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Bone-Specific Antibodies in Sera from Patients with Celiac Disease: Characterization and Implications in Osteoporosis

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Osteopenia and osteoporosis are well-known complications detected in celiac disease patients with still obscure pathogenesis. In the present study we investigated the presence of circulating anti-bone autoantibodies in patients with celiac disease and explored their role in the associated bone disease. We evaluated serum samples from 33 patients at the time of diagnosis and from 20 of them after treatment. Sera from patients with inflammatory bowel disease ($n = 9$), nonceliac osteoporotic ($n = 18$), and healthy individuals ($n = 10$) were used as controls. The presence of IgA specific anti-bone antibodies was first investigated using indirect immunofluorescence on cryosections of fetal rat tibia (20-day pregnancy). Furthermore, samples were homogenized and total tissue extracts were subjected to Western blot analysis to confirm immunoreactivity. At diagnosis, sera from 51.5% (17/33) of celiac patients had antibodies that recognized antigenic structures in chondrocytes and the extracellular matrix along mature cartilage, bone interface, and perichondrium of fetal rat bone. Among controls, only two osteoporotic patients showed very low titles of anti-bone autoantibodies. The immunostaining was localized in areas where an active mineralization process occurred and was similar to the distribution of the native bone tissue transglutaminase. The frequency of patients with positive baseline titers of anti-bone antibodies diminished significantly after treatment ($P = 0.048$). Western blot assays confirmed the presence of autoantibodies in sera from patients with a positive immunofluorescence staining. Autoantibodies recognized a major protein band on tissue extracts with a molecular weight of 77–80 kDa, which could be displaced when sera were preadsorbed with human recombinant tissue transglutaminase. We provide original evidence that patients with celiac disease have IgA-type circulating autoantibodies against intra- and extracellular structures of fetal rat tibia. Our findings suggest that these antibodies recognize bone tissue transglutaminase as the autoantigen, and based on the localization of the immunoreactivity we speculate that they might have an active role in the pathophysiology of celiac disease-associated bone complications.

KEY WORDS: Celiac disease; osteoporosis; autoantibodies; tissue transglutaminase.

INTRODUCTION

Celiac disease (CD) is a gliadin-induced enteropathy characterized by the production of a series of antibodies with different specificities. Many studies have explored the prevalence and pathogenic role of antibodies to dietary proteins such as gliadin, milk protein, and so forth (1–4). More recently, specific autoantibodies (antireticulin, antiendomysial, and antijejunum) have acquired special relevance mainly based on their diagnostic role and, possibly, their pathogenic importance (5, 6). Furthermore, sera from patients with active CD display a series of non-organ-specific and organ-specific autoantibodies against different tissue structures (anti-smooth muscle, anti-nuclear, anti-thyroid, anti-gastric parietal cells, etc.) (7). Although they still have an unknown clinical significance, it seems possible that these organ-specific autoantibodies might determine an autoimmune reactivity against antigenic structures that affect functionality (7).

Osteopenia and osteoporosis are well-recognized complications of CD and constitute a major problem through their association with bone fractures mainly in the peripheral skeleton (8). Many factors have been sug-

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gested to play a role in the development of osteopenia in patients with CD, including calcium and vitamin D malabsorption, malnutrition, and menopause (9, 10). Secondary hyperparathyroidism has been proposed to be a key factor in bone affectation. However, bone demineralization has been demonstrated in patients with no evidence of malabsorption (11, 12). Therefore, the precise pathophysiological and molecular mechanisms inducing bone disease in celiac patients are still partially known. It is now well established that CD is caused by a T-cell-mediated hypersensitivity with the subsequent release of imbalance quantities of proinflammatory and anti-inflammatory cytokines (13). Because bone loss is an effect observed in a tissue distant from the primary site of inflammation (small intestine), we speculated that the effect could be attributable, at least partially, to systemic immunological factors. Research from our laboratory and from other groups has recently suggested that cytokines such as interleukin-6 (IL-6), IL-1β, IL-1ra, and the insulinlike growth factor I (IGF-1) might play a role in the osteopenia associated with CD (14, 15). However, until now the role of CD-specific and -nonspecific antibodies in osteopenia and osteoporosis has not been explored.

Antiendomysial antibodies are a hallmark of CD. Recently, Dieterich *et al.* (16) identified tissue transglutaminase (tTG) as the main antigen eliciting antiendomysial antibodies. It has been hypothesized that a neoepitope (including gliadin and tTG) would promote the onset or most probably aggravate the celiac lesion inducing an immune response against both antigens. Interestingly, tissue transglutaminase, which belongs to a very ubiquitous family of enzymes catalyzing a Ca^{2+}-dependent acyl-transfer reaction in which new γ-amide bonds are formed, has been shown to be relevant in bone calcification (17–19). Hence, it has been demonstrated that native bone tTG has a specific and key role in modulating maturation of bone/cartilage matrix and facilitating its stabilization and finally mineralization (18, 19). This effect has been attributed to the tTG cross-linking activity, which generates deamidation and negative charges on specific bases, in which calcium and hydroxyapatite will deposit (19).

As far as we know, no attempts have been made to search for specific autoantibodies against bone in sera from celiac patients. The presence of these autoantibodies might play a critical role in the pathogenesis of bone alterations in CD patients. In the present study we investigated the presence of circulating anti-bone antibodies in sera from patients with CD, by analyzing the immunoreactive profile of these autoantibodies using two simultaneous experimental approaches (immunofluorescence staining and Western blot analysis). Furthermore, we attempted to determine whether tTG was the main target of these autoantibodies. Finally, we explored the potential relationship between anti-bone antibodies and the presence of bone impairment in these patients.

