not from other Cereals.* All TCC recognized most of the wheat preparations used, although some formulations were more stimulatory than others (Table 3). All gluten-specific TCC recognized the gliadin moiety of gluten. In contrast, proteins from rye, barley, and oat were not recognized by any of the TCC tested (Table 3). We then examined whether the TCC could respond to any of a panel of seven overlapping peptides covering the NH<sub>2</sub>-terminal 58 amino acid residues of  $\alpha$ -gliadin. Neither the T cell lines nor the clones tested showed any reactivity towards these peptides (Table 3).

*Diversity of TCR Usage.* TCR usage of the gliadin-specific



**Figure 3.** APC from donors carrying both DQA1\*0501 and DQB1\*0201 induce a gluten-specific response of the TCC tested. Legends and data presentation as in Fig. 2. (N.t.) Not tested in this experiment.



**Figure 4.** The gluten-specific TCC recognized gluten presented by a transfectant expressing DQ(α1\*0501, β1\*0201). The 10th IHWS B-LCL No. 9037 (carrying DQA1\*0501 and DQB1\*0301) was transfected with DQB1\*0201, giving the transfectant 9037DQB. (RG and RN) Local B-LCL carrying DQA1\*0501 and DQB1\*0201. (Hatched bars) T cell responses in the presence of APC alone. (Solid bars) T cell responses to gluten in the presence of APC and show mean incremental cpm (experimental mean value – mean of [<sup>3</sup>H]thymidine incorporation of APC alone).

**Table 4.** HLA-DQ ( $\alpha$  1\*0501,  $\beta$  1\*0201) May Present Gluten to TCC When It Is Encoded in Both Cis and Trans

	APC			TCC response				
	HLA-DR	DQA1	DQB1	5.14		5.48		B-LCL alone
		0501	0201	No Ag	With Ag	No Ag	With Ag	
CD280 (Auto)	DR3/6	ND	ND	0.2 ± 0	<b>11.2 ± 2.4</b>	0.1 ± 0	<b>11.1 ± 2.1</b>	0.1 ± 0
CD114	DR3/3	+	+ ( <i>cis</i> )	1.8 ± 0	<b>16.8 ± 1.5</b>	1.3 ± 0.3	<b>35.0 ± 2.6</b>	1.0 ± 0.1
CD052	DR5/7	+	+ ( <i>trans</i> )	0.6 ± 0.2	<b>3.9 ± 0.6</b>	0.5 ± 0	<b>13.7 ± 0.3</b>	0.4 ± 0.1
CD268	DR4/5	+	-	0.7 ± 0.1	1.1 ± 0.7	0.7 ± 0.1	1.4 ± 0.5	0.7 ± 0
CD225	DR7/8	-	+	0.7 ± 0.3	0.5 ± 0	0.6 ± 0	1.7 ± 0.1	0.5 ± 0
No. 9038	DR5/5	+	-	0.3 ± 0	0.3 ± 0	0.4 ± 0.1	0.7 ± 0.4	0.3 ± 0
No. 9047	DR7/7	-	+	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0	0.5 ± 0.1	0.4 ± 0.1

The proliferative response to a peptic-tryptic digest of gluten (2 mg/ml) was investigated. Irradiated B-LCL from local donors or HLA homozygous 10th IHWS B-LCL were used as APC. CD052 denotes a CD patient. CD268 and CD225 denotes her healthy parents (5). Values show mean cpm ( $\times 10^{-3}$ ) of triplicate cultures  $\pm$  SD. Positive values are given in boldface. Only the presence or absence of DQA1\*0501 and DQB1\*0201 is indicated. *Cis* and *trans* denotes the chromosomal location of these genes as carried by the given APC.

TCC was examined by sequencing of TCR  $\alpha$  and  $\beta$  genes. The TCC carried a number of different V $\alpha$  and V $\beta$  segments (Table 5). Two TCC carried two functionally rearranged  $\alpha$  chain genes, it is not known which were expressed. V $\alpha$ 6.2, V $\alpha$ 12.1, V $\alpha$ 22.1, and V $\beta$ 6.7 were all found twice. Apart from this, it was evident that a wide range of T cells with differing TCR variable segments were able to recognize gluten presented by DQ2. We also compared the CDR3 (V-(D)-J) junction of the TCR, and found different sequences here also.

### Discussion

This report shows that gluten-reactive T cells may be isolated from the small intestinal mucosa of CD patients. The major proportion of these T cells was found to be restricted

by the CD-associated HLA-DQ( $\alpha$ 1\*0501, $\beta$ 1\*0201) heterodimer.

