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Oral mucosa of coeliac disease patients produces antiendomysial and antitransglutaminase antibodies: the diagnostic usefulness of an *in vitro* culture system

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SUMMARY

Background
Antiendomysial (EmA) and antitransglutaminase (anti-tTG) antibodies are the most specific indirect marker of coeliac disease (CD). It is not known whether the oral mucosa of patients with CD is able to produce these antibodies or not.

Aims
To evaluate the ability of the oral mucosa of patients with CD to produce antibodies in an *in vitro* culture system.

Patients and methods
Twenty-eight patients with new diagnosis of CD (15 adults and 13 children) and 14 adult subjects with other diseases (controls) were studied. All underwent oral mucosa biopsy and subsequent EmA and anti-tTG assays on the mucosa culture medium.

Results
Sensitivity and specificity of EmA and anti-tTG assayed in the oral mucosa culture medium for CD diagnosis were 54% and 100% and 57% and 100%, respectively. The CD clinical presentation, such as the presence of oral mucosa lesions, did not influence the results of the EmA and anti-tTG assays in the oral mucosa culture medium. There was an association between positivity of antibodies and greater severity of the oral mucosa lymphocyte infiltration.

Conclusion
This study demonstrates that the oral mucosa contributes to EmA and anti-tTG production in untreated patients with CD.

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INTRODUCTION

Celiac disease (CD) is one of the most common chronic diseases among Caucasians, with a frequency in the general population between 1:85 and 1:300 in both Europe and the US.[1–5] According to current criteria, CD diagnosis is based on the combination of clinical, histological and serologic data.[6, 7] In fact, it is necessary to demonstrate intestinal villous atrophy and the presence of circulating antiendomysial (EmA) and/or antitransglutaminase (anti-tTG) antibodies on a gluten-containing diet and the subsequent disappearance of symptoms on gluten-free diet, although there is evidence that a percentage of patients with CD can have minimal intestinal histology damage and/or negative serum EmA and anti-tTG at CD diagnosis.[8–11]

In any case, an endoscopic approach with intestinal biopsy for the histological diagnosis is still necessary,[12] although this requires an invasive procedure. A site which could be studied with less invasiveness is the mouth. In fact, previous reports on the oral mucosa showed that the challenge performed in treated patients with CD, either supramucosally with gliadin powder or by submucosal injection of dissolved gliadin, determined an inflammatory reaction.[13, 14]

The aims of this pilot study were to evaluate: (i) whether the oral mucosa of patients with CD is able to produce EmA and anti-tTG in an *in vitro* culture system or not, (ii) the possible clinical usefulness of EmA and anti-tTG *in vitro* production evaluated by oral mucosa culture.

PATIENTS AND METHODS

Patients with new CD diagnosis observed in three centres (one for paediatric and two for adult patients), between June 2004 and May 2005, were randomly invited to enter the study. Out of a total of 105 new diagnoses, 51 patients were asked to participate and 28 subjects accepted and were recruited. Of these, 15 were adults (10 F, 5 M, median age 39 years, range 19–73 years) and 13 children (10 F, 3 M, median age 7 years, range 1–16 years). The CD clinical presentation was typical (malabsorption syndrome) in 14 patients, atypical (absence of intestinal symptoms) in 12 and silent in two (patients identified through screening programs).[7]

In all patients, CD diagnosis was based on serum EmA and anti-tTG positivity associated with evidence of intestinal villi damage (villous height/crypt depth ratio < 3), and in all cases on a subsequent gluten-free diet clinical symptoms disappeared and EmA and anti-tTGs became negative.