MATERIALS AND METHODS

Celiac Disease Patients and Controls

We evaluated serum samples from 33 patients with CD (29 female; 24 to 65 years old) with different bone mineral density (BMD) determined at the time of diagnosis, 9 patients diagnosed with inflammatory bowel disease (6 female; 22 to 41 years old), 18 subjects with clinical and densitometric evidences of osteoporosis (16 female; 43 to 60 years old) but not celiac disease, and 10 healthy control subjects (5 female; 36 to 52 years old). While 13 patients had normal BMD (>-1 *z* score) at the time of diagnosis, 7 patients had osteopenia (-1 to -2 *z* score) and 13 had osteoporosis (<-2 *z* score). Sera from 20 CD patients were reevaluated after treatment (median time on a gluten-free diet: 13 months). Serum samples corresponding to healthy controls were obtained from the medical staff of the Gastroenterology Hospital. Sera from control patients with idiopatic osteoporosis and patients with inflammatory bowel disease were provided by the Service of Osteopathies of the San Martin Hospital (UBA) and the IBD Section of the Gastroenterology Hospital, respectively. All the patients gave a written consent for this study. Care was taken to be sure that no other related diseases, which could potentially induce osteopenia or osteoporosis, were present in CD patients. Celiac disease was diagnosed by currently accepted criteria based on the presence of severe mucosal atrophy on small bowel biopsy while on gluten-containing diet. Moreover, diagnosis also included a positive serology and a positive clinical and/or histological response to a gluten-free diet. Gluten-free diet produced negative tests or reduced titers of CD-related serology in all patients. Only well-established CD cases were included. Osteoporosis in nonceliac patients was diagnosed based on the presence of a minimal traumatic fracture history and BMD values lower than -2 *z* score.

Bone mineral density was measured using a dual-energy X-ray absorptiometer as reported previously (9). Scans of the whole body and lumbar spine were performed in every study and reported as BMD at lumbar spine (L2 to L4). Results were reported as absolute values (g/cm^2) or *z* score that represents the number of

SD separating an individual value from the corresponding mean normal value, corrected by sex and age.

Fetal Rat Bone Samples and Immunohistochemical Methods

Tibia bones obtained by dissection from 20-day-old Wistar rat fetuses were preserved frozen until assays were performed. Fetal tibia is an adequate material for unfixed processing and cutting without prior abrasive handling. Histological analysis of fetal bone was performed by hematoxylin-eosin staining of paraffin-embedded sections. For immunohistochemistry, cryosections (4 μm) were cut, mounted on Silane-coated slides (Sigma; St. Louis, MO), and stored frozen until use. Sera diluted in PBS (1:5) were incubated with sections of tibia bone at room temperature for 30 min. After washing, sections were incubated with a 1:80 dilution of FITC-labeled goat anti-human IgA (Kallestad Sanofi Diagnostics Pasteur Inc., Chaska) at room temperature. After 30 min, sections were washed and examined under a fluorescence microscope. Positive sera were diluted until a negative immunofluorescence pattern was obtained. To assess expression of bone-specific tTG we also used the indirect immunofluorescence method. A mouse IgG1 anti-guinea pig liver tTG monoclonal antibody (mAb) (Neomarkers, Freemont; CA; CUB 7402 MS-224-P) diluted in PBS (1:10) was incubated overnight at 4°C with rat tibia cryosections. Dilution of this primary antibody was selected after titering the sera at concentrations ranging from 1:10 to 1:50 dilutions. Specificity and absence of cross-reactivity was checked by preadsorbing human antisera with human tTG. Secondary FITC-conjugated IgG anti-mouse antibody (Chemicon; Temecula; CA) diluted 1:20 was incubated for 1 hr at room temperature. No reactivity was found when the first antibody step was omitted for control purposes or when the anti-mouse polyclonal IgG was incubated with rat tissue sections.

Antitissue Transglutaminase and Endomysial Antibodies Assays

IgA antitransglutaminase (anti-tTG) antibodies were determined using a commercial ELISA test (INOVA Diagnostic Inc. San Diego; CA) (normal values $<$20 AU/ml). Endomysial antibodies (EmA) were assessed by indirect immunofluorescence on monkey esophagus tissue slides using a commercial kit (INOVA Inc. San Diego, CA). The sections were incubated with serum samples diluted in PBS (1:5). Both procedures were performed as previously described (20)

Western Blot Analysis

Fetal rat tibia bones were homogenized with 0.1 M Tris HCl pH = 8.3, 0.3 M NaCl, 50 mM $CaCl_2$, 5 mM dithiothreitol and protease inhibitors: phenylmethanesulfonyl fluoride (1 mM), benzamidine HCl (10 mM) and 6-amino-hexanoic acid (0,1 M). Thirty micrograms of tissue protein extracts were electrophoretically separated in 10% SDS-polyacrylamide minigels (BioRad Labs. Richmond, CA) under reducing conditions according to the Laemmli gel method for Western blot analysis (21). Gels were electrotransferred to Hybond-C nitrocellulose membranes as previously described (22). Blotted membranes were blocked overnight at 4°C with 10% skim milk powder in TBS (20 mM Tris pH = 7.4, 125 mM NaCl). After 18 hr membranes were incubated for 3 hr at room temperature with sera corresponding to untreated and treated CD patients and healthy subjects. Serum samples were tested at 1:50 dilution. A peroxidase-labeled goat IgG anti-human IgA (Sigma Chemical Co., St Louis, MO) was used as secondary antibody at 1:3000 dilution. The reaction was visualized using 4-chloro-1-naphtol-H_2O_2 as substrate and chromogen.

