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Characterization of the Anti-Tissue Transglutaminase Antibody Response in Nonobese Diabetic Mice[1]

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Type 1 diabetes mellitus is an autoimmune disorder characterized by destruction of insulin-producing pancreatic β cells by T lymphocytes. In nonobese diabetic (NOD) mice, a role has been hypothesized for dietary gluten proteins in the onset of diabetes, and because gluten dependence is the major feature of celiac disease, together with production of Abs to the autoantigen tissue transglutaminase (tTG), we looked for the presence of anti-tTG Abs in the serum of NOD mice and, to establish their origin, analyzed the Ab repertoire of NOD mice using phage display Ab libraries. We found significant levels of serum anti-tTG Abs and were able to isolate single-chain Ab fragments to mouse tTG mainly from the Ab libraries made from intestinal lymphocytes and to a lesser extent from splenocytes. Data from NOD mice on a gluten-free diet suggest that the anti-tTG response is not gluten-dependent. The intestinal Ab response to tTG is a feature of NOD mice, but the underlying mechanisms remain obscure. *The Journal of Immunology,* **2005, 174: 5830–5836.**

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Nonobese diabetic (NOD)[3] mice develop spontaneous autoimmune disorders including insulin-dependent or type 1 diabetes mellitus (T1DM), Sjögren's syndrome, systemic lupus erythematosus, and arthritis (1). T1DM, the most characterized NOD disease, is the result of selective destruction of insulin-producing pancreatic β cells by autoimmune T lymphocytes (2). Failed tolerance of T cells has been associated with up to 19 genetic loci (1) although a major role is played by the I-A^{g7} MHC class II haplotype (3). Furthermore, B lymphocytes were shown to be critical in the breakdown of tolerance, suggesting that their ability to present diabetic Ags to T cells as APCs (3–5) may be important in pathogenesis, a concept strengthened by the observation that NOD mice have early naive self-reactive B cell clones which recognize T1DM Ags (6). Environmental factors are commonly considered triggers of autoimmune processes and NOD mice have been evaluated for many possible risk factors including wheat gluten, the withdrawal of which from the diet delays the onset of T1DM (7).

Human celiac disease (CD), or gluten-sensitive enteropathy, is thought to be caused by a disordered mucosal immune response to wheat gliadin and related prolamines of other toxic cereals (8). It affects genetically susceptible individuals, with HLA genes playing a major role (9). Autoimmunity is a recognized feature of CD; in fact, disease-specific Abs have long been shown to be directed against components of the extracellular matrix, recently identified as tissue transglutaminase (tTG) (10), an enzyme catalyzing Ca^{2+}-dependent cross-linking between glutamine residues in peptides and polyamines, and primary amino groups. In addition to cross-linking, tTG can modify proteins by amine incorporation and deamidation, as well as by acting as an isopeptidase in a Ca^{2+}-dependent manner (11). Ab levels against gliadins and tTG in CD patients increase upon exposure to glutens, and decrease on a gluten-free diet. Moreover, CD appears to be closely associated with a series of additional autoimmune conditions, such as T1DM, thyroiditis, myasthenia, autoimmune hepatitis, and cerebellar ataxia (12).

In a recent work, we reported the production and analysis of phage Ab libraries obtained either from PBLs or from lymphocytes infiltrating the intestinal mucosa of three CD patients (13). We were able to isolate single-chain Ab fragments (scFvs) to tTG from all intestinal lymphocytes libraries but from none of those obtained from PBLs. By way of contrast, Abs against gliadin were obtained from all libraries, indicating that the humoral response against tTG occurs at the local level, whereas the anti-gliadin response occurs both peripherally and centrally.

Phage Ab libraries have been widely used to investigate the Ab response in other human autoimmune diseases such as thyroid disease (14), systemic lupus erythematosus (15), Sjögren's syndrome (16), paraneoplastic encephalomyelitis (17), myasthenia gravis (18), idiopathic thrombocytopenic purpura (19), and T1DM (20), and the specificities of selected Abs mirror those in the serum. Cloned Abs, specific for autoantigens, have been sequenced to examine the possibility of preferential V gene usage, and somatic mutation, supporting the idea that the response in patients is Ag driven.

Because anti-tTG autoantibodies are a major feature of CD, with their appearance directly linked to gluten exposure, and T1DM in NOD mice can be delayed by gluten withdrawal, we looked for the presence of anti-tTG Abs in the serum of NOD mice and related it

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[3] Abbreviations used in this paper: NOD, nonobese diabetic; T1DM, type 1 diabetes mellitus; CD, celiac disease; tTG, tissue transglutaminase; scFv, single-chain Ab fragment; h tTG, human tTG; m tTG, mouse tTG; CDR, complementarity-determining region; SPL, spleen; INT, intestine.