Furthermore, 14 adult patients (8 F, 6 M, median age 36.5 years, range 21–70 years) with symptoms compatible with CD diagnosis but negative for serum EmA and anti-tTG assays were enrolled. They were being investigated for the presence of one or more of the following symptoms: weight loss or failure to thrive, anaemia, chronic diarrhoea, or abdominal pain. All underwent oesophago-gastro-duodenoscopy (OGDS) with duodenal histology examination, oral mucosa biopsy and EmA and anti-tTG assays in both the duodenal and oral mucosa culture medium. All showed normal histology of the intestinal mucosa and were considered control subjects. In these cases, the complete diagnostic work-up may also have included routine haemato-chemical assays, a thyroid study, serum autoantibodies assay, abdominal ultrasonography and/or computed tomography, colonoscopy, small-intestine barium examination, H2 breath test, duodenal fluid microbiological evaluation and bone marrow biopsy.

All the adult patients included in the study were followed as out-patients, whereas the children were hospitalized.

The protocol was approved by the Ethics Committee of the University Hospital of Palermo and informed consent was obtained from the patients involved in the study or from the parents in the case of the paediatric patients.

Serology for CD diagnosis

Serum IgA was measured by ELISA to exclude IgA deficiency. IgA EmAs and anti-tTGs were assayed using commercial kits as previously described.[15]

Intestinal and oral biopsies and histology study

Following general anaesthesia (in children) or conscious sedation with meperidine/pethidine 1 mg/kg i.v. and midazolam 0.05–0.1 mg/kg i.v. (in adults), OGDS were performed with standard paediatric or adult video-colonoscopes (Olympus or Fujinon or Pentax, Milan, Italy). Six duodenal biopsy samples were obtained from each patient. Four samples underwent routine histology evaluation, and two specimens were cultured for 72 h at 37 °C with a commercial reagent set (anti-Endomysium-biopsy, Eurospital), as

described previously.[16, 17] One sample was cultured in the presence of the 31–43 gliadin peptide (0.1 g/L) and the other without its addition. Culture supernatants were collected and stored at −80 °C until used. IgA EmA antibodies in undiluted supernatants were determined by the same commercial reagent set used for serum EmA.

IgA anti-tTG antibodies were determined using a commercial ELISA as recently described.[16] In brief, recombinant human-t TG (h-tTG) antigen diluted in phosphate-buffered saline was used to coat the wells. The culture medium was diluted 1:5. The conjugate was diluted to obtain reliable optical density (OD). Absorbance was read in a microplate reader at 450 nm. Anti-tTG values in the supernatants were expressed as OD. Normal values were taken as <300, representing a value >2 s.d. above the mean of 200 healthy individuals. The intra-assay CV for the IgA h-tTG autoantibody ELISA on culture medium was 4.2% (n = 20), and the inter-assay CV was 5.9% (n = 20).

Oral mucosa biopsy specimens were taken at the same time as the OGDS in children and under local anaesthesia (xylocain adrenalin) in adults. The specimens were taken in the mouth, at the second molar tooth region, beneath the occlusal line. Each sample was divided into four parts: two were fixed to be processed for histology evaluation and two were cultured with the procedure described above for the EmA assay in the duodenal mucosa culture medium. Before culture, biopsy samples were weighed (minimal weight required 5 mg/fragment) and washed.

Previous studies in which IgA EmAs and anti-tTG antibodies were first assayed on fresh medium and then 6 months later after storage at −80 °C, had shown that storage did not alter the results obtained, as EmA and anti-tTG results were identical.[16, 17] IgA EmA and anti-tTG assays on the culture media were performed by personnel unaware of the clinical and laboratory data of the patients.

Biopsy specimens of the intestinal mucosa adequate in size were immediately oriented with a stereomicroscope and subsequently embedded in paraffin.[15–17] The slides were stained with haematoxylin and eosin and graded by conventional histology according to Oberhuber *et al.*[18]

Biopsy specimens of the oral mucosa were embedded in paraffin and the slides stained with haematoxylin and eosin; lamina propria lymphocytes infiltration was arbitrarily graded as mild when ≤10 lymphocytes × 5 HPF (magnification 40×) were counted, and severe when there were >10 lymphocytes × 5 HPF.

