Epidermal Transglutaminase (TGase 3) Is the Autoantigen of Dermatitis Herpetiformis

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Abstract

Gluten sensitivity typically presents as celiac disease, a common chronic small intestinal disorder. However, in certain individuals it is associated with dermatitis herpetiformis, a blistering skin disease characterized by granular IgA deposits in the papillary dermis. While tissue transglutaminase has been implicated as the major autoantigen of gluten sensitive disease, there has been no explanation as to why this condition appears in two distinct forms. Here we show that while sera from patients with either form of gluten sensitive disease react both with tissue transglutaminase and the related enzyme epidermal (type 3) transglutaminase, antibodies in patients having dermatitis herpetiformis show a markedly higher avidity for epidermal transglutaminase. Further, these patients have an antibody population specific for this enzyme. We also show that the IgA precipitates in the papillary dermis of patients with dermatitis herpetiformis, the defining signs of the disease, contain epidermal transglutaminase, but not tissue transglutaminase or keratinocyte transglutaminase. These findings demonstrate that epidermal transglutaminase, rather than tissue transglutaminase, is the dominant autoantigen in dermatitis herpetiformis and explain why skin symptoms appear in a proportion of patients having gluten sensitive disease.

Key words: gluten sensitive enteropathy • celiac disease • IgA • immune complex • skin

Introduction

Gluten sensitive enteropathy (GSE)* is evoked and maintained by gluten, the adhesive mass of water-insoluble proteins found in many cereals. The clinical appearance of GSE is typically celiac disease (CD), a common chronic small bowel disorder; however, in certain individuals it is associated with the skin disorder dermatitis herpetiformis (DH). This is a bullous skin disease with polymorphic papules and blisters typically located over the extensor surfaces of the major joints and characterized by granular IgA deposits in the papillary dermis. Gastroenterological symptoms in DH are generally mild or clinically completely absent (1), however, inflammatory small bowel changes can often be found by histological examination even in the absence of clinical signs. The enteropathy in DH is morphologically identical with that in CD suggesting identical or very similar etiology and pathomechanism in both DH and CD (1). Further, both occur in the same genetic background being primarily associated with the HLA class II genes HLA-DQA1*0501, DQB1*02, and to a lesser extent with the HLA-DQA1*03, DQB1*0302 genes (for a review, see reference 2).

Both CD and DH patient sera show a typical IgA staining pattern when applied to tissue sections containing reticulin fibers such as endomysium. Recently, tissue transglutaminase (TGc, EC 2.3.2.13) was shown to be the predominant autoantigen in these sections (3, 4) and ELISA tests based upon this protein have been shown to be useful for the diagnosis of GSE (5, 6, 7, 8). TGc is a member of the transglutaminase (TG) family, which in man consists of nine distinct proteins present in a wide variety of cell types (Table I; references 9–27). TG family members show conservation especially of certain enzymatically relevant domains (10, 19). The active members catalyze a posttranslational modification linking low molecular weight amines to proteins, or induce an isopeptide bond between or within polypeptide chains leading to a cross-linked supramolecular

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**Abbreviations used in this paper:* AU, arbitrary unit(s); CD, celiac disease; CI, confidence interval; DH, dermatitis herpetiformis; EMA, endomysium Ab; GSD, gluten sensitive disease; GSE, gluten sensitive enteropathy; TG, transglutaminase; TGc, tissue (cellular, type 2) transglutaminase; TGe, epidermal (type 3) transglutaminase; TGk, keratinocyte (type 1) transglutaminase; TGx, type 5 transglutaminase.

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Table I. *Comparison of Transglutaminases (References 9–27)*

TG	FXIIIa	TGk	TGc	TGe	TGp	TGx	TGy	TGz	Band 4.2
Gene name	F13A1	TGM1	TGM2	TGM3	TGM4	TGM5	TGM6	TGM7	EPB42
Chromosomal localization	6p24-25	14q11.2	20q11-12	20q11	3p21-22	15q15.2	20q11	15q15.2	15q15.2
Number of amino acids without the 1. Methionine	731	816	686	692	683	719	706	709	690
Molecular weight (kD)	~77	~106	~78	~77	~77	~81	~79	~80	~72
Primary molecular features	Exists as zymogen with 2 catalytic a subunits and 2 b subunits	Exists as zymogen, both cytosolic and membrane associated	Monomeric	Exists as zymogen	Monomeric	Monomeric (occurs as splice variants)	Unknown	Unknown	Monomeric, lacks enzymic activity
Presence in the skin (mRNA)	Yes	Yes	Yes	Yes	No	Yes	No?	Yes	Yes
Presence in other cells, tissues, or organs	Blood plasma, platelets, monocytes-macrophages, hepatocytes, chondrocytes, placenta	Keratinocytes, epithelial cells in stratified squamous epithelia, endometrium, ducts of pancreas and mammary glands	Widespread	Mouse: brain, stomach, spleen, small intestine, esophagus, testis, skeletal muscle, Human: kidney and lung.	Prostate only	Widespread	Unknown	Widespread	RBCs, platelets, fetal liver and kidney, adult brain, adult kidney?

FXIIIa, factor XIII a-subunit; TGy, transglutaminase type 6; TGz, transglutaminase type 7; band 4.2, erythrocyte protein band 4.2; RBC, red blood cell.

protein network (for reviews, see references 9 and 11); further, under special circumstances they are also able to deamidate glutamine residues.

The discovery of TGc as the main endomysial autoantigen failed to explain why only a proportion of gluten sensitive patients show symptoms of DH and whether there is a difference in the antigenic repertoire between CD and DH. By comparing the Ab responses to skin transglutaminases we could show that CD and DH are diseases where the main autoantigens are distinct but share common epitopes. This explains the similarities in pathology while also clarifying why certain gluten sensitive patients present with dermatological symptoms.

Materials and Methods

Mass spectrometry, SDS-PAGE, and endomysium Ab (EMA) tests were performed as described previously (8).

Production of Recombinant Human Transglutaminases. Human TGc was expressed recombinantly in the human embryonic kidney cell line 293-EBNA as a COOH-terminal fusion protein with the eight amino acid Strep II tag, and purified via streptavidin affinity chromatography as described previously (8).