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to gluten exposure. The origin of these Abs was analyzed using phage Ab libraries. In the present work, we describe the production of phage Ab libraries from the splenic and intestinal lymphocytes of NOD mice and their screening for reactivity to tTG as well as the diabetes autoantigen IA-2 (tyrosine phosphatase).

Materials and Methods

Animal strains

The studies were performed on female NOD and BALB/c mice purchased at the age of 3–4 wk from Charles River. Experiments were also performed on NOD mice from a colony reared and maintained on a gluten-free diet (GFD). GFD (Mucedola) was prepared with potato flour, peanut powder, dry nonfat milk, and soy oil. All mice were maintained under strict pathogen-free conditions and had free access to drinking water.

Plasmids and bacteria

DH5αF′ (*F′/endA1 hsdR17* (r^{K-} m^{K+}) *supE44 thi-1 recA1 gyrA* (*Nal^r*) *relA1 D* (*lacZYA-argF*)*U169 deoR* (*F80dlacD*(*lacZ*)*M15*) was used for phage propagation; HB2151 (*K12, ara* Δ(*lac-pro*)*, thi/F′ proA*$^+$*B*$^+$, *lacI^q*ZΔ*M15*) was used to make soluble scFv. pDAN5 (21) was used for scFv display. Molecular biology enzymes were purchased from Invitrogen Life Technologies, New England Biolabs, or Promega.

Ags

Human tTG (h tTG) was produced as previously described (22). The mouse tTG (m tTG) gene was obtained by amplifying cDNA from an intestinal specimen with specific primers (the restriction sites are underlined): Mttg-sense AGC TCG CTG CAG GTA TGG CAG AGG AGC TGC TCC TG and Mttg-antisense GAA GCG AAT TCT TAG GCC GGG CCG ATG ATA AC, and cloned as *Pst*I-*Eco*RI fragment into pTrcHisB.

Human IA-2 (aa 630–976) was amplified with primers: IA-2 sense GCA TGC TCG AGC GAC CTG TGC CGC CAG CAC ATG G and IA-2 antisense GA AGC GAA TTC TCA CTG GGG CAG GGC CTT GAG and cloned as *Xho*I-*Eco*RI fragment into pTrcHisB. Protein purification was performed as described in Ref. 23. The protein fractions were controlled by ELISA using scFv, mAbs, and human sera essentially as reported below for other ELISA. Purified α-gliadin was prepared as described (24).

Lymphocyte RNA preparation and library construction

Total RNA was prepared as in Ref. 25 from biopsied tissues from NOD mice treated directly. Synthesis of cDNA was performed using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). Ig V-regions were amplified using the specific V-region primers described in Ref. 26.

Selection and testing of phage Abs

The rescue of phagemid particles was as described in Ref. 27. Panning was performed by adding phages diluted to 2% nonfat milk in PBS (MPBS) to immunotubes (Nunc) coated with purified h tTG, m tTG, human IA-2 and α-gliadin (10 μg/ml), washing 20 times with PBS, 0.1% Tween 20 (PBST) and 20 times with PBS, followed by elution with 1 ml of *Escherichia coli* cells at 0.5 OD_{600} for 30′ at 37°C. The panning procedure was repeated up to three times. After selection, 96 individual clones from each selection were rescued by helper phage and screened for reactivity to their respective Ags as well as irrelevant Ags by microtiter plate ELISA (27). Briefly, phagemid from culture supernatant were diluted 1/1 with 4% MPBS, added to microtiter plate wells and detected with an anti-M13 peroxidase-conjugated mouse mAb (Amersham Biosciences). scFv 2.8 previously isolated (13) and mAb CUB 4702 (Neomarkers) were used as positive controls.

Characterization of Ab isotype

A single scFv reactive to tTG (clone G6-INT) was sequenced and the complementarity-determining region 3 (CDR3) used to design the sense primer: GCT AGG GAT TAC GGG AGC. The antisense primers specific for mouse Ab classes IgA, IgG, and IgM located at the C terminus of the CH2 domain were: GTG CCA GAC TCA GGA TGG for IgA, GAT GGT TCT CTC GAT GGG for IgG and TCT TCA AGA AGG TGA GAC C for IgM. Primer pairs were used to amplify spleen and intestine cDNAs from the NOD mouse used for the construction of the scFv libraries to selectively reveal the presence of clone G6-INT like Abs with different constant regions belonging to the three Ab isotypes.

Assay of anti-transglutaminase NOD Abs

NOD and BALB/c mouse sera were collected and anti-tTG IgG and IgA Abs were assayed by ELISA. Briefly, serum dilutions (1/100) were added in duplicate to wells coated with m tTG (10 μg/ml). The presence of Abs was detected with biotin-conjugated rabbit anti-mouse IgG Abs (Sigma-Aldrich). After adding streptavidin-peroxidase complex (Sigma-Aldrich), the reaction was developed with 1 mg/ml *o*-phenylenediamine/HCl and 0.06% H_2O_2 as substrates. Results were expressed as OD at 490 nm.