Inhibition of Autoantibodies Using Human Recombinant Tissue Transglutaminase

Serum samples from CD patients diluted in PBS (1:20; 1:40, and 1:80) were preincubated overnight at room temperature with 10, 20, and 100 μg of human recombinant tTG (Eurospital; Trieste, Italy). Tissue transglutaminase-pretreated or untreated sera were tested on fetal rat tibia substrates using Western blot analysis and immunofluorescence as described above.

Ethical Issues and Statistics

At the time of blood extraction, patients were informed of the aim of the study and gave their consent to be included. Rats were handled according to ethical norms and institutional guidelines. Results were expressed as mean $\pm$ SEM or median and range and the 25 and 75 percentiles as appropriate. The proportion of positive anti-bone autoantibodies cases at baseline and after a gluten-free diet were compared using the Fisher's exact test. Titers of antibodies between groups were compared using the ANOVA and Kruskal–Wallis tests and those in the same population were compared using the pair *t*-test. For the association of variables, the Spearman rank test or logistic regression were used.

Table I. Epidemiological and Clinical Characteristics of Patients at Diagnosis

Characteristic	
Number of patients	33
Sex (female/male)	29/4
Age at diagnosis (yr) (mean ± SEM)	37 ± 3
Body weight (kg) (mean ± SEM)	54.5 ± 2.0
Body mass index (kg/cm^2) (mean ± SEM)	20.8 ± 0.6
Lumbar spine bone mineral density absolute values (g/cm^2) (mean ± SEM)	0.99 ± 0.04
Z-score (mean ± SEM)	−1.06 ± 0.3
Endomysial antibodies: *N* patients with positive results	28/33 (84%)
Antigliadin antibodies type IgA: *N* patients with positive results	18/33
Values (UA/mL) (mean ± SEM)	33 ± 6
Antigliadin antibodies type IgG: *N* patients with positive results	31/33
Values (UA/ml) (mean ± SEM)	46 ± 5
Anti-tTG antibodies type IgA: *N* patients with positive results	31/33
Values (UA/ml) Median (range)	71 (1–451)

RESULTS

Clinical Characteristics and Demography of Patients

Table I shows some clinical characteristics and epidemiological data from patients at the time of diagnosis. All patients included were classically symptomatic at diagnosis. Patients with a negative EmA test ($n = 5$) were confirmed as having celiac disease by a satisfactory response to a gluten-free diet and an improved posttreatment biopsy. All had low levels of total serum IgA (cutoff value: 80 mg/ml). However, three cases had positive titers of anti-tTG antibodies (21, 26, and 32 AU/ml). All these cases had very mild IgA deficiency with values of total serum IgA ranging between 30 and 60 mg/ml. Compared with baseline data, treated patients significantly increased body weight and had a significant reduction of antigliadin antibodies and anti-tTG titers. Most patients were found to be negative for EmA after treatment.

Detection and Distribution of Anti-bone Antibodies

The histological characteristics of fetal rat tibia shows the histological characteristics at light microscopy of the epiphyses growth zone of fetal rat tibia (20-day pregnancy) (Fig. 1). We identified different areas of the cartilage (for interpretation, see Fig. 1). Figure 2 shows the immunoreactive pattern corresponding to IgA anti-bone antibodies present in sera from CD patients.

Anti-bone autoantibodies were found to bind different structures in the fetal rat tibia. Staining was detected both at the intra- and extracellular levels on cryostat sections. While intracellular staining was detected at the level of chondrocytes (Fig. 2A) with a gradient of intensity in direct association with the maturation of the cells, the extracellular staining was present in the perichondrium (Fig. 2B) and in the cartilage/bone matrix following the trabecular design of the zone of provisional calcification. Preadsorption of CD sera with human recombinant tTG did not abrogate immunofluorescence staining on fetal rat tibia substrate but was effective to block EmA on monkey esophagus substrate (data not shown).

Detection and Distribution of Tissue Transglutaminase in Fetal Tibia

The immunofluorescence pattern of expression of native bone tTG using a monoclonal anti-TG mouse

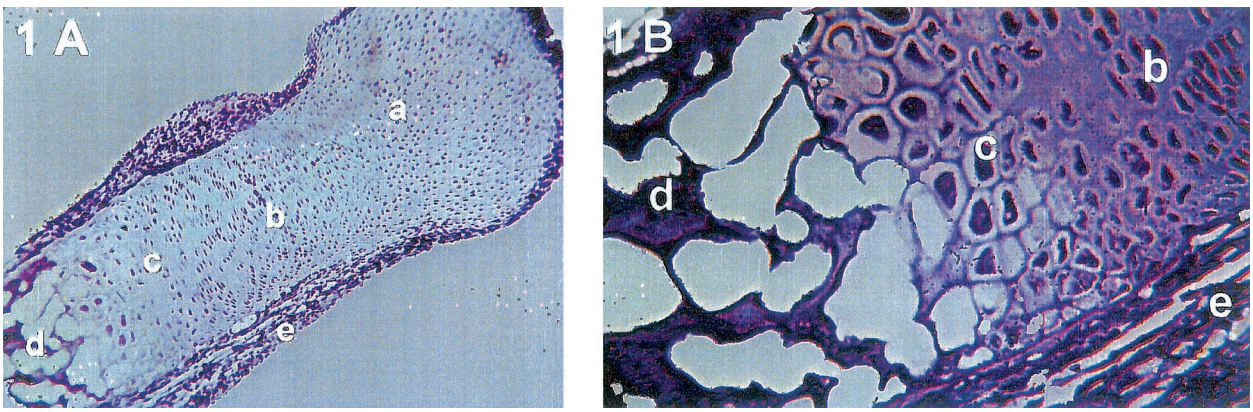

Fig. 1. Histological characteristics of fetal rat tibia (hematoxilin-eosin staining) in a paraffin-embedded section (4 μm) (A, 160× magnification; B, 400× magnification). Different stages of the cartilage can be appreciated, which include reserve zone (a); proliferation zone (b); hypertrophic zone (c); provisional calcification zone (d); and perichondrial zone (e).