To express human epidermal (type 3) transglutaminase (TGe), a method similar to that for TGc was used. Total RNA from human keratinocytes was reverse transcribed and the cDNA coding for the TGe proenzyme amplified by PCR using the forward primer 5′-ATTAAGCTTGCCGCCACCATGGCTGCTCTAGGAGTC, and the reverse primer 5′-ATTGCGGCCGCTTCGGCTACATCGATGGACAAC. The forward primer introduced a HindIII restriction site and a Kozak translation initiation sequence while the reverse primer inserted a NotI restriction site and removed the stop codon. The PCR product was digested with the HindIII/NotI restriction enzymes and inserted at the same restriction sites into the episomal eukaryotic expression vector pCEP-Pu/TGc/C-Strep (8), producing pCEP-Pu/TGe/C-Strep. The correct insertion and sequence of the full construct

was verified by cycle sequencing. The plasmid was electroporated into human embryonic kidney cells (293-EBNA; Invitrogen) and transfected cells were selected with puromycin. Expression of the proenzyme, which has an additional COOH-terminal Strep II fusion tag, was confirmed by immunoblotting using a rabbit polyclonal serum raised against the Strep II tag (IBA). The protein was isolated by affinity chromatography using StrepTactin® (IBA) as described previously (8, 28).

Transglutaminase Activity Assay. TGe and TGc activity was measured by incorporation of [1,4-^{3}H]putrescine as described previously (8). The TGe was activated by partial proteolytic digestion preincubating it 20 min at 37°C together with either 45.4 μg/ml (0.5 U/ml) proteinase K (Sigma-Aldrich), or 45.4 μg/ml (55.4 U/ml) trypsin 1:250 (Sigma-Aldrich), or 1.18 mg/ml (1 U/ml) dispase (Life Technologies).

Production of Rabbit Sera against Human TGe. Rabbits were immunized with the COOH-terminally tagged human TGe proenzyme. TGe Abs were affinity purified by binding to Sepharose 6B (Amersham Pharmacia Biotech) coupled TGe and tested for cross-reactivity against TGc, keratinocyte TG (TGk), and factor XIII.

Sera and Patients. All patients had been examined at the Gastroenterological Departments of Internal Medicine or Pediatrics and the Department of Dermato-Venereology of Semmelweis University, Budapest. The diagnosis of CD was confirmed by EMA positivity and jejunal biopsy while DH was proven by skin biopsy using both conventional and immunohistochemical techniques. Sera were obtained from 59 patients with DH (including 43 samples from untreated patients, and 16 from patients on a complete or incomplete gluten-free diet) and 104 with CD (including 36 samples from untreated patients, and 68 from patients on a complete or incomplete gluten-free diet). Sera from 79 patients with non-CD gastrointestinal diseases, 47 with other diagnoses, and 30 from healthy individuals including 20 healthy relatives of CD patients were also included. Mean ages and sex ratios of the patients are detailed in Table II. No individual in this study had IgA deficiency. All serum samples were stored at −78°C until assayed.

TGe and TGc ELISAs. The ELISA method was as for the human TGc and described previously (8). Briefly: 96-well microtiter plates (MaxiSorp; Nunc) were coated with 1 μg per well of either human TGc or TGe in 100 μl of 50 mM Tris/HCl (pH 7.5) containing 5 mM $CaCl_2$ at 4°C overnight (at least 9 h). No blocking was used. After each step the wells were washed by 50 mM Tris/HCl (pH 7.5) containing 10 mM EDTA and 0.1% Tween 20 (TET). Sera were diluted to various concentrations with TET, and incubated on the plates for 1.5 h at room temperature. Bound IgA was detected by peroxidase-conjugated Ab against human IgA (Dako), diluted 1:4,000 in TET, and incubated for 1 h at room temperature. The color was developed by 100 μl of 60 μg/ml 3,3′,5,5′-tetramethylbenzidine substrate in 100 mM sodium acetate (pH 6.0) containing 0.015% H_2O_2 at room temperature. The reaction was stopped by adding 100 μl of 20% H_2SO_4. For the TGc, the color reaction was always stopped after 5 min; for the TGe, it was stopped after 15–20 min according to kinetic measurements so that the OD of the standard serum reached at least 0.6, but did not exceed 1.1. The absorbance was read in an ELISA reader at 450 nm. All serum samples were examined in triplicate, and triplicates of a negative and two positive reference sera were included in each assay. The Ab concentrations were expressed in arbitrary units (AU), i.e., as percentages of one of the positive reference sera. To semiquantitatively compare the IgA levels measured in the TGc and TGe ELISA assays, the standard serum was assayed in an identical manner against wells coated either with TGe or TGc. These were compared with standardized amounts of human IgA (Sigma-Aldrich). The protein coating efficiency of the ELISA plates was first determined by BCA protein quantification (Pierce Chemical Co.).

Inhibition ELISA. The ELISA method was as described above, but before their addition to the coated ELISA plate, the test sera were diluted to a fixed concentration and incubated with a dilution series of TGc or TGe. Sera and protein were mixed together for 90 min in a shaking incubator at 37°C. The fixed serum dilution was chosen in each case to obtain the greatest OD difference between the IgA Ab titers of the sera with and without

Table II. *The Patients' Number, Sex, Age at the Time of Blood Sampling, and Serum Ab Concentrations Against TGc and TGe*

				Median Ab cc. against TGc (in AU)		Median Ab cc. against TGe (in AU)	
Diagnosis	No. of patients	Male/ female	Mean age (yr) (min.–max.)	Median	95% CI	Median	95% CI
CD	104	40/64	12.5 (0.9–66)	—	—	—	—
CD, untreated	36	12/24	17.8 (1.4–66)	88.1	68.2–98.1	65.8	47.6–96.1
CD, on a GFD	68	28/40	9.7 (0.9–34.3)	22.7	16.6–41.0	20.3	15.2–26.6
DH	59	31/28	30.8 (6.2–73.5)	—	—	—	—
DH, untreated	43	23/20	32.8 (6.2–73.5)	63.3	54.8–77.6	70.1	54.6–75.7
DH, on a GFD	16	8/8	25.6 (10.3–45)	24.3	18.0–68.1	27.8	21.3–43.6
Controls	156	75/81	10.2 (0.5–55.5)	11.1	10.7–11.7	13.6	12.6–14.7
GI diseases	79	40/39	5.4 (0.5–27.6)	11.0	10.4–11.4	12.6	10.4–14.4
Other diagnoses	47	22/25	11.0 (0.7–53.2)	11.2	10.3–12.5	13.6	12.8–14.9
Healthy individuals	30	13/17	21.9 (0.7–55.5)	13.0	9.8–14.7	15.4	13.6–18.4
All samples	319	146/173	14.8 (0.5–73.5)	—	—	—	—

cc., concentrations; GFD, gluten-free diet; GI, gastrointestinal.

preincubation (1:500–1:4,000 for inhibition of the TGc ELISA, 1:125–1:1,000 for the TGe ELISA). These diluted sera were then incubated with a dilution series containing different amounts of TGc or TGe in 160 μl TET. The color reaction was stopped at 5 min for the TGc-coated ELISA and 15 min for the TGe ELISA.