Immunohistochemistry

Phage immunohistochemistry was performed on histological sections of mouse muscle prepared according to standard techniques. Phage-scFv were added to the sections, incubated for 30′ at room temperature in a moist chamber, followed by peroxidase-conjugated mAb anti-M13 and diaminobenzidine (DAB) as substrate.

Fragments of NOD mouse proximal jejunum were embedded in OCT compound (Tissue Tek, Miles Laboratories), frozen in isopenthane chilled in liquid nitrogen and stored at −170°C. Cryostat sections (5 microns thick) were placed on aminopropyltriethoxysilane-coated slides, allowed to dry in air overnight and then fixed in acetone for 30 min at 4°C. Indirect immunodetection using mouse mAbs could not be used in immunohistochemical studies of murine tissues without severe background staining. To detect tissue transglutaminase in jejunum cryosections, we performed a modification of this method in which mouse mAb CUB 7402, recognizing both human and mouse tTG, was used.

Before application to the section, mouse IgG1 mAb CUB 7402 (CovalAb), diluted 1/200 in TBS and biotinylated rabbit anti-mouse IgG1 serum (BD Pharmingen), diluted 1/25 in TBS, were allowed to complex in vitro. After blocking of the unbound secondary antiserum with mouse IgG1 negative control (Serotec), diluted 1/50 in TBS, standard immunohistochemistry was performed. Slides were incubated in a humid chamber with the in vitro complex for 1 h at room temperature, and then, after washing, incubated with streptavidin-HRP peroxidase-conjugated (DAKO) diluted 1/500 in TBS for 30 min at room temperature. Finally, two washings with TBS, pH 7.4, were performed, and the color (brown-orange) was developed using freshly prepared substrate solution consisting of 0.05% 3-amino-9-ethylcarbazole (Sigma-Aldrich) and 0.3% H_2O_2 in 50 mmol/L sodium acetate buffer, pH 5.0. The reaction was stopped in water after 3 min, and the sections were slightly counterstained in Mayer's hemalum (Sigma-Aldrich).

Results

Antigens

To analyze the anti-tTG response of NOD mice, m tTG was cloned into pTrcHisB and subsequently expressed. The protein was purified by metal affinity chromatography and analyzed by electrophoresis and Western blotting, revealing a high degree of purity (Fig. 1). The immunoreactivity of the protein fraction was assayed by ELISA using sera from 10 CD patients, CUB 4702, an anti-tTG mAb, and scFv (clone 2.8) cross-reactive to rodent tTG isolated in a previous study (13). Human IA-2, considered to be a specific Ag

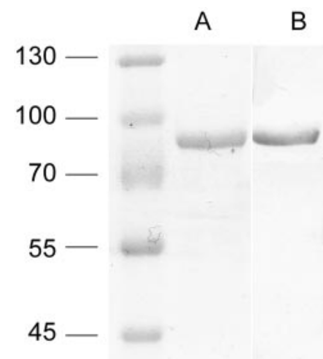

FIGURE 1. SDS PAGE of recombinant purified m tTG. *A*, Blue Coomassie-stained gel. *B*, Western blotting of the same sample. Primary Ab, mAb CUB 4702; secondary Ab, goat anti-mouse Ig conjugated with alkaline phosphatase.

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of the corresponding autoimmune response in NOD mice (28), was cloned and expressed as described for mouse tTG to provide a reference Ag. The ELISA was performed on the IA-2 purified fraction using human sera from 10 diabetic patients. The results are reported in Table I. The mouse tTG was well-recognized by all the human sera (OD >0.5) as well as CUB 4702 and the scFv to h tTG cross-reactive to rodent tTG (OD >0.5). The IA-2 fraction was challenged with sera from 10 diabetes patients and from controls, and showed good levels of sensitivity and specificity with 7 sera of 10 with an OD >0.5. Both proteins were deemed suitable for further analysis.

NOD serum Abs to tTG

To investigate the anti-tTG response in the NOD mouse model, 23 (IgG) and 26 (IgA) serum samples from NOD mice were assayed on m tTG by ELISA. Almost all the sera from NOD mice tested showed an appreciable response to the Ag with some sera with an OD value higher than 1, whereas all 6 (IgG and IgA) BALB/c control mice showed little or no response. This finding, reported in Fig. 2, suggested the need to further analyze the nature of the Ab response with particular emphasis to the site of synthesis of the Abs to tTG, using phage display Ab cloning and selection.