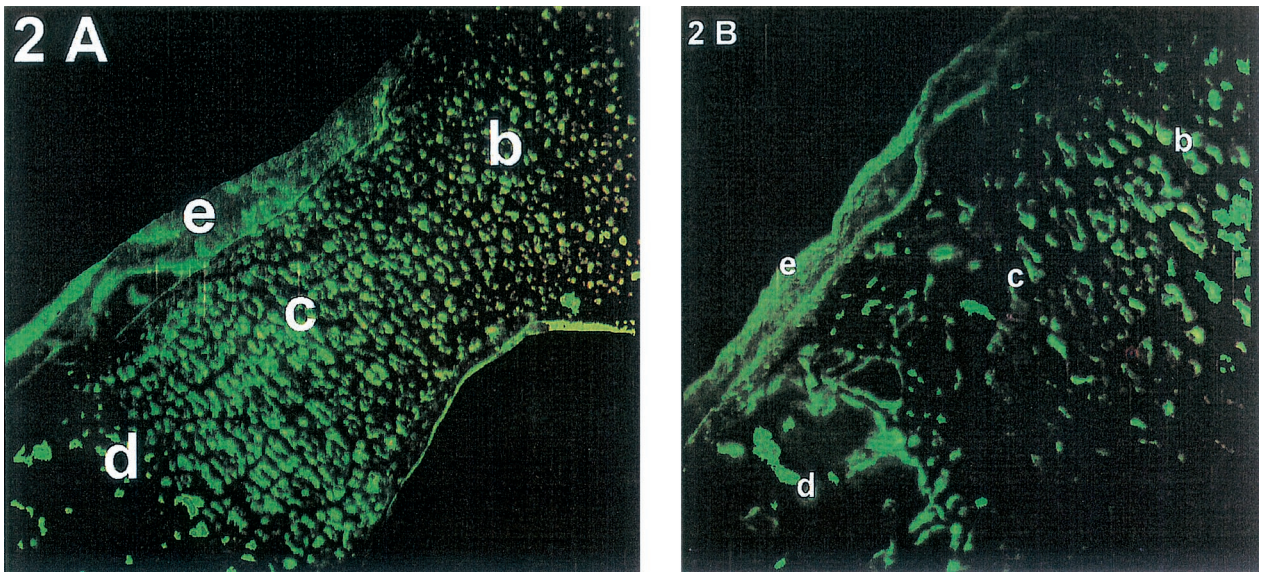

Fig. 2. Binding of anti-bone antibodies corresponding to a representative positive serum (titer 1/1280) from a CD patient to fetal rat tibia substrate in a nonfixed cryosection (A, 160× magnification; B, 400× magnification). Immunofluorescence staining can be visualized at different levels in the cytoplasm of chondrocytes in differentiation stages (a–c) or in the extracellular milieu (d, e). Cytoplasmatic staining shows a progressive gradient of intensity from the reserve zone to the hypertrophic zone. Extracellular staining is located in the core of trabeculae in provisional calcification stage. Preadsorption of human sera with human recombinant tTG does not displace immunoreactivity (data not shown).

antibody in a cryostat section of fetal rat tibia is shown in Fig. 3 (focalized on bone matrix and perichondrium). The enzyme was detected intracellularly in chondrocytes in the maturation/proliferation zone of fetal rat tibia (not shown). Similar to the immunoreactive profile shown by anti-bone autoantibodies, the expression pattern appears to reach maximal intensity in the bone matrix and perichondrium areas, in which provisional calcification has been produced. The enzyme was also found in the extracellular space showing a similar staining pattern as anti-bone antibodies where the enzyme was distributed in cores of trabeculae corresponding to the provisional calcification zone of the cartilage.

Prevalence and Titers of Anti-bone Antibodies

Seventeen of 33 (51.5%) serum samples from untreated CD patients had antibodies that recognized antigenic structures in chondrocytes and the extracellular matrix along mature cartilage, bone interface, and perichondrium of fetal rat bone (median titer 1:20; range 1:10 to 1:1280). Only those titers higher than 1:5 were considered to be positive. Among controls, only two osteoporotic patients had low titers of anti-bone autoantibodies (both 1:10). Sera from patients with inflammatory bowel disease were found to be negative for the presence of antibodies. In all control cases, CD was excluded based on the negative results for EmA and anti-tTG (7 and 8 AU/ml for both cases) antibodies (normal value, <20 AU).

Sera from a group of 20 CD patients assessed at the time of diagnosis were reassessed after a period of more than 1 year on a gluten-free diet. While at baseline 13 of 20 patients (65%) had positive titers of the autoantibody, only 4 (20%) of them were positive after treatment ($P = 0.048$). Titers of anti-bone antibodies correlated significantly with those of anti-tTG (r, 0.64; $P < 0.0001$). Titers of anti-bone antibodies did not show significant difference comparing patients with normal BMD and those with values of osteopenia and osteoporosis (Table II). Values for anti-tTG antibodies did not show differences among subgroups (Table II). However, patients with the greatest titers of autoantibodies (1:1280 in some patients and 1:160 in others) had BMD in the range of osteoporosis.