Gluten-specific proliferative responses were found in T cell lines established from ex vivo gluten-stimulated biopsies from treated CD patients and propagated in the absence of gluten. Of the six treated patients tested, we succeeded in establishing gluten-reactive T cell lines from three individuals. It may be relevant that those three individuals had an irregular small intestinal morphology (they had not fully recovered on a presumptive gluten-free diet), whereas the three patients from whom we were unable to establish gluten-specific T cell lines had either a normal or nearly normal small intestinal morphology. Most recently, we investigated T cells isolated from biopsies from a CD patient being not DR3, DQ2, but rather DR4,DQ8. We and others (26, 27) have found that the few

**Table 5.** TCR Usage of Gluten-specific TCC Examined

TCC	TCR- $\alpha$			TCR- $\beta$		
4.19	V $\alpha$ 12.1	CALSEKGGYQKVTFG	J $\alpha$ 02 (AG212)	V $\beta$ 1.2	CASSVSPIGPEQYFG	J $\beta$ 2.7
5.14	V $\alpha$ 6.2	CAMRDLKTSYDKVIFG	J $\alpha$ MTC5	V $\beta$ 8.1	CASRSLEWGIGEGQPQHF	J $\beta$ 1.5
5.48	V $\alpha$ 17.1	CALSEAGANSKLTFG	J $\alpha$ 07	V $\beta$ 6.7b	CASSILQGGDTQYFG	J $\beta$ 2.3
	V $\alpha$ 12.1	CAAIHGGSQGNLIFG	J $\alpha$ 06			
1.27	V $\alpha$ 6.2	CAMREGQAGNQAGTALIFG	J $\alpha$ S	V $\beta$ 20.1	CAWSTTGWDTGELFFG	J $\beta$ 2.2
	V $\alpha$ 22.1	CALSDLTDSWGKLVFG	J $\alpha$ RB2			
1.50	V $\alpha$ 20.1	CLVGGNSGGGADGLTFG	J $\alpha$ W	V $\beta$ 6.7a	CASSFRALDVNEQFFG	J $\beta$ 2.1
1.63	V $\alpha$ 22.1	CALSDASQGGSEKLVFG	J $\alpha$ AC9	V $\beta$ 22.1	CATNQRAYNEQFFFG	J $\beta$ 2.1

TCR genes were sequenced using a modified PCR technique (25). The CDR3 region sequences are given from the conserved cysteine residues in V $\alpha$  and V $\beta$  to the conserved FG pair of residues in J $\alpha$  and J $\beta$ . The nomenclature of V $\alpha$ , J $\alpha$ , V $\beta$ , and J $\beta$  is according to Obata and Kashiwagi (50).

CD patients who do not carry DQ( $\alpha 1^*0501, \beta 1^*0201$ ) often instead carry DQ( $\alpha 1^*0301, \beta 1^*0302$ ) (DQ8). This patient had an irregular small intestinal morphology, and also in this case were gluten-specific responses found, but restricted by HLA-DQ8 (Lundin, K. E. A., et al., manuscript in preparation). Thus, an increased number of T cells in the biopsies may well facilitate establishment of gluten-specific T cell lines. In keeping with this notion, the CD biopsy specimens showing higher frequencies of CD25<sup>+</sup> T cells following ex vivo challenge with gluten (13) do also have subtle morphological changes (Halstensen, T. S., et al., unpublished results).

In one case, a weak gluten-specific response was also found for a T cell line from an untreated patient. The main difference in the handling of biopsies from treated and untreated patients is that biopsies from individuals on a gluten-containing diet were not challenged, since many of the T cells in the untreated lesion already are activated. It is conceivable that establishment of gluten-specific T cell lines has a better chance of success using biopsies from treated patients, since the intense immunological activation in ongoing lesions may also harbor many T cells not specific for gluten, but activated nonspecifically. Findings compatible with this view have been reported from studies of allograft rejection (28) and experimental allergic encephalomyelitis in rats (29).

Another explanation for our failure to detect strong proliferative gluten-specific responses in T cell lines from the established lesions may be that gluten-specific T cells could have lost their proliferative, antigen-responsive potential, but still could provide other T cell functions. Such findings have been done for gut-associated T lymphocytes in bacterial infections in primates (30). Further, activated CD4<sup>+</sup> T cells in the lamina propria of untreated CD patients, although they express the CD25 activation antigen, do not express the Ki-67 marker, suggesting that cell division does not take place (31).