Library construction

The scFv libraries were constructed from either splenic (SPL) lymphocytes or intestinal (INT) lymphocytes from a 20-wk-old NOD mouse. V_H and V_L chains were amplified from SPL and INT cDNA by PCR using a set of oligonucleotides that recognize all mouse V genes (see *Materials and Methods*). V_H and V_L amplificates were assembled by PCR and cloned into the phagemid vector pDAN5 (21) to obtain the primary libraries. The two libraries ranged in size from around 5×10^6 to 10^7, and 20 clones picked at random from each were shown to contain full-length scFv with a different *Bst*NI restriction pattern, confirming the diversity and integrity of the libraries. The libraries are here reported as NOD 1 (from SPL) and NOD 2 (from INT). For reference, two libraries were also made from the splenic (BALB/c 1) and intestinal (BALB/c 2) lymphocytes of a BALB/c mouse. These two libraries were compared with those derived from the NOD mouse.

Library selection

Abs were affinity-selected on purified recombinant m tTG and human IA-2. After every cycle of selection, the eluted phages were reamplified for the next cycle. After the second and third cycles, 96 individual clones were assayed using phage ELISA for the Ag used for the selection. Positive clones were analyzed by *Bst*NI fingerprinting and sequencing to determine the number of different clones, and these results are reported in Table II. Interestingly enough, after the second round, 3 different scFv to m tTG were obtained from the INT library, and 2 clones to IA-2 were isolated from the SPL library after three rounds. No positive clones to IA-2 were obtained from the INT library even after three cycles of selection, whereas 2 clones to m tTG were obtained from the SPL library only after the third round. This result suggests that anti-tTG Abs were more common in the INT library than in the SPL one. The failure to select anti-IA-2 Abs from the INT library indicates that failure was not due to lack of efficiency in isolating rare scFv but rather to the lack of such Abs in the library. Although it was possible to select anti-tTG Abs from the NOD libraries, they represented only 4% (SPL library after three rounds) to 6% of scFvs (INT library after two rounds) tested. This is in contrast to selection from intestinal CD libraries, when almost 30% of positives, on an average, are seen after only a single round (13). This indicates that although Abs against tTG are produced in the NOD intestine and spleen, they appear to be at far lower levels than those found in CD. No scFv to either IA-2 or tTG were selected from the BALB/c libraries. The libraries were also selected for anti-α-gliadin Abs and 2 and 4 clones were isolated only from the spleen libraries of the NOD and control mouse, respectively. This result is also in agreement with the CD libraries (13), with no significant presence of anti-α-gliadin clones in the libraries from intestinal lymphocytes.

Table I. *Reactivity of mAb, scFv, and human sera to cloned Ags tested by ELISA*

Sample	h tTG	m tTG	Human IA-2
CUB 4702	+[a]	+	−
scFv tTG 2.8	+	+	−
CD sera	+	+	−
T1DM sera	−	−	+

[a] + = OD > 0.5.

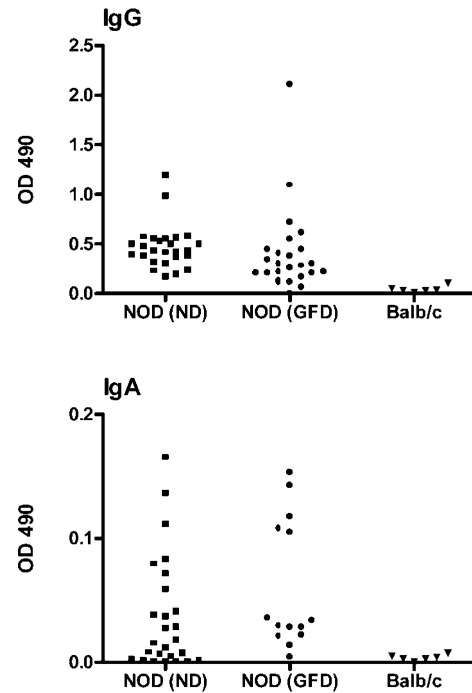

FIGURE 2. ELISA of sera from NOD mice on normal gluten-containing diet (ND) or on gluten-free diet (GFD) and from BALB/c mice for IgG (*upper*) and IgA (*lower*) to m tTG. Secondary Ab, goat anti-mouse Ig conjugated with peroxidase. Data represent mean values of experiments in triplicate.

V family usage and somatic mutations of anti-transglutaminase scFvs

To investigate the genetic origins of the selected scFv to tTG, the Ab V chain genes were sequenced and the V_H and V_L families were assessed together with the specific V genes (Table III, columns 3 and 7) by screening against the IMGT, the international ImMunoGeneTics database (⟨http://imgt.cines.fr⟩) (29). The amino acid sequence of the complementarity determining regions (CDR3) are reported in columns 6 and 10, whereas the numbers of mutations determined by comparing the V sequence to the closest germline sequence are reported in columns 4 and 8. As far as the V_H/V_L pairing is concerned, it is interesting to note that the same V_H (scFv A11 and G6) is paired with different V_Ls and the same

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Table II. *Abs to mtTG and human IA-2*

Library	Source[a]	Tested	m tTG Round[b]	m tTG Positive	m tTG Different[c]	Human IA-2 Round	Human IA-2 Positive	Human IA-2 Different	Gliadin Round	Gliadin Positive	Gliadin Different
NOD 1	SPL	96	3	4	2	2	2	2	3	4	2
NOD 2	INT	96	2	6	3	3	0		3	0	
BALB/c 1	SPL	96	3	0		3	0		3	0	
BALB/c 2	INT	96	3	0		3	0		3	6	4

[a] Ab libraries from SPL or INT lymphocytes of NOD and BALB/c mice.
[b] Rounds of affinity selection.
[c] Number of different clones determined by *Bst*NI fingerprinting and sequencing.