Immunoreactive Profile of Anti-bone Autoantibodies by Western Blot Analysis

Western blot analysis confirmed the presence of anti-bone antibodies and a positive correlation between IgA antibodies and osteoporosis in patients with active CD at the time of diagnosis (Fig. 4, lanes 1 and 2). After treatment with a gluten-free diet, anti-bone immunoreactivity was completely or partially abolished (Fig. 5, lanes 3 and 4, respectively). Sera from CD patients,

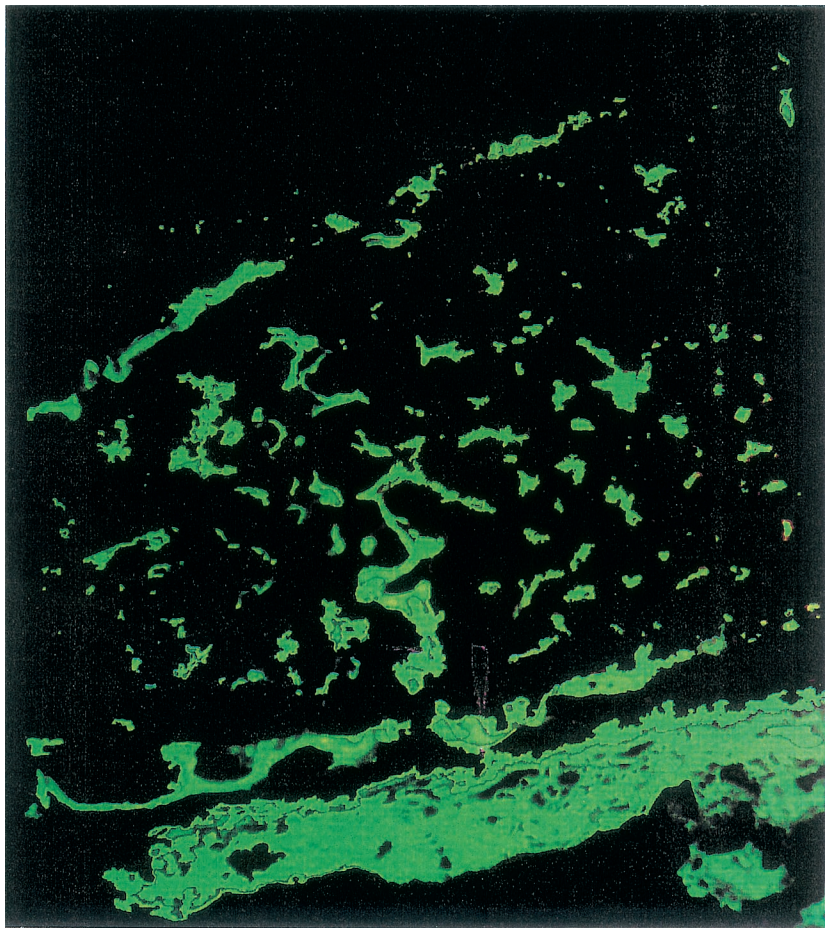

Fig. 3. Expression of bone-specific tTG in the fetal rat tibia (400× magnification). The photograph is focused on the provisional calcification zone.

which were previously preadsorbed with human recombinant tTG showed no reactivity with fetal rat tibia extracts (lanes 5 and 6), suggesting that bone immunoreactivity was raised against tTG. As controls, nonceliac patients suffering from osteoporosis and healthy controls did not show any immunoreactivity (Fig. 4, lanes 7 and 8, respectively). Representative immunoreactive profiles of nonceliac and celiac patients suffering from osteoporosis are shown.

Table II. Anti-bone Antibody Titers and Anti-tTG Values for Patients Grouped According to the Baseline Densitometric Values

	Bone mineral density		
	Normal (n = 15)	Osteopenia (n = 7)	Osteoporosis (n = 11)
Anti-bone antibodies (titers)			
Median	1:5	1:5	1:10
Range	0–1:40	1:5–1:40	0–1:1280
Percentiles			
25	0	1:5	1:5
75	1:20	1:40	1:160
Anti-tTG values (AU/ml)			
Median	66	71	188
Range	6–451	22–251	1–451
Percentiles			
25	13	28	4
75	252	243	282

DISCUSSION

The pathophysiological mechanisms inducing osteopenia and osteoporosis in CD patients are still unknown. Most studies in this area have focused on the metabolic pathway resulting from malabsorption (8, 9). Based on the fact that CD is due to an immunological disturbance, others and we have recently explored whether products of this imbalance could be implicated in the associated bone disease (14, 15). Herein, we used an indirect immunofluorescence approach and immunoblotting to investigate the presence of circulating anti-