Affinity Purification of Patient Abs Directed Exclusively against TGe. TGc or TGe was coupled to CNBr activated Sepharose 4B, 50 μg of coupled protein per patient sample was used in the purifications described below. 80 μl of serum was diluted 1:10 with 10 mM Tris (pH 7.5) and was circulated over a TGc column for 1 h. For a number of high titer samples the efficiency of anti-TGc Ab depletion was assayed at this step. To obtain TGe specific Abs and remove any traces of TGc immunoreactivity, the flow through now depleted of TGc reacting Abs was then applied to a TGe column in the same manner. The TGe columns were washed with 250 μl of 10 mM Tris (pH 7.5) followed by 250 μl of 10 mM Tris, (pH 7.5) containing 0.5 M NaCl. Bound Abs were eluted with 250 μl 100 mM glycine (pH 2.5) or 250 μl 100 mM triethylamine (pH 11.5) directly into 250 μl 1 M Tris (pH 8.8). These solutions were then dialyzed against PBS (pH 7.4). The Abs eluted from the TGe column were tested in the TGc and TGe ELISAs as described above.

Direct Immunofluorescence. 6-μm cryostat tissue sections of human jejunal biopsy samples, human skin, or the aboral part of monkey esophagus were used for staining. Bound IgA was detected by α-chain specific, affinity purified, FITC-conjugated, goat anti–human IgA Abs (Sigma-Aldrich) at a dilution of 1:100 in phosphate-buffered saline (PBS, pH 7.4).

For localization of TGe, the affinity purified rabbit antisera raised against the recombinant TGe proenzyme was diluted 1:100 in PBS, followed by incubation with Cy3-labeled goat antisera raised against rabbit immunoglobulins (Sigma-Aldrich), diluted 1:800 in PBS. For TGc and TGk, mouse mAbs (Neomarkers, Ab-3 [a mix of mAbs CUB7402 and TG100], and Biomedical Technologies, mAb BC.1, respectively) were diluted 1:100 and 1:50 in PBS followed by incubation with Cy3- or FITC-labeled sheep anti–mouse Abs diluted 1:800 or 1:400, respectively.

Statistics. The optical densities (and thus titers given in AU values) had Gaussian distribution neither in the control group nor among CD or DH patients, thus for description of Ab concentrations, medians with their 95% confidence intervals (95% CI) are presented (29). For description and comparison of the two ELISA systems, the areas under the receiver operating characteristic curves are given. For comparison between patient groups, Mann-Whitney's nonparametric, unpaired, two-tailed test is shown (30). To describe the correlation of titers, the Spearman's correlation coefficient with its 95% CI and correlation analysis for unpaired data of nonnormal distribution was used (29, 30). For comparison of the TGc and TGe Ab inhibition assays, Wilcoxon's two-tailed signed rank test for pairs was performed (30).

Results

Production and Purification of Recombinant TGe

The human TGe was expressed in the 293-EBNA human embryonic kidney cell line as a fusion proenzyme with the Strep II tag. The protein was purified in a single step, and eluted as one 80 kD band when visualized by Coomassie-staining after SDS-PAGE (Fig. 1). The molecular mass calculated for the tagged human TGe proenzyme is 78.0 kD (the COOH-terminal tag having a mass of 1.2 kD). Although the recombinant human TGc and TGe have approximately the same calculated molecular mass (78.4 kD for TGc), the human TGc migrated slower than expected when visualized by SDS-PAGE (8; Fig. 1). Mass spectrometry of the TGe fusion protein gave a molecular mass of 77.8 kD. The yield from the lysate of a confluent cell monolayer in a cell culture dish of 13 cm diameter was ~100 μg of purified protein. In cell lysates, the activity of the recombinant human TGe was 2.5 times higher than the background activity of transglutaminases present in untransfected 293-EBNA cells. The freshly purified human TGe proenzyme showed ~2% of the activity of the same amount of human TGc. The human TGe activated with different proteases (proteinase K, trypsin, or dispase) showed similar or higher activity than the TGc, which is similar or higher than the activity of the commercially available guinea pig TGc enzyme (Sigma-Aldrich).

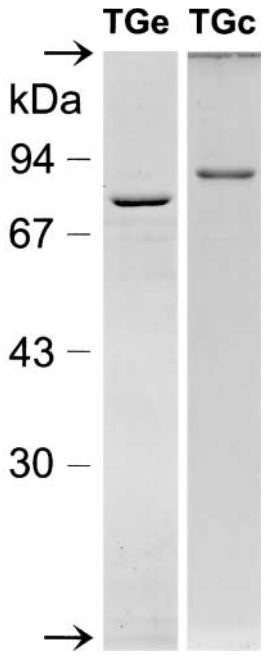

Figure 1. SDS-PAGE analysis of TGc and TGe after purification. Positions of molecular mass standards (kD) are indicated on the left. Arrows show the start and end lines of the gel.