V_Ls (scFv D9 and G6) are paired with different V_Hs. Because V_H and V_L regions are assorted randomly during Ab library construction, it is likely these coselected V_H/V_L pairs represent cases where either H or L chain can provide binding specificity, and the combination of these chains may mirror the in vivo pairing.

As for the difference with respect to the germlines, all the scFv V genes were shown to be highly mutated. In particular, major differences were seen in the V_H CDR3 region, which is created by the juxtaposition of D_H and J_H segments as well as by removal and addition of nucleotides by exonucleases and TdT. In Fig. 3, the mutations with respect to the germline of the V_H chain of clone G6 are reported for reference.

Ab class identification of the scFv to tTG

As the Ab constant regions are lost during scFv amplification and assembly, we set up a specific test to characterize the Ab classes expressed as constant regions in a specific Ab to tTG. To this purpose, the clone G6-INT was taken as reference and its V_H CDR3 sequence used to design a sense primer for specific amplification of the sequence of this particular Ab. This clone was chosen because of the low number of CDR3 AT nucleotides, which may affect the specificity of recognition of the cDNA template. The antisense primers were designed to selectively recognize sequences at the C-terminal end of mouse IgA, IgG and IgM isotypes. All the oligo pairs were first tested on cDNA from intestine and spleen of wild-type mice with no evident amplificate (not shown). When the same cDNA from the intestine of the NOD mouse used in the phage library construction was used, two PCR bands for IgA and IgM were revealed in electrophoresis (Fig. 4). The two bands corresponded to the molecular mass predicted for the amplificates. A similar result was obtained using the cDNA from the NOD spleen, except that an additional product was also obtained for IgG. In general, these results demonstrate that the very same anti-tTG Ab undergoes isotype switching in both intestine and spleen. In addition, the IgG isotype is present only in the spleen, whereas, to judge from the intensity of the electrophoretic bands, the IgA seems more concentrated in the intestine, as expected in the intestinal secretory epithelium.

Reactivity of phage ScFv to tTG by ELISA and immunohistochemistry

Phage-scFv were derived from the INT clones recognizing m tTG, and the reactivity to cloned mouse and human tTG was tested by ELISA (Fig. 5). All the scFv were shown to be able to recognize m tTG, but neither native human or denatured mouse tTG, despite the high homology between the two molecules. This was interpreted as indicating the possible recognition of a conformational epitope, the structure of which is lost upon denaturation. In fact, mAb CUB 4702, that recognizes a linear epitope, showed only a limited reduction in Ag recognition. This feature, shared by all the scFv, was also shown by the scFv to tTG isolated from human intestinal lymphocytes, as reported above. The reactivity to m tTG was also analyzed by phage immunohistochemistry on mouse muscle histological sections, revealing a recognition pattern typical of the tTG distribution in this tissue (Fig. 6).

Immunostaining of tTG

We analyzed the presence and distribution of tTG in histological sections of NOD mouse proximal jejunum by immunodetection with an immunocomplex formed by mAb CUB 7402 preincubated with biotinylated rabbit anti-mouse IgG Abs (see *Materials and Methods*). This method allowed us to reduce the nonspecific staining of NOD Abs present in situ in the histological section by the secondary anti-mouse Abs. We found strong expression of tissue transglutaminase in the subepithelial basal membrane (Fig. 7), while a moderate staining seemed to be associated with the extracellular matrix. There was also a strong staining at the pericryptal and muscularis mucosae level. No differences were noted in both overall morphology and tTG (expression and/or distribution) between NOD and BALB/c mice (not shown).