Evidence indicates that CD4<sup>+</sup> T cells in the lamina propria play an important role in the development of the celiac lesion (2, 3, 8, 13, 32, 33). However, the antigen specificity and HLA restriction of such T cells have previously not been established. The gluten-specific, HLA-DQ-restricted T cells obtained in this study were most probably derived from the lamina propria, because lamina propria CD4<sup>+</sup>, TCR- $\alpha/\beta$ <sup>+</sup> T cells are specifically stimulated to express CD25 by gluten challenge of biopsies ex vivo (13). Our T cells were first positively selected for their CD25 expression and later shown to be CD4<sup>+</sup>, TCR- $\alpha/\beta$ <sup>+</sup>. Second, there is strong expression of DQ molecules on lamina propria APC, but not on epithelial cells (34), and our T cells were predominantly HLA-DQ restricted. Moreover, the T cells are most likely relevant for CD since only T cells in small intestinal biopsies from CD patients but not from healthy individuals, can be induced to express CD25 by gluten challenge (13).

The HLA association of CD is well-established and is primarily conferred by the DQ( $\alpha 1^*0501, \beta 1^*0201$ ) heterodimer (4–7). By using an extensive panel of HLA homozygous or heterozygous B-LCL as APC, we found that the gluten-specific responses of the T cells studied were restricted by the CD-associated DQ( $\alpha 1^*0501, \beta 1^*0201$ ) heterodimer. Using TCC,

gluten-specific responses were seen when the APC expressed this molecule encoded by DQA1\*0501 and DQB1\*0201 genes in *cis* or in *trans* position. These studies are in agreement with our previous studies using alloreactive TCC recognizing DQ( $\alpha 1^*0501, \beta 1^*0201$ ) (5). Also in the present study, we observed weaker responses of the TCC when the restricting DQ( $\alpha 1^*0501, \beta 1^*0201$ ) heterodimer was encoded by genes in *trans* than in *cis* position. The reason for this could be lower density of the *trans*-encoded molecule, since this appears to correlate with T cell response (35).

The DQ( $\alpha 1^*0501, \beta 1^*0201$ )-restricted, gluten-specific T cells all carried diverse TCR. Thus, we were unable to demonstrate a predominant TCR usage by such T cells, in contrast to what has been found in experimental models of MHC class II-associated diseases in rodents (36). Interestingly, studies so far have failed to show any association between germline TCR polymorphisms and susceptibility to CD (7, 37).

The diversity of the TCR used may indicate that the involved T cells may recognize not a single but rather a range of gliadin peptides. Wheat gliadins consist of  $\alpha/\beta$ ,  $\gamma$ , and  $\omega$  gliadins, all of which may cause disease (38). Considerable interest has been paid to peptides from the NH<sub>2</sub>-terminal region of  $\alpha$ -gliadin, since they have been shown to sustain the pathology of the established CD lesion using organ culture assays with biopsies from untreated CD patients (39, 40). Interestingly, we have recently defined a T cell epitope in this region with DQ( $\alpha 1^*0501, \beta 1^*0201$ )-restricted T cells from the peripheral blood of a CD patient (Gjertsen et al., manuscript submitted for publication). However, neither the TCC nor the TCC lines described here showed reactivity to any of the seven peptides tested. At least two possible explanations for this finding are apparent. First, since there is genetic variability between  $\alpha$ -gliadins (16–18), these findings do not allow us to definitively exclude that the T cells actually recognize peptides from this region. Second, although disease-relevant epitopes can be found in the NH<sub>2</sub>-terminal region of  $\alpha$ -gliadin (39, 40), other regions in both  $\alpha$ -gliadin (39, 41) and other gliadins have been suggested to be implicated in the disease (38, 42–44).

Not only wheat, but also rye and barley, and possibly also oat, have been considered capable of perpetuating CD (1). However, whereas the prolamins of wheat and the other cereals have many similarities, their sequences are generally not identical (45). This may explain why our T cells did not recognize other cereals than wheat.

One of the major uncertainties regarding HLA-associated diseases is the level at which the HLA association is operating (46–48). It has been argued that HLA-DQ molecules should be mainly responsible for shaping the T cell repertoire rather than presenting antigen to mature T cells (48). The present report strongly suggests that HLA-DQ molecules can efficiently present antigenic peptides to T cells in the target organs. With regard to CD, it is conceivable that gluten-specific, HLA-DQ-restricted T cells *in situ* secrete lymphokines which cause the immunopathology. At least two different not mutually exclusive pathways could be involved in this process. IFN- $\gamma$  may cause death of mucosal epithelial

cells (49), and this cytokine could be found in supernatants after activation of the TCC studied (Nilsen, E. M. et al., unpublished results). Alternatively, activated T cells could secrete an unknown lymphokine causing crypt cell hyperplasia (8, 33). Thus, one possible mechanism behind the DQ association in CD may be preferential presentation of gluten

to T cells in the small intestine by the disease susceptibility DQ( $\alpha 1^*0501, \beta 1^*0201$ ) molecule itself. Important questions which remain to be answered are why this particular DQ molecule may serve this function and why not all individuals carrying this particular DQ molecule develop CD.

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