CD and DH Patient Sera Contain IgA Abs against TGe and TGc

We have previously described a TGc ELISA based upon the human recombinant protein (8), ELISAs were performed against TGe or TGc using the same antigen concentration for coating, serum dilution, and positive and negative reference sera. As signals in the TGe ELISAs were for every serum significantly lower than against TGc, color development in the TGe ELISA was allowed to continue 3–4 times longer than that in the TGc ELISA until the positive reference serum reached similar ODs in both ELISAs. The results are expressed as a percentage (AU) of the signal of the positive reference serum in both assays. These ELISA results show that patients with both DH and CD have serum IgA Abs reacting against TGe and TGc (Fig. 2). To allow a comparison of the levels of IgA directed against either TGc or TGe, the reference serum (100 AU in the above assays) was assayed under identical conditions upon

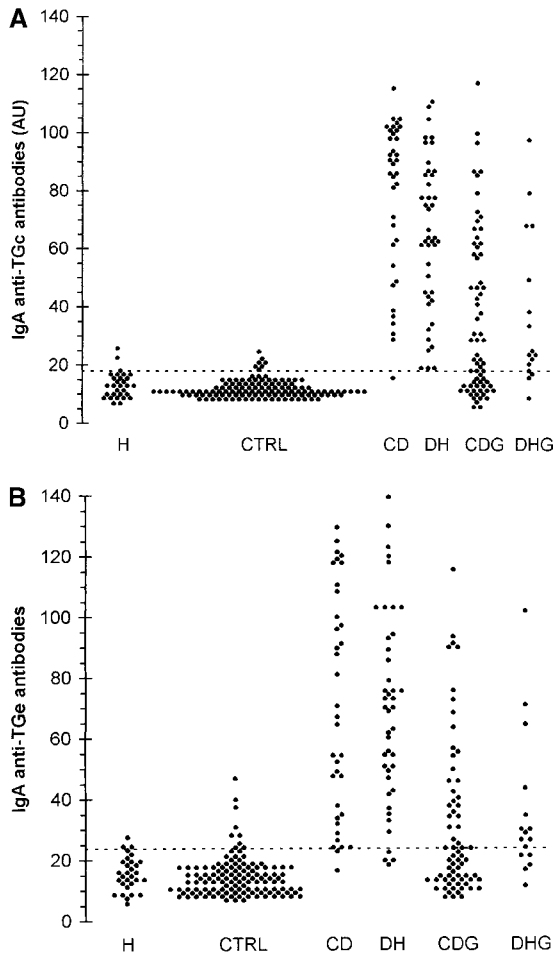

Figure 2. Analysis of serum anti-TGe and anti-TGc IgA. Serum concentrations of IgA Abs (in AU) against human TGc (A) and TGe (B) in healthy individuals (H), other controls (CTRL), patients having untreated CD or DH, as well as those on a complete or incomplete gluten-free diet (CDG and DHG, respectively). 100 AU corresponds to 16.78 μg IgA/ml of serum in the TGc ELISA and 2.45 μg IgA/ml in the TGe ELISA.

TGe and TGc coated ELISA wells in parallel to wells coated with a known amount of IgA. The anti-TGc IgA signal corresponded to 16.78 μg/ml of serum while the concentration of anti-TGe IgA in the positive reference serum was 2.45 μg/ml. In the TGe ELISA, the mean intra- and interassay coefficients of variation for the positive standard serum used for AU calculation were 2.7 and 19.1%, respectively. The mean intra- and interassay coefficients of variation (using Ab concentrations given in AU) for the other sera tested in the human TGe ELISA were 4.7% (n = 334) and 16.4% (n = 74), respectively.

The median Ab concentrations (in AUs) from the TGe and TGc ELISAs with their 95% CIs are presented in Table II. Although the confidence intervals overlapped, the median Ab concentration against TGc was significantly higher in CD than in DH patients (P = 0.0188). However, there was no significant difference in the Ab levels against TGe between CD and DH patients. The median Ab concentrations against TGc and TGe were significantly higher in untreated CD or DH patients when compared with the controls ($P < 0.0001$ in each case). Differences between the control subgroups were not significant. Both CD and DH patients had reduced Ab activity against TGe when on a gluten-free diet, results similar to those observed for TGc Abs.

The two ELISAs showed good linear correlation (r_S = 0.851, 95% CI: 0.818–0.878, $P < 0.0001$, data not shown). Indeed, the human TGe ELISA seemed to be suitable for diagnosis of GSE. The area under the receiver operating characteristic curve was 0.982 (in the TGc ELISA it was 0.997). In the TGe ELISA, a cutoff value of 23.7 AU, chosen based upon the analysis of the receiver operating characteristic curve, gave a specificity and a sensitivity of 92.3% (95% CI: 88.9–95.7%) and 92.4% (95% CI: 89–95.8%), respectively. The coincidence of the human TGe assay with the clinical diagnosis of CD or DH was 217/235 (92.3%), giving 12 false-positive and 6 false-negative results (Fig. 2 B). Four of the false-negative patients had DH, two of them were EMA negative. All the other DH or CD patients were positive for EMA.

For comparison, the TGc ELISA using a cut-off value of 18 AU (8) gave in this study a specificity and a sensitivity of 94.2% (95% CI: 91.2–97.2%) and 98.7% (95% CI: 97.2–100%), respectively. The coincidence of the human TGc assay with the clinical diagnosis was 225/235 (95.7%), giving one false-negative and nine false-positive results (Fig. 2 A). The false-negative serum and three of the false-positive sera were also falsely detected in the TGe ELISA.

These results suggest that either GSE patients have Abs cross-reacting between different transglutaminases or that specific Abs against both TGc and TGe occur in GSE and that Abs directed against TGe, as those against TGc, are maintained by the ingestion of gluten.

Inhibition ELISAs Show Differences in Ab Avidity to TGe between DH and CD Patients

To discover the significance of Ab cross-reactivity between these enzymes within the two patient groups, we performed inhibition studies. ELISA plates were coated with either human TGc or TGe, and the patient sera were preincubated with various concentrations of either of the two transglutaminases. Initial experiments allowed us to find appropriate serum dilutions giving results within a linear range for the given ELISA. The degree of inhibition produced by the preincubation with either of the two proteins was compared with control samples where the sera had been preincubated with buffer alone. The results are presented as reduction in the optical density given as percentage of the controls. Two examples of these inhibition ELISAs performed over a range of inhibitor concentrations with typical CD and DH sera are shown in Fig. 3. For group analysis of 36 CD and 34 DH patients, results of inhibition with 32 ng and 1 μg of the relevant transglutaminase are shown in Fig. 4.