STATISTICAL ANALYSIS

We followed the STARD checklist for studies on the diagnostic accuracy of tests.[19] The sensitivity, specificity and diagnostic accuracy values of the diagnostic procedures examined were calculated by standard statistical methods.[20] The Fisher exact test was used to compare the different sensitivity, specificity and diagnostic accuracy values of the assays. The chi-squared test for trend was calculated to compare the frequency of CD cases showing mild, moderate or severe intestinal villous atrophy with or without EmA or anti-tTG positive assays in the culture medium.

RESULTS

None of the patients enrolled showed IgA deficiency. The final diagnoses of the patients with negative serum EmA and normal intestinal histology were: multiple food intolerance (three cases), Crohn's disease (two cases), sideropenic anaemia (three cases), major recurrent aphtous stomatitis (3 cases), liver cirrhosis (two cases), duodenal ulcer (one case).

The results of the IgA EmA and anti-tTG assayed on the duodenal and oral mucosa culture were identical and remained unchanged when the 31-43 peptide was added to the culture medium. A cross-tabulation of the EmA assay results on the culture medium of the oral and duodenal biopsies obtained in paediatric and adult patients, according to the final diagnoses, is shown in Table 1. EmA assayed on the duodenal mucosa culture medium gave results completely concordant with the final diagnoses, as they were positive in all patients with CD and negative in all patients not suffering from CD. EmA assayed on the oral mucosa culture medium was positive in 15 of the 28 patients with CD (53.6%).

Also anti-tTG antibodies assayed on the duodenal mucosa culture medium were positive in all patients with CD and negative in all patients not suffering from CD. Anti-tTG antibodies assayed on the oral mucosa culture medium were positive in 16 of the 28 patients with CD (57%) (Figure 1).

Table 2 shows the sensitivity, specificity and diagnostic accuracy of the EmA and anti-tTG assays on the duodenal or oral mucosa culture medium in CD diagnosis. As shown in Table 3, CD clinical

Table 1. Cross-tabulation of the antiendomysial (EmA) assay results on the culture medium of the duodenal and oral biopsies, in adult and pediatric subjects, according to the final diagnoses

	EmA assay on duodenal mucosa culture medium			EmA assay on oral mucosa culture medium		
	Celiac disease (CD) diagnosis (*n*)	Non-CD diagnosis (*n*)	Total no.	CD diagnosis (*n*)	Non-CD diagnosis (*n*)	Total no.
Adults						
Positive test	15	0	15	9	0	9
Negative test	0	14	14	6	14	20
Total	15	14	29	15	14	29
Children						
Positive test	13	0	13	6	0	6
Negative test	0	0	0	7	0	7
Total	13	0	13	13	0	13

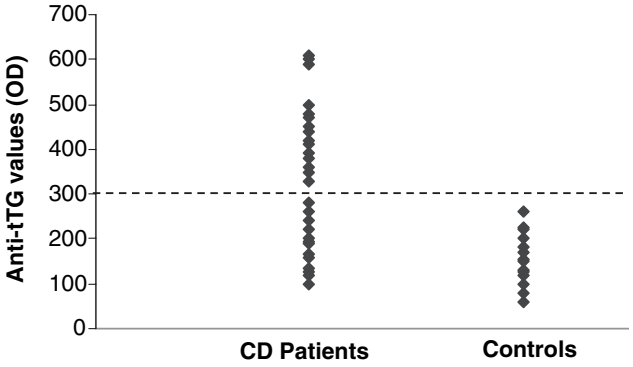

Figure 1. Individual values of antitransglutaminase antibodies assayed in the oral mucosa culture medium, in 28 celiac patients and in 14 patients not suffering from celiac disease. Values are expressed as optical density (OD). Dotted line indicates the upper normal limit.

presentation did not influence the behaviour of the EmA and anti-tTG assays in the oral mucosa culture medium. The presence of oral mucosa lesions did not influence the EmA and anti-tTG assays on the oral biopsy culture medium either. In fact, we observed recurrent aphtous stomatitis in four patients with CD and in four adult controls (three with final diagnosis of major aphtous stomatitis and one with Crohn's disease); only one of the four patients with CD and none of these four controls were positive for EmA and anti-tTG antibodies.