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Table III. *V family usage and somatic mutations of anti-tTG Abs: summary of the features of five scFv selected against tTG*

No.[a] Source[b]	No. Clones	V_H Gene[c]	Mutation[d]	CDR3 Length[e]	CDR3 Sequence[e]	V_L Gene[c]	Mutation[d]	CDR3 Length[e]	CDR3 Sequence[e]
A11-INT	1	*IGHV 14S2*01*	9	5	DYGRY	*IGKV 6–23*01*	18	9	EQYSSSPLT
D9-INT	4	*IGHV 1S136*01*	36	12	YGSSHAGYFDVW	*IGKV 3–10*01*	5	9	QQNNEDPWT
G6-INT	1	*IGHV 14S1*01*	9	5	DYGSY	*IGKV 3–10*01*	5	9	QQNNEDPWT
H1-SPL	1	*IGHV 1S61*01*	7	9	ETKTGTFDY	*IGKV 2–109*01*	10	9	AQMLKFPRT
G7-SPL	1	*IGHV 2S1*01*	15	8	MVTWYFDV	*IGKV 6–13*01*	9	9	QQYGTSPLT

[a] Reference numbers of selected clones.
[b] Ab libraries from SPL or INT lymphocytes of NOD mouse.
[c] V_H and V_L family and gene segment.
[d] Number of mutations with respect to the germ line.
[e] Length and sequence of CDR3.

```
1                5                    10                   15                   20                   25
CAG GTG AAG CTG CAG CAG TCA GGG GCT ... GAG CTG GTG AGG CCT GGG GCT TCA GTG ACG CTG TCC TGC AAG GCT
G-- --C C-- --- --- --- --T --A C-- ... --- --- --- -A- --- --- --- --- --- -A- A-- --- --- --- ---

    ___________________ CDR1_______________________
                 30                   35                   40                   45                   50
TCG GGC TAC ACA TTT ACT GAC TAT GAA ... ... ... ... ATG CAC TGG GTG AAG CAG ACA CCT GTG CAT GGC CTG
--T --A --- --- --C --- --- --- -TT ... ... ... ... --- --- --- --- --- --- -AG --- -G- --G --- --T

                       __________________ CDR2 _________________
                 55                   60                   65                   70                   75
GAG TGG ATT GGA GCT ATT GAT CCT GAA ACT GGT GGT ACT ... ... GCC TAC AAT CAG AAG TTC AAG ... GGC AAG
--- --- --- --- TA- --- T-- --- T-C -A- -A- --- --- ... ... -AG --- -C- G-- --- --- --A ... --- ---

                 80                   85                   90                   95                   100
GCC ATA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC ATG GAG CTC CGC AGC CTG ACA TCT GAG GAC TCT GCC
--- -C- --- --- TT- --- --- --- --- --- --- --- --- --- --T --- A-- --- --- --C --- --- --- --- A-G

                     ________________________________CDR3_______________
             104                                                          118
              C   T   I   Y   G   S   S   H   A   G   Y   F   D   V   W   G   T   G
GTC TAT TAC TGT ACA ATA TAC GGT AGT AGT CAC GCT GGG TAC TTC GAT GTT TGG GGC ACA GGG
--- --- ---
```

FIGURE 3. The nucleotide and deduced amino acid sequences of the V_H chain of the scFv clone G6 to tTG is compared with the putative germline sequence of the mouse gene IGHV 14S1*01. All the differences are reported in the lower line only at the positions where they differ from the germline V_H chain.

Effect of GFD

To investigate the role of dietary gluten in the production of anti-tTG autoantibodies, sera were obtained from NOD mice from a colony maintained for several generations on GFD. Serum levels of both IgG and IgA anti-tTG Abs were comparable to those measured in NOD mice on a normal gluten-containing diet, but significantly higher than those present in the serum of BALB/c mice (Fig. 2).

Discussion

Much evidence suggests that the gut immune system plays a crucial role in the pathogenesis of T1DM. In humans, glutamic acid decarboxylase (GAD)-reactive T cells express gut-associated homing receptors (30) and T cells derived from human diabetic pancreas show mucosal homing properties (31). Similar observations have been made in NOD mice in which lymphocytes expressing mucosal adhesion molecules were found to infiltrate the pancreatic islets (32). Very recently, evidence has been produced indicating that initial priming of diabetogenic T cells takes place in the gut, whereas pancreas-draining lymph nodes may serve as an amplification site of the autoimmune response (33). The involvement of the mucosal immune system is accompanied by signs of inflammation in the structurally normal intestine of patients with T1DM, consisting of enhanced expression of DR and DP HLA molecules in the villous epithelium and crypts (34). In a parallel way inflammatory signs have been noted in the small intestine of NOD mice[4] in which enhanced expression of class II histocompatibility molecules on the intestinal epithelium suggests mucosal hyperexpression of proinflammatory cytokines with a higher expression of mRNA for IFN-γ. At the same time a disordered immune response to gliadin is also a feature of T1DM. We have recently shown that a subset of T1DM patients react to rectal instillation of gliadin with lymphocyte infiltration (35) and that in T1DM the small intestinal mucosa shows signs of T cell activation when challenged in vitro with gliadin (36). These data appear even more interesting in consideration of the strong linkage between CD and T1DM. CD is characterized by an aberrant mucosal immune response to wheat gliadin, but also by association with other autoimmune diseases; tTG autoantibodies represent one of the hallmarks of the disease, most evidence suggesting that they are primarily produced at the gut level (13). In the effort to define the links between CD and T1DM more precisely, and with the hope that NOD mice could represent an animal model, so far lacking, for CD, we decided to investigate the anti-tTG response in NOD mice, focusing our attention on the site of their production. In this paper, we show that NOD mice have significant amounts of tTG autoantibodies in their serum and that those autoantibodies are synthesized in both spleen and intestine.