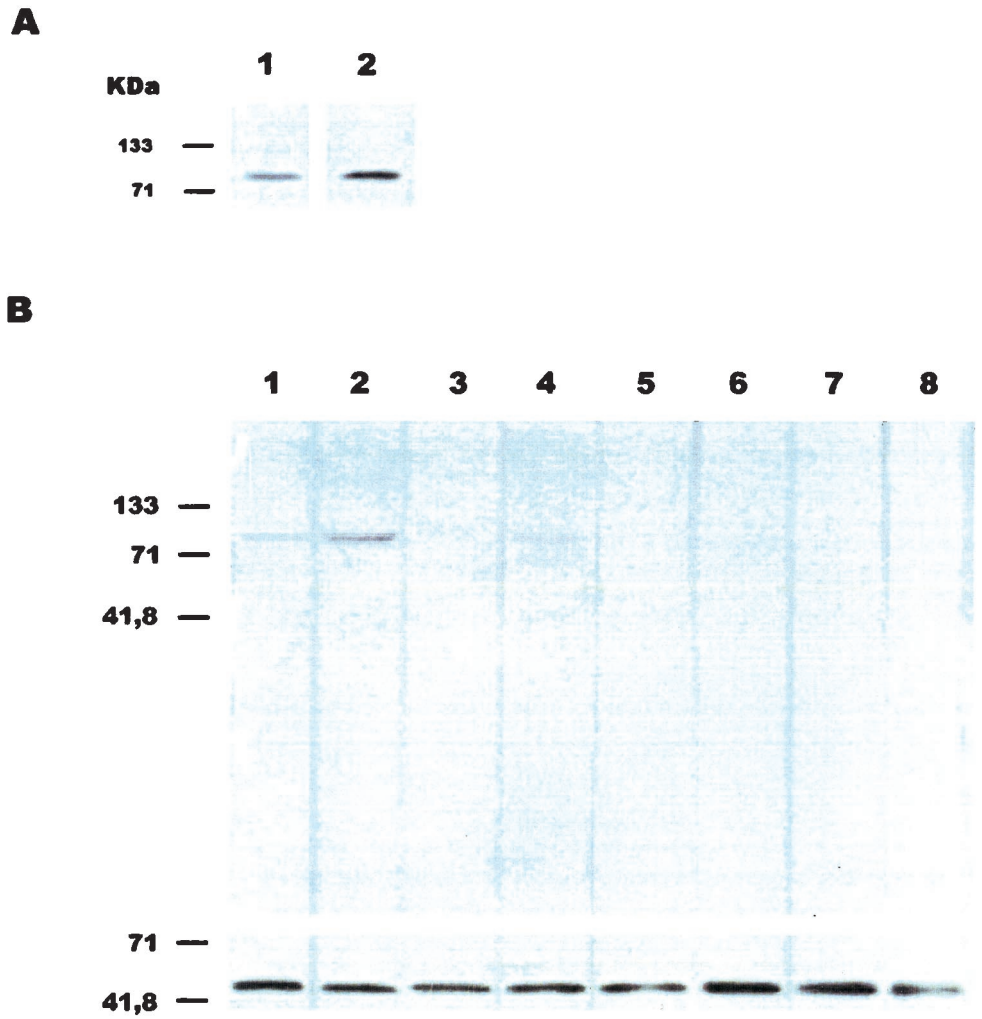

Fig. 4. Coomassie blue staining of protein profile (lane 1) and immunoreactive pattern (lane 2) of human tTG incubated with an anti-human tTG mAb (A). Immunoreactive profile of fetal rat tibia extracts incubated with (a) sera from untreated celiac patients with osteoporosis (lanes 1 and 2); (b) sera from the same patients after one year of a gluten-free diet (lanes 3 and 4, respectively); (c) sera from the same untreated patients previously adsorbed with human tTG (lanes 5 and 6); (d) sera from nonceliac patients with osteoporotic manifestations (lanes 7 and 8) (B). The reactive sera profile from the same patient under different conditions is shown in lanes 1, 3, and 5 or in lanes 2, 4, and 6, respectively. To confirm equal protein loading in each lane, samples also were blotted using an anti-rat α-tubulin (DM1A; lower panel, B). The result is a representative of two independent experiments.

bodies in sera from CD patients, which could bind to diverse structures of fetal rat tibia. We also aimed to partially characterize the antibodies and the antigenic structures responsible for the observed immunoreactivity. In spite of the possibility of species-specific differences, the rationale for using fetal rat tibia as substrate for detection of anti-bone antibodies was established by at least two facts. First, fetal rat tibia (20- to 28-day pregnancy) is a well-defined cartilage, where different stages of bone formation can be identified and which presents an intense and active mineralization process. On the other hand, fetal tibia results in adequate material for unfixed processing and cutting without prior abrasive handling.

In the present study we show evidence of the presence of bone-specific antibodies in sera from most patients with active CD, but not from the great majority of healthy and disease controls. Furthermore, serum titers of antibodies correlated with the degree of bone impairment of untreated patients and improved significantly or were negative after successful treatment with a gluten-free diet. Additionally, baseline titers of anti-bone antibodies determined by indirect immunofluorescence staining correlated significantly with values of anti-tTG

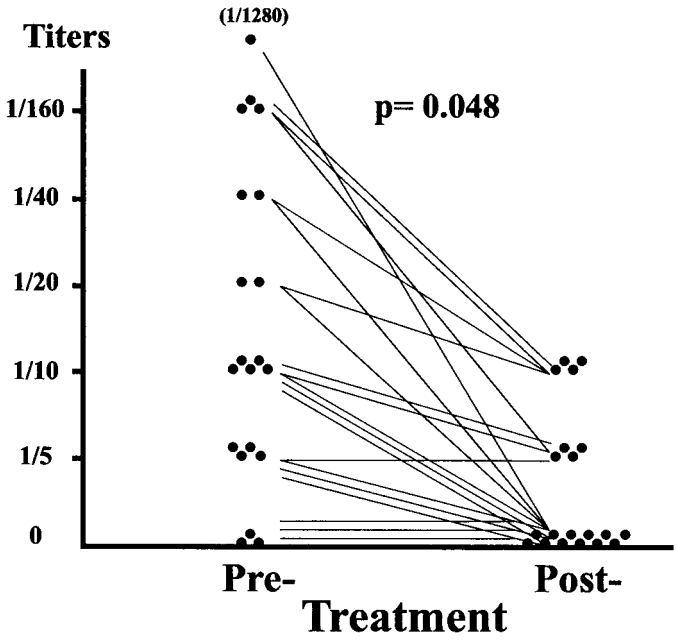

Fig. 5. Titers of anti-bone antibodies by indirect immunofluorescence staining at baseline and after treatment. Titers of anti-bone antibodies from 20 untreated celiac disease patients decreased after a period of a gluten-free diet, as shown by indirect immunofluorescence staining of fetal rat tibia incubated with sera from CD patients. The baseline frequency of cases with positive titers decreased significantly after treatment ($P = 0.048$).