Inhibition of Abs against TGc. We analyzed 34 sera of DH patients and 36 CD patients. The sera were diluted as described above and preincubated with human TGc or TGe

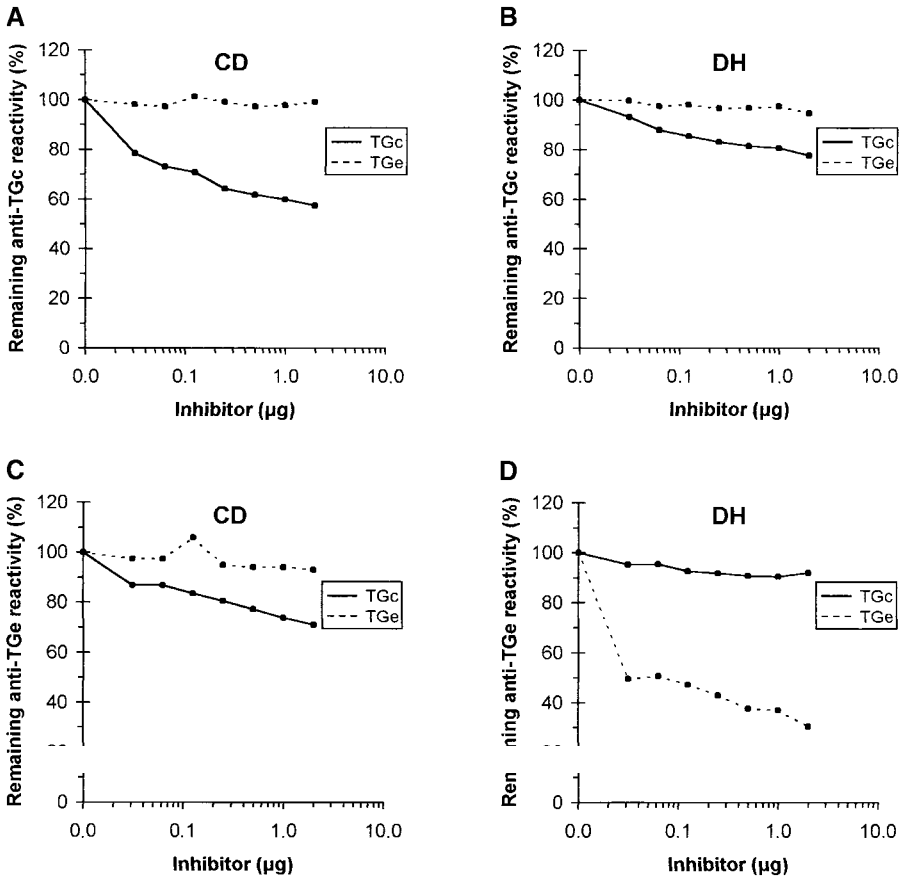

Figure 3. Transglutaminase inhibition ELISAs, typical examples of inhibition curves. Each diagram shows the effect of preincubation on the remaining IgA Ab reactivity in one single serum sample from a patient with untreated CD (A and C) or DH (B and D). On the vertical axis is the remaining IgA Ab reactivity against TGc (A and B) or TGe (C and D) given in percentage of the buffer control, on the horizontal axis are inhibitor amounts on a logarithmic scale used for preincubation. The control was preincubated with buffer only, the other samples with a serial dilution of TGc (continuous line) or TGe (dashed line). The TGe is seen to be an effective inhibitor of IgA Abs against TGe only in DH patients (D), but not in individuals with CD (C) (see group analysis in Fig. 4, statistics in the text). TGc has the greatest inhibitory effect on IgA Abs against TGc in CD patients (A).

in various concentrations before addition to ELISA wells coated with TGc. While incubation with even such low amounts as 32 ng of TGc (always in a volume of 160 µl, see Materials and Methods for details) effectively inhibited the reactivity of the sera from both the DH and CD patients with the coated TGc, the TGe failed to block the TGc reactivity (Fig. 3, A and B). Only at very high concentrations (above 10 µg) did preincubation with TGe produce any inhibition in the TGc ELISA (data not shown). There was no significant difference in the inhibition when comparing the results from DH or CD patient groups in these experiments.

Inhibition of Abs against TGe. Sera from DH and CD patients were diluted to the chosen dilution and preincubated with TGc or TGe before addition to ELISA wells coated with TGe. Here the human TGe, at 32 ng, effectively inhibited the reactivity of sera from DH patients with human TGe (Figs. 3 D and 4), but failed to inhibit that from CD patients (Figs. 3 C and 4). At higher concentrations, the inhibitory effect of preincubation with TGe increased with sera from DH patients and also a slight inhibition of the reactivity of sera from CD patients occurred which was more apparent upon the addition of very high amounts of TGe (up to 8 µg, results not shown). The difference between CD and DH patient groups upon inhibition with TGe was highly significant ($P < 0.0001$; Fig. 4).

At low concentrations, the human TGc produced only very marginal inhibition. However, when at high concentrations (at or above 1 µg), it could inhibit the reactivity of IgA Abs to TGe in both disease groups (Fig. 3, C–D, and

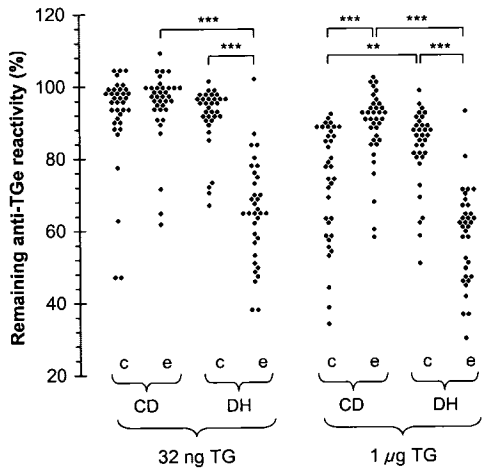

Figure 4. Effect of preincubation of sera from patients with CD ($n = 36$) or DH ($n = 34$). On the vertical axis, remaining IgA Ab reactivity against TGe is indicated in percentage of the buffer control. The four dot diagrams on the left show the inhibitory effect of preincubation with 32 ng of TGc (c) or TGe (e) on the remaining IgA Ab reactivity of sera from patients with CD and DH. The four dot diagrams on the right demonstrate the same using 1 µg of TGc or TGe for preincubation. The asterisks on top of connecting lines show the degree of significance in the difference between the two groups of samples so linked: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Fig. 4), although CD sera were more strongly inhibited than DH sera ($P \leq 0.0054$). These results provide evidence of various IgA Ab populations directed against both common and different epitopes on the two molecules and suggest that in DH patients there are IgA Abs with a high avidity directed against TGe.