Figure 2 shows the number of lamina propria lymphocytes in the oral mucosa of patients with CD and controls. A greater severity of inflammation of the oral mucosa was observed in the patients with CD with positive EmA or anti-tTG in the oral mucosa culture medium (Table 4). In fact, the frequency of positive assays was significantly higher in the patients with severe inflammation: $P < 0.01$ (Fisher's test).

DISCUSSION

Although nowadays there is general agreement that CD must be considered a systemic disease and may affect several extra-intestinal organs, the intestinal mucosa examination is still considered the corner stone of a definitive CD diagnosis.[6, 7, 12, 21] However, the small bowel biopsy is an invasive procedure which many patients – especially if asymptomatic or suffering from only mild symptoms – find hard to accept. In this respect, a study of the potential diagnostic use of the oral mucosa could be of great interest. In fact, the oral mucosa is very often damaged in CD[22] and previous studies in patients with CD on gluten-free diet showed that both the oral supramucosal application and submucosal injection of gliadin powder or gliadin peptides induced significant immunological changes.[13, 14]

As previous studies have demonstrated that EmA and anti-tTG assays on duodenal mucosa culture medium have an excellent diagnostic accuracy in CD diagnosis,[16, 17, 23] we aimed to evaluate the ability of the oral mucosa in patients with CD to produce EmA and anti-tTG in a culture system, comparing results with those obtained on duodenal mucosa culture.

Our results showed that the oral mucosa of patients with a new CD diagnosis, still on a gluten-containing

Table 2. Sensitivity, specificity and diagnostic accuracy in celiac disease (CD) diagnosis of the antibodies assays on the culture medium of the duodenal and oral mucosa biopsies. The study groups included 15 adult patients with CD, 13 pediatric patients with CD and 14 adult controls

	Sensitivity	Specificity	Diagnostic accuracy
Antiendomysial (EmA) assay in duodenal mucosa culture medium			
Adults	100%	100%	100%
Children	100%	Not evaluated	Not evaluated
Total	100%	–	–
EmA assay in oral mucosa culture medium			
Adults	60%	100%	79%
Children	46%	Not evaluated	Not evaluated
Total	53.6%	–	–
Antitransglutaminase (anti-tTG) assay in duodenal mucosa culture medium			
Adults	100%	100%	100%
Children	100%	Not evaluated	Not evaluated
Total	100%	–	–
Anti-tTG assay in oral mucosa culture medium			
Adults	60%	100%	79%
Children	54%	Not evaluated	Not evaluated
Total	57%	–	–

Table 3. Results of antiendomysial (EmA) and antitransglutaminase (anti-tTG) assays in the oral mucosa culture medium according to the different clinical presentations in 28 celiac disease (CD) patients

	Typical CD presentation	Atypical CD presentation	Silent CD
Number of EmA positive assays	7 cases	7 cases	1 case
Number of EmA negative assays	7 cases	5 cases	1 case
Number of anti-tTG positive assays	8 cases	7 cases	1 case
Number of anti-tTG negative assays	6 cases	5 cases	1 case

The CD clinical presentation was considered typical when there was malabsorption syndrome, atypical in the absence of intestinal symptoms, and silent in the absence of symptoms.

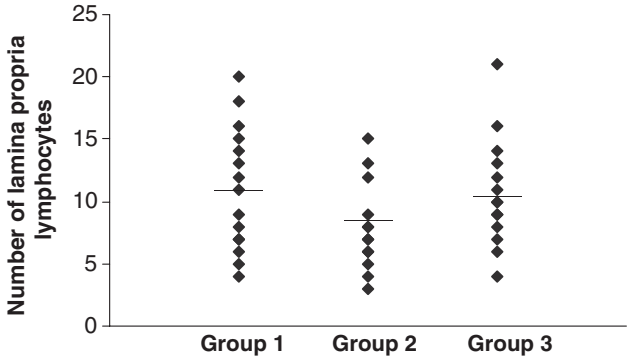

Figure 2. Number of the lamina propria lymphocytes in the oral mucosa in 15 celiac disease (CD) patients with positive antiendomysial (EmA) assay in the oral mucosa culture medium (GROUP 1), in 13 patients with CD with negative EmA assay in the oral mucosa culture medium (GROUP 2) and in 14 patients without CD (GROUP 3). Lines indicate the mean values.