antibodies detected by indirect ELISA. The immunostaining in bone substrates was demonstrated both on intracellular and extracellular locations. Thus, while intracellular staining was detected in hypertrophic chondrocytes of the proliferation/maturation area and around the cartilage/bone interface, the extracellular staining was localized in the matrix of the provisional calcification zone of the epiphysis and the perichondrium. All these areas of fetal bones are characterized by the presence of a very active provisional mineralization process. Using a monoclonal antibody to tTG on the same rat tibia substrates, staining was shown at the same areas and with a similar pattern to that observed for anti-bone antibodies. Taken together, these findings suggest that both antibodies might share the same epitopes. The immunoblotting assay confirmed the presence of antibodies in sera from CD patients and immunoreactivity was drastically reduced or abolished in treated cases. The assay also confirmed that anti-bone antibodies were not present in sera from healthy and disease controls. It also was shown that the bone antigenic protein that reacted with anti-bone antibodies exhibited a molecular weight of approximately 77–80 kDa in agreement with that shown by human recombinant tTG. Interestingly, neutralization of circulating anti-tTG (EmA) from sera of CD patients using human recombinant tTG completely abolished the presence of an immunoreactive band by Western blot analysis. Contrasting with these findings, when sera from CD patients with osteoporosis were incubated with recombinant human tTG, we could not observe a complete inhibition of immunoreactivity (data not shown) on rat tibia, but complete inhibition of EmA was detected on monkey esophagus substrate.

Under normal conditions, the bone remodeling process is influenced by interactions that include systemic and local factors. In the bone disorder of CD patients there is an imbalance between bone resorption and formation (with the former exceeding the latter). This process seems to be driven by a secondary hyperparathyroidism with high levels of parathyroid hormone (PTH) (8, 9). In addition to the suspected pathogenic effect of PTH, we have recently suggested that the hormone might participate in osteoporosis-generating changes in the bone structure and in the regional muscle mass and strength, all factors potentially affecting bone resistance to trauma (23). The likelihood that systemic immunological factors might affect distant tissues from the primary site of inflammation was highlighted by recent studies. It was suggested that circulating IL-6 and IL-1β, among other cytokines, might increase bone resorption (14). Hence,

the present results allow us to speculate that anti-bone antibodies could play a pathophysiological role in the osteopenia and osteoporosis associated with CD. To our knowledge, this hypothesis has not been suggested previously. We based this proposal on a clinical finding such as the presence of bone disease in clinically silent CD patients, who do not show signs of malabsorption (8, 11). While the presence of anti-bone antibodies in sera from CD patients was clearly established in this study, characterization of the specific autoantigen(s) eliciting this immunoreactivity is crucial in order to gain insight into the potential role of these autoantibodies in celiac osteopathy. The recent identification of tTG as the antiendomysial autoantigen (16) and the recognized physiological role for bone-specific tTG allowed us to hypothesize that EmA could be the anti-bone antibody and tTG might be the autoantigen. Interestingly, calcifying bones show a restricted expression of tTG (18, 19). This enzyme first appears as an intracytoplasmatic molecule during the maturation process of hypertrophic chondrocytes (18). Externalization of tTG is produced before the onset of mineralization, when it has a specific and key role in influencing maturity of the bone/cartilage matrix and producing stabilization and mineralization of the substrate (18). According to these findings, one may hypothesize that tTG could be one of the targets for anti-bone antibodies. If so, a pathogenic role might be suggested in bone demineralization of CD patients. In this context, *in situ* neutralization of the enzyme would prevent deposit of calcium and hydroxyapatite. Interestingly and further supporting this hypothesis, we observed similar staining patterns for both anti-bone antibodies and bone-specific tTG. In order to determine whether tTG is indeed the autoantigen recognized by anti-bone antibodies, we performed Western blot analysis and immunofluorescence tests using sera from CD patients that were previously preabsorbed with human recombinant tTG. We could not find immunoreactivity in preabsorbed sera using the Western blot assay. In contrast, when sera from CD patients with osteoporosis were preincubated with recombinant human tTG, we could not observe an inhibition of immunoreactivity (data not shown). These observations suggest that other target autoantigens might be involved in bone immunoreactivity or that sera from CD patients may recognize other conformational epitopes absent in denatured/dissociated proteins processed by Western blot analysis. Alternatively, the bone matrix may have plentiful binding sites for the tTG used for blocking, thus serving as a mediator of the autoantibody binding rather than an inhibitor.

Consistent with the idea that anti-bone antibodies and EmA are not the same antibodies is the observation that the prevalence of positive EmA and anti-tTG antibodies (84% and 91%, respectively) was higher than that shown for anti-bone antibodies (51.5%). However, this difference is not unusual for this type of antibody and previously has been observed comparing sensitivity for EmA and antireticulin antibodies (24). The most probable explanation for this discrepancy is the possibility of species-specific and organ-specific differences between substrates, as suggested by Maki (24). It also is likely that this difference may be related to the fact that osteoporosis is in part associated to other nonimmunological mechanisms. Accordingly, only a percentage of celiac disease patients will show the presence of anti-bone autoantibodies.