Purification of TGe Abs from DH Sera

To discover if there are Ab populations exclusively directed against TGe present in either DH or CD patients, we affinity purified TGe specific Abs from patient sera. Sera from 20 CD patients and 18 DH patients were applied to columns of Sepharose 4B to which TGc had been covalently coupled. To test the efficiency of the removal of TGc Abs from the sera, the flow through fractions from this column were compared with a dilution series of the starting sera. This was performed for a number of high titer sera and showed a reduction of the TGc titer by some 98–99%. To isolate anti-TGe IgA, the immunodepleted (flow through) fraction was applied to columns carrying TGe, and after washing, the Abs binding to TGe were eluted. The eluates were compared with the unprocessed, precolumn sera for anti-TGe and anti-TGc immunoreactivity in the relevant ELISAs.

The removal of TGc immunoreactivity was highly effective with the eluates from the TGe column showing little or no reactivity in the TGc ELISA (Fig. 5). In the TGe ELISA, however, the eluates from DH patients showed in almost all cases significant levels of TGe immunoreactivity, while those of CD patients generally failed to give a signal

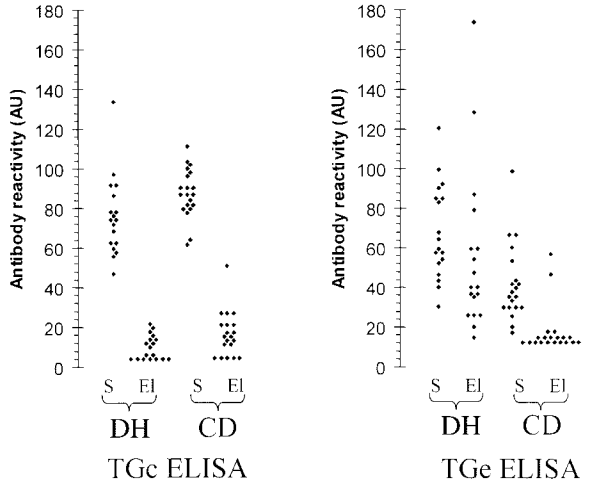

Figure 5. Affinity purification of the Ab population directed against TGe. Sera from CD and DH patients were circulated over columns of Sepharose 4B to which TGc had been covalently coupled. The immunodepleted fraction was then applied to columns carrying TGe. Unprocessed serum samples (S) and eluates from the washed TGe column (El) were compared for TGe and TGc immunoreactivity. The depletion worked with high efficiency and there was little or no reactivity in the TGc ELISA. In the TGe ELISA, the eluates from DH patients showed significant levels of TGe immunoreactivity, while those of CD patients generally failed to give a signal. Hence patients with DH have significant levels of Abs directed specifically against TGe which do not cross react with TGc, these Abs are absent in CD.

(Fig. 5) displaying clear evidence for the existence of a TGe-specific Ab population which is usually present in DH but absent from the vast majority of CD patients.

TGe Is Present within the IgA Precipitates in DH Skin

The antigenic component of the granular IgA precipitates occurring in the skin of DH patients was investigated for the presence of transglutaminases. A rabbit antiserum directed against the tagged human recombinant TGe proenzyme was produced and the specificity of the purified antiserum was verified in ELISA and immunoblots. This serum gave no cross-reaction with human TGk or factor XIIIa and a slight reactivity to human TGc, which was approximately a 100-fold lower than that to TGe. In normal human skin, the anti-TGe Ab stained solely the epidermis in a tapering manner being most intensive in the upper keratinocyte layers and quite different to the expression seen for TGc (results not shown). In addition to the epidermal signals, identical to those seen in normal skin, immunostaining of the skin from 8 DH patients with this antiserum revealed that TGe is found in aggregates within the dermal papillae (Fig. 6, A and C). This staining could be blocked efficiently by preincubation of the sera with TGe but not TGc or TGk (results not shown). Dual staining for the presence of IgA showed that TGe and IgA colocalized within these precipitates (Fig. 6 A) while TGc and TGk were absent from the aggregates and had the expression pattern seen in normal skin (TGc: Fig. 6 C; TGk: not shown). To verify that an alteration in the TGe staining pattern is a specific finding for DH skin, skin from patients suffering from linear IgA dermatosis was also analyzed. In this disease, IgA also accumulates within the skin but binds to the dermo-epidermal basement membrane. Here no TGe staining of the dermis or at the basement membrane was found and no colocalization with the IgA signal occurred (Fig. 6 B). Hence the IgA precipitates found in DH are immunocomplexes containing TGe which accumulate specifically in this disease.

Discussion

CD and DH are closely related diseases both induced by a sensitivity to gluten. As they share an identical jejunal pathology, genetic background, similar pathomechanism, common diagnostic analysis, and shared dietary possibilities for therapy, we suggest the term "gluten sensitive disease" (GSD) for both of these forms of condition showing manifestation on gluten challenge, disappearance of symptoms on gluten withdrawal, and recurrence of disease upon gluten intake. Both forms of GSD can be subdivided into clinical manifestations of different severity, as they can also present with unspecific or even absent clinical signs or symptoms. Moreover, epidemiological studies show that the majority of GSD patients actually have very mild or atypical symptoms or often clinically silent disease (31).