diet, was able to produce EmA and anti-tTG in the culture medium, with or without *in vitro* gliadin stimulation. In fact, EmA and anti-tTG assays were positive in 15 and 16 out of 28 patients with CD respectively. This result is in keeping with the theory that the mouth is part of the gut-associated lymphoid tissue (GALT) system[24] and, although its epithelium is very different from that of the gut, the dissemination of the immune effector cells involves the oral mucosa and can determine a gliadin-induced response in patients with CD.

However, in general, the sensitivity of CD-specific antibodies assay in the oral mucosa culture medium was low, with EmA assay more sensitive in adults (60%) than in children (46%). One child negative for EmA assay on oral culture medium resulted to be positive for anti-tTG assay. The sensitivity of the assays was not influenced by clinical presentation as it did

Table 4. Results of antiendomysial (EmA) and anti-transglutaminase (anti-tTG) assays in the oral mucosa culture medium according to the severity of the lymphocytes infiltration in the lamina propria of the oral mucosa

	Mild inflammation	Severe inflammation
Number of EmA positive assays	7 cases	8 cases
Number of EmA negative assays	10 cases	3 cases
Number of anti-tTG positive assays	7 cases	9 cases
Number of anti-tTG negative assays	10 cases	2 cases

Lamina propria lymphocytes infiltration was graded as mild when ≤10 lymphocytes × 5 HPF (magnification 40×) were counted, and severe when there were >10 lymphocytes × 5 HPF.
The frequency of EmA positive assays was significantly higher in the patients with severe inflammation: $P < .01$ (Fisher's test).

not differ between patients with typical, atypical or silent disease. Furthermore, the presence of 'macroscopic' oral manifestations was not relevant as four patients with CD were suffering from recurrent aphtous stomatitis at the moment of the study but only one of these was positive for both EmA and anti-tTG assays on oral mucosa culture medium. There was a positive correlation between severity of the lymphocytes infiltration in the lamina propria of the oral mucosa and EmA or anti-tTG positivity in the culture medium. This aspect is similar to the well-known observation that there is a positive correlation between the severity of intestinal histology and the presence of serum EmA in CD.[17, 25]

Antiendomysial and anti-tTG assays on the oral mucosa culture medium showed a specificity of 100%, as we did not observe false positive results. It must be underlined that even the presence of recurrent aphtous stomatitis – present in four controls – did not determine false positive EmA/anti-tTG results. Obviously, as we included few control patients, further studies are needed to confirm the absolute specificity of the EmA or anti-tTG assays on the oral mucosa culture medium.

A previous study of the oral mucosa histology of untreated patients with CD did not show any significant histological alterations, which could help diagnosis and the oral gluten challenge performed on patients with CD on gluten-free diet showed a sensitivity of 73% and specificity of 80%.[26] However, the immuno-histochemical methods used in previous studies[13, 14] were more cumbersome and time-consuming than the simple antibodies assay in the culture medium. Furthermore, the evidence that anti-tTG antibodies can be effectively assayed in the oral mucosa culture medium opens the way to an easy ELISA.

As regards antibodies assay on duodenal mucosa culture medium, our results are in keeping with all previous reports, which have underlined the excellent diagnostic value of this assay.[13, 14] The intestine is the main site of EmA production and both EmA or anti-tTG can also be assayed in the whole gut lavage fluid[27] or in the duodenal mucosa by immuno-histochemistry,[28, 29] giving relevant diagnostic help in cases of seronegative patients with CD.

In conclusion, this study demonstrated for the first time that the oral mucosa contributes to EmA and anti-tTG production in untreated patients with CD. As the search for easier diagnostic methods than the small intestine biopsy procedure is important, the antibodies assay on oral mucosa culture medium merits further evaluation.

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