In conclusion, we show original evidence that most patients with CD have circulating autoantibodies against bone structures and that bone tTG may to be one of the autoantigens involved in sera immunoreactivity. High titers of antibodies correlate with the most severe bone impairment and are significantly reduced after treatment. Due to the coincidental localization of anti-bone antibodies with areas of active mineralization, we suggest a possible role for these antibodies in bone disorders. However, further studies are necessary to characterize in depth the implicated self-antigen and to identify its pathogenic role in osteopenia and osteoporosis associated with CD.

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REFERENCES

1. Marsh MN: The natural history of gluten sensitivity: Defining, refining and redefining. Q J Med 85:9–13, 1995
2. Marsh MN: Gluten, major histocompatibility complex, and the small intestine: A molecular and immunobiologic approach to the spectrum of gluten-sensitivity (“celiac sprue”). Gastroenterology 102:330–354, 1992
3. Maki M, Collin P: Coeliac disease. Lancet 349:1755–1759, 1997
4. Scott H, Kett K, Halstensen TS, Hvatum M, Rognum TO, Brandtzaeg P: The humoral immune system in coeliac disease. *In* Coeliac Disease, MN Marsh (ed). Oxford, Blackwell Scientific Publications, 1992, pp 239–282
5. Chorzelski T, Beutner EH, Suley J, Tchorzewska H, Jablonska S, Kumar V, Kapuscinska A: IgA antiendomysium antibody. A new

immunological marker of dermatitis herpetiformis aand coeliac disease. Br J Dermatol 111:395–397, 1984
6. Schuppan D: Current concepts of celiac disease pathogenesis. Gastroenterology 119: 234–242, 2000
7. Pedreira S, Vazquez H, Sugai E, Niveloni S, Smecuol E, Mazure R, Flores D, Mauriño E, Bai JC: Clinical significance of anti-smooth muscle antibody (SMA) fluorescence in patients with celiac disease. Gastroenterology 118; A363, 2000
8. Vazquez H, Mazure R, Gonzalez D, Flores D, Pedreira S, Niveloni S, Smecuol E, Mauriño E, Bai JC: Risk of fractures in celiac disease patients: A cross-sectional, case–control study. Am J Gastroenterol. 95:183–189, 2000
9. Gonzalez D, Mazure R, Mautalen C, Vázquez H, Bai JC: Body composition and bone mineral density in untreated and treated patients with celiac disease. Bone 16:231–234, 1995
10. Corazza GR, Di Sario A, Cecchetti L, Tarozzi C, Corrao G, Bernard M, Gasbarrini G: Bone mass and metabolism in patients with celiac disease. Gastroenterology 109:122–128, 1995
11. Mazure R, Vázquez H, Gonzalez D, Mautalen C, Pedreira S, Boerr L, Bai JC: Bone mineral affectation in asymptomatic adult patients with celiac disease. Am J Gastroenterol 89:2130–2134, 1994.
12. Mustalahti K, Collin P, Sievanen H, Salmi J, Maki M: Osteopenia in patients with clinically silent coeliac disease. Lancet 354:744–745, 1999
13. Beckett CG, Dell'Olio D, Kontakou M, Przemioslo RT, Rosen-Bronson S, Ciclitira PJ: Analyses of interleukin-4 and interleukin-10 and their association with the lymphocytic infiltrate in the small intestine of patients with coeliac disease. Gut 39:818–823, 1996
14. Fornari MC, Pedreira S, Niveloni S, Gonzalez D, Diez RA, Vazquez H, Mazure R, Sugai E, Smecuol E, Boerr L, Maurino E, Bai JC: Pre- and post-treatment serum levels of cytokines IL-1β, IL-6 and IL-1 receptor antagonist in celiac disease. Are they related to the associated osteopenia? Am J Gastroenterol 93:413–418, 1998
15. Baldimarson T, Arnqvist HJ, Toss G, Jarnerott G, Nystrom F, Strum M: Low circulating insulin-like growth factor 1 in coeliac disease and its relation to bone mineral density. Scan J Gastroenterol 34:904–908, 1999
16. Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO, Schuppan D: Identification of tissue transglutaminase as the autoantigen of coeliac disease. Nat Med 3: 797–801, 1997
17. Aeschlimann D, Paulsson M: Transglutaminases: Protein cross-linking enzymes in tissues and body fluids. Thromb Haemostasis 71:402–415, 1994
18. Aeschilmann D, Kaupp O, Paulsson M: Transglutaminase-catalyzed matrix cross-linking in differentiating cartilage: Identification of osteonectin as a major glutaminyl substrate. J Cell Biol 129:881–892, 1993
19. Aeschilmann D, Watterwald A, Fleisch H, Paulsson M: Expression of tissue transglutaminase in skeletal tissues correlates with events of terminal differentiation of chondrocytes. J Cell Biol 120:1361–1370, 1993
20. Sugai E, Salveggio G, Vazquez H, Viola M, Mazure R, Pizarro B, Smecuol E, Flores D, Pedreira S, Mauriño E, Gomez JC, Bai JC: Tissue transglutaminase antibodies in celiac disease: Assessment of a commercial kit. Am J Gastroenterol 95:2318–2322, 2000
21. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970
22. Towbim H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354, 1979
23. Ferretti JL, Mazure R, Tanoue P, Marino A, Cointri G, Niveloni S, Pedreira S, Mauriño E, Zanchetta J, Bai JC: Combined analysis of metabolic and structural (QCT) indicators explains the musculoskeletal affectation in celiac disease. (Abstr) Gastroenterology 118: 363A, 2000
24. Maki M: Use of serological antibody tests in celiac disease. *In* D Branski, P Rozen, MF Kagnoff (eds). Res Basel, Karger; 1992, vol 19, pp 108–129