GSD is the result of three processes culminating in the intestinal mucosal damage of CD and in the skin defects of DH. Both are hereditable conditions with strong associa-

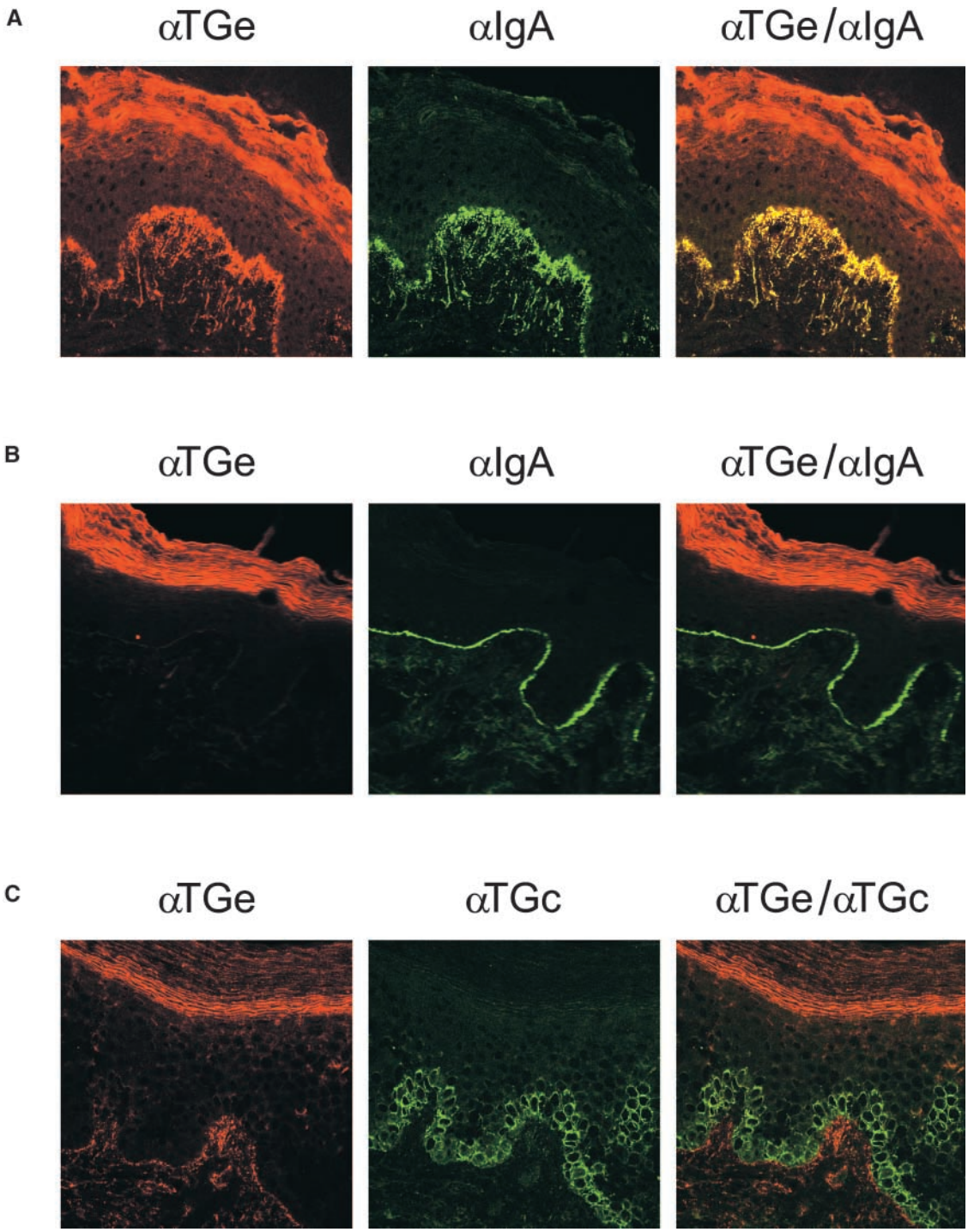

Figure 6. Colocalization of IgA and TGe in the papillary dermis of DH patients. (A) In DH patients, TGe shows normal distribution pattern in the epidermis; typical precipitates are present, however, in the upper dermis. In these precipitates, the TGe (red) and the IgA (green) colocalize as visualized with a confocal microscope (yellow). (B) The TGe precipitates are absent in linear IgA dermatosis, a bullous skin disease with linear IgA precipitates in the upper dermis, suggesting that TGe deposits are specific markers for DH. In addition, this also demonstrates the absence of cross-reactivity of anti-TGe Abs with IgA. (C) The TGe (and the IgA, data not shown) precipitates do not colocalize with TGc.

tions to identical HLA haplotypes; however, it does not appear that genetic factors alone decide the clinical outcome as monozygotic twins may exhibit any combination of manifest CD, DH, or clinically silent GSD (1, 32) proving environmental factors are also significant. The main environmental factor in GSD is the ingestion of gluten in cereals, however, Abs against these proteins are not perfect diagnostic markers for the disease and are found in a range of other

gastrointestinal disorders without any evidence that they play a pathogenic role in these conditions (33, 34). However, the third factor, namely that the patients' immune system produces Abs reacting with the endomysium, is found present in every form of GSD and is highly specific (35). The standard serological diagnosis for GSD depends upon the staining of endomysial tissue with IgA Abs, and it was shown that the antigen within these sections is TGc (3, 6, 7, 36, 37). The presence of autoantibodies to TGc has been shown to be linked to disease activity with the titer decreasing when patients are placed upon a gluten free diet and increasing upon subsequent gluten challenge (6, 7). Our aim was to understand why GSD appears as two distinct clinical entities.

Our initial hypothesis was that there was immunoreactivity specifically in the DH patient population against a further transglutaminase expressed in the skin. Four transglutaminases have been isolated from the skin, TGe and TGk are both produced by epidermal cells, as is TGc, which is also found together with factor XIIIa in the dermis. To discover if any of these proteins are antigens in DH we produced ELISAs based upon human transglutaminases. Initial ELISA studies using human recombinant TGk as well as the commercially available human factor XIIIa showed that there was no specific immunoreactivity in either CD or DH patient sera against these enzymes (results not shown). However, both patient groups had Abs recognizing TGe as well as TGc. The results from the TGc and TGe ELISAs showed a good correlation and indeed the specificity and sensitivity of the TGe ELISA came close to that of the TGc based test. However, in CD patients, the median Ab concentration against TGc was higher than against TGe, and this was reversed for DH patients (Table II), although because of the overlapping confidence intervals this tendency cannot be judged to be a true distinction. Further, the immunoreactivity for both proteins and in both disease groups showed a reduction in titer when the patients were placed upon a gluten free diet. This is in agreement with known clinical improvement seen in DH patients on a gluten free diet and the common background of both diseases.