In fact, the selection of Abs from the two NOD mouse libraries originating from spleen and intestinal lymphocytes on purified tTG resulted in the isolation of 2 scFv clones after three rounds of selection from the spleen library and 3 clones after only two rounds from the intestinal one. This suggests an increased presence at the intestinal level of B lymphocytes synthesizing anti-tTG Abs, because the spleen library needed more rounds of selection to enrich the specific Abs. These findings are similar to those obtained in CD (13), where we also found that anti-tTG Abs are mainly synthesized at the intestinal level. Although it was impossible to analyze Ab synthesis in the spleens of CD patients, no anti-tTG scFvs could be isolated from Ab libraries derived from PBLs. In the present study, we also found serum Ab reactivity to tTG in almost all the NOD mouse sera analyzed, thus ruling out the possibility that the phage Ab libraries might be biased by sampling from the occasionally positive NOD mouse. Furthermore, the extensive somatic mutations found in the V regions of all the isolated

[4] F. Maurano, G. Mazzarella, D. Luongo, R. Stefanile, R. D'Arienzo, M. Rossi, S. Auricchio, and R. Troncone. Small intestinal enteropathy in NOD mice fed a gluten-containing diet: a model for coeliac disease?

FIGURE 4. PCR amplificates of cDNAs from intestine and spleen of a NOD mouse, using primers specific for the scFv A11 and Ab isotypes A, G, and M.

FIGURE 5. Phage ELISA of three scFv from NOD mouse intestine (INT) and two scFv from NOD mouse spleen (SPL) isolated on mouse tTG, one human scFv (H 2.8) to human tTG, cross-reactive to rodent tTG and mAb CUB 4702 to tTG. Secondary Abs, anti-M13 peroxidase-conjugated monoclonal mouse Ab for the phage-scFvs and goat anti-mouse Ig conjugated with peroxidase for mAb CUB 4702. D-mouse tTG, denatured mouse tTG. Data represent mean values ± SEM of experiments in triplicate.

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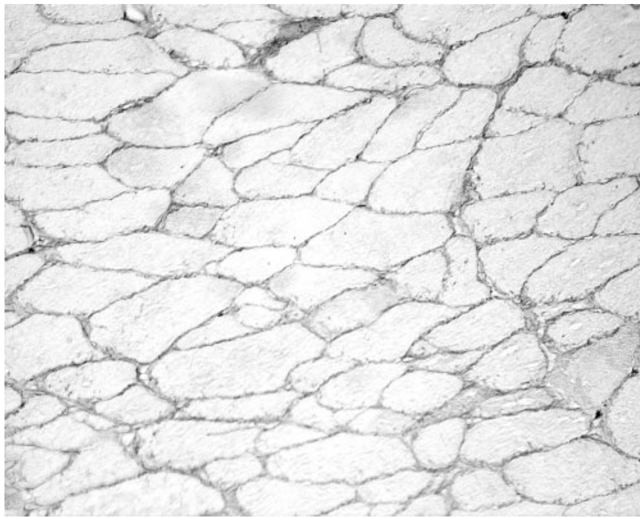

FIGURE 6. Immunoreactivity of phage scFv (clone G6-INT) on histological sections of mouse muscle. Secondary Abs, anti-M13 peroxidase-conjugated mouse mAb.

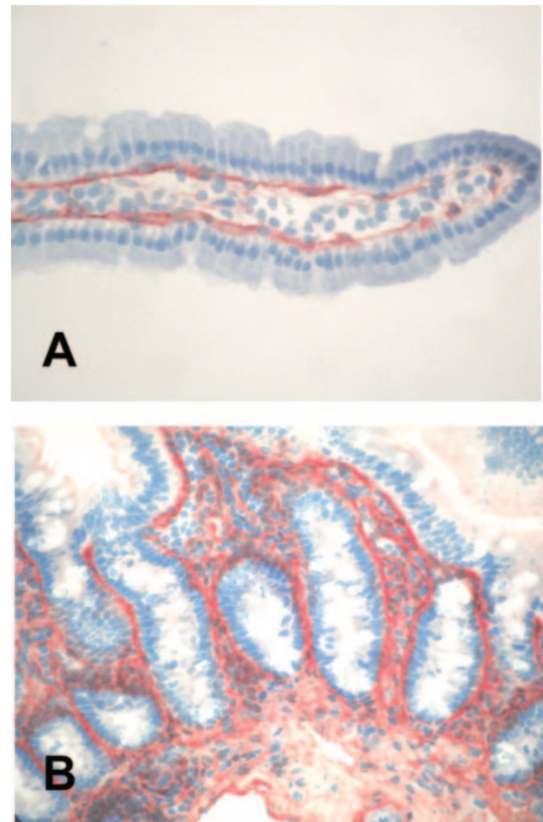

FIGURE 7. Immunolocalization of tTG on histological section of NOD mouse proximal jejunum. 5A, villus; 5B, cryptae. Primary Ab, mAb CUB 7402 to tTG. Secondary Ab, rabbit anti-mouse IgG and peroxidase-anti-peroxidase mouse complex isotype IgG1.