Members of the transglutaminase family share a high degree of sequence conservation especially in their active sites. In the case of the TGe and TGc there is an overall conservation of 38% at the amino acid level, but with up to 64% homology in certain regions (19). Phylogenetically, TGe and TGc seem to be more related to each other than to TGk or factor XIIIa (10). Cross-reacting Abs against TGc or TGe in GSD patients are therefore not surprising; however, we could use ELISA blocking experiments to show differences in avidity for the different TGs between the two patients groups. As expected, TGc inhibited the reactivity of the sera from both CD and DH patients in the TGc ELISAs showing that anti-TGc immunoglobulin species are present in both diseases. In the TGe ELISA, however, inhibition with TGe could be invoked only in DH patient sera suggesting the presence of high affinity anti-TGe Abs in DH, and the presence of only of low affinity TGe reactive Abs in CD. Recently three new members of the TG gene family, type 5 transglutaminase (TGx), TGy, and TGz (Table I), have been described (10). We were unable to test these transglutaminases in our study, thus the possibility of cross-reactivity with other TGs cannot be completely excluded.

Our results prove the presence of two Ab populations in GSD, one against only TGe (detected in patients with DH only, see Fig. 5), and one directed against common epitopes of TGe and TGc (detected in both CD and DH, see Fig. 4). A third population against only TGc may also be present, but was not investigated. As shown by the differences in the IgA levels against TGc and TGe in the standard serum, the concentration of IgA Abs directed against epitopes present on TGe is much lower than that directed against TGc. This means that both in DH and CD patients, only a fraction of the Abs directed primarily against TGc have cross-reactivity with TGe. In addition, DH patients develop a higher avidity Ab population directed against only TGe. This Ab fraction also is much smaller than that against TGc. This explains why there is no apparent difference between sera of CD or DH patient groups in either the TGc or TGe ELISAs (Fig. 2, A and B) and why the TGc and TGe ELISA results from patient sera correlate. While the Ab population directed against only TGe (found in DH patients and having high avidity), can be inhibited with very small amounts of TGe, those primarily directed against TGc, (having low avidity against TGe) can only be inhibited with high amounts of TGe. Accordingly in DH patients typically a two-step inhibition curve is seen (Fig. 3 D). This further explains why preincubation of CD serum (which has little or no high avidity TGe Abs) with TGc has a greater impact on reactivity to TGe than preincubation with TGe itself (Fig. 3 C).

While affinity purification of sera of GSD patients showed that the presence of TGe-specific IgA is a hallmark of DH rather than CD, a small number of patients (10%) deviated from the bulk of results in both the blocking assay and in their behavior upon purification. These DH patients, having Ab response characteristic for CD, might currently be showing transition from CD into DH. The CD patients, behaving rather as expected for DH patients, might be expected in later life to show symptoms of DH, if they continue gluten intake.

The diagnosis of DH depends upon the finding of IgA deposits within the dermal papillae, and in the majority of patients EMAs can also be detected (38). The latter is typically shown with the labeling of the endomysium, which has earlier been shown to colocalize with the TGc staining pattern (6, 37). Our observation that the major Ab population both in CD and in DH is directed against TGc, supports the finding that the endomysial signal seen on monkey esophagus is of TGc origin and indeed we found that TGe was present only in the epithelial cells of monkey esophagus, but not in the endomysium (results not shown). The epidermal staining pattern with the TGe antiserum supported previous reports on its distribution (39), TGe being present in the epidermis in a tapering manner, with maximum staining of the upper epidermal layers. It was also found present in hair follicles. In DH skin, the epider-

mal and hair follicle staining was indistinguishable from that seen in normal skin, but the dermal IgA containing aggregates also stained strongly for TGe. TGc, which was found present in the basal keratinocytes, was absent from these aggregates.

Our hypothesis for the etiology and pathogenesis of DH is that TGc-gluten complexes initiate an IgA autoantibody response (40), but fail to produce high affinity anti-TGc immunoglobulins, so resulting initially in a silent CD. These Abs cross react with TGe, but are of low avidity to it. After prolonged gliadin provocation (DH patients usually show symptoms later in life than CD patients), specific cross-reacting Ab populations develop in patients who will go on to acquire DH. These Abs have a low affinity to TGc, but extremely high affinity to TGe. Whether they arise against TGe as a primary antigen or are the result of epitope spreading cannot be answered at the moment. Why only a proportion of patients develop specific Abs against TGe and why these patients show only a very mild form of enteropathy also remains to be elucidated.

We speculate that the skin pathology may be evoked by the dermal deposition of circulating immune complexes containing IgA and TGe. Possibly the TGe is active, resulting in covalent cross-linking of the complex to certain dermal structural elements. This could be the basis for the stability of these immune complexes, as it is known that the IgA deposits in DH skin stay detectable up to a decade after the introduction of a completely gluten-free diet (1). It would also explain why it has not been possible to extract the IgA immune complexes from the skin of DH patients. Inflammation of the skin might eliminate the covalently bound immune complexes. Indeed often the IgA granules are present perilesionally but not in areas of blister formation. This circulating immune complex hypothesis for DH is supported by a number of findings. First, TGe is expressed in several tissues in the body (Table I), and thus the antigen might originate from organs other than the skin. We failed to detect TGe in the human jejunum with our rabbit antiserum (results not shown), although the mRNA for the TGe proenzyme was demonstrated in mouse jejunal tissue extracts (17). We did, however, detect TGe mRNA in other human organs including the kidney (results not shown). Further, the skin histology in DH has features in common with other dermatoses induced by circulating immune complexes (41), and although the main site of immune complex deposition is the upper dermis, they are also present in vessel walls. In DH, asymptomatic IgA immune complex depositions can be detected in the kidney (42), a situation often seen in systemic diseases caused by circulating immune complexes, and indeed DH-associated IgA nephropathy has been reported (43). The fact that Abs in DH sera do not bind to the normal human papillary dermis again suggests that the deposits derive from circulating immune complexes. The factors that induce the classical distribution pattern of skin lesions in DH patients, localized mainly on extensor aspects, are as yet unknown. Here, however, we have shown that high affinity anti-TGe IgA maintained by gluten is present in DH patients and not in patients suffering from CD and that TGe is present in the skin IgA aggregates typical of DH.

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