Abs indicate clonal proliferation of a highly selected B cell population, and is consistent with an Ag-driven process, supporting a role for early Ag stimulation by tTG. The complex immunological process controlling the production of these Abs is confirmed by the analysis of the V_H chain of the clone G6, taken for reference, the isotype switching of which involves all three of the Ig classes considered, although the two lymphocyte populations seem to undergo differing regulation, because synthesis of IgG was shown to occur in the spleen only. We also examined the characteristics of the scFv, bearing in mind that this approach can have the drawback that V_H-V_L pairings occurring in vivo are not usually recreated in vitro, because V_H and V_L genes are paired at random. However, the limited number of scFv clones isolated and the crossed pairing of some of the V chains to form functional scFv suggest that they are derived from an oligoclonal Ab response, and it seems reasonable to suppose that the in vitro pairings reflect those occurring in vivo. This hypothesis is somewhat strengthened by the fact that all the scFv have the same pattern of recognition of m tTG, h tTG, and denatured m tTG. We obtained a similar result in phage Ab libraries derived from CD patients where we found that the isolated scFv recognized a conformational epitope sensitive to denaturation (23).

The main result of this study is the demonstration of significant titers of anti-tTG Abs in NOD mice. Although this finding is considered a hallmark of CD, it is not completely disease-specific. As a matter of fact, titers of anti-tTG Abs have been reported to be raised in other conditions, including inflammatory bowel diseases and autoimmune liver disease (37), although their level appears to be far lower than that found in CD. It is likely that some properties of these Abs may be different in different clinical situations; in CD, for example, a preferred use of some V_H genes has been reported (13) and preliminary data from our laboratory suggest the presence of anti-tTG Abs, but with a different V_H gene use in the intestinal mucosa of type 1 diabetes patients. The other important aspect of the anti-tTG response is the dependence upon gluten. Although there is no doubt that anti-tTG Abs in CD patients are closely related to gluten exposure, the specific role of gluten in the production of anti-tTG Abs in NOD mice remains to be clarified. The presence of tTG autoantibodies of comparable levels in the serum of NOD mice from a colony reared on GFD appears to question the gluten dependence of the phenomenon. For CD the most accepted hypothesis to explain the anti-tTG response is that gliadin-specific T cells drive such a response. Gluten peptides are a natural substrate of tTG and in some circumstances, tTG can bind covalently with gluten peptides forming complexes (11) (38). In CD, it has been suggested that B cells expressing anti-tTG Abs internalize and process these complexes, thereby acting as APCs, in the context of HLA DQ2 (DQA1*0501-DQB1*0201) or DQ8 (DQA1*0301-DQB1*0302) (39). If in NOD mice the mucosal T cell response to gliadin is not essential, as indicated by the data on mice on GFD, it is possible that alternative Ags, perhaps critical for NOD autoimmunity, may be complexed with tTG and drive the anti-tTG autoantibody response.

The interesting aspect is that, similarly to CD, the main site for the production of anti-tTG Abs is the small intestinal mucosa, this finding suggesting once again a local immunoregulatory defect. In NOD mice the inflammatory state of the small intestine could also in this case favor the local production of autoantibodies.

The question remains whether the anti-tTG response in NOD mice may be one of the factors in the onset of T1DM. Several studies have indicated that NOD B lymphocytes play a major role in the pathogenesis of T1DM, being required for activation of diabetogenic T cells (40) through Ig-mediated capture of T1DM-related Ags. In particular, Noorchashm et al. (3) have shown that Ag presentation by B cells is mediated by I-A^{g7} MHC class II. The existence in NOD mice of early autoimmune B cells, which may act as APCs for autoimmune Ags (6) and the finding that B cells expressing anti-insulin Abs contribute to the outcome of diabetes (41), also strongly support this hypothesis.

In conclusion, we have shown that NOD mice produce anti-tTG Abs at the gut level. This is further proof of a disordered intestinal immune response in such mice. The appearance of such Abs is

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clearly independent of gluten exposure, but may nevertheless be induced by exposure to alternative Ags. Although it is uncertain at this stage to what extent anti-tTG Abs contribute to the onset of type 1 diabetes, a closer analysis of the anti-tTG response in NOD mice may give further insight into the biology of the NOD phenotype.

Disclosures

The authors have no financial conflict of interest.

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