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Production of Anti-Endomysial Antibodies in Cultured Duodenal Mucosa:

Usefulness in Coeliac Disease Diagnosis

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Background: Although anti-endomysial antibodies (EmA) have been found in the supernatants of cultured intestinal mucosa from patients with coeliac disease (CD), in no study has the clinical reliability of this new diagnostic tool been investigated. Our aims were to evaluate the clinical usefulness of the in vitro production of EmA in CD diagnosis in consecutive patients with suspected CD, and to evaluate the reliability of the in vitro challenge in CD patients on a gluten-free diet (GFD). **Methods**: For the former aim, consecutive patients who were due to undergo intestinal biopsy for suspected diagnosis of CD were enrolled; according to the final diagnosis, these patients were divided into two groups: Group 1 comprised 91 newly diagnosed CD patients (40 males; age range 7 months to 84 years), Group 2 included 100 subjects with diseases other than CD (44 males; age range 9 months to 76 years). For the latter aim, we also studied 21 CD patients on a gluten-free diet after 16–123 months (8 males; age range 3–51 years), with normal intestinal architecture (Group 3) and 22 patients who served as controls (12 males; age range 4–60 years) with gastroesophageal reflux disease-like symptoms (Group 4). All patients underwent determination of serum anti-gliadin (AGA) and EmA antibodies, histology evaluation of the intestinal biopsies and EmA assay in the supernatants of in vitro gliadin-challenged duodenal mucosa. **Results**: EmA assay in the supernatants showed a sensitivity and specificity of 96% and 100%, respectively; these were not significantly different from those observed for serum EmA (88% and 99%, respectively). However, EmA assay in the supernatants was useful in CD patients with mild intestinal histology lesions (infiltrative/hyperplastic type): in this subgroup it was positive in 9/12 of cases, but serum EmA was positive in only 2/12. As regards the reliability of the in vitro gliadin challenge, EmA production in supernatants was recorded only in 10/21 CD patients on a gluten-free diet. The patients with a positive in vitro challenge had a higher number of intra-epithelial lymphocytes than patients with a negative challenge. **Conclusions**: 1) EmA assay in the medium of cultured intestinal biopsy can detect gluten-sensitive enteropathy, characterized by an infiltrative/hyperplastic histological pattern, which is often associated with negative serum EmA. 2) The in vitro challenge in CD patients on a gluten-free diet detects EmA production in the culture medium only in half of the cases and other studies must be performed to evaluate whether EmA production after in vitro challenge can be considered a reliable test for confirming CD diagnosis.

Key words: Anti-endomysial antibodies; coeliac disease; culture; gliadin-challenge ; intestinal histology; sensitivity; specificity

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During the past few decades there has been an increase in the number of coeliac disease (CD) diagnoses, and population studies indicate a CD prevalence much higher than previously reported (1, 2). Certainly, this phenomenon is in part due to the advances in diagnostic means. These include the very recently described detection of anti-endomysial antibodies (EmA) in in vitro cultured intestinal explants from untreated CD patients and the opportunity to perform an in vitro gliadin challenge (3), thus avoiding the complaints and the psychological impact of the classical challenge. However, few studies have evaluated this method and few patients have undergone this new diagnostic test (3–5). Furthermore, the possibility of performing the in vitro challenge—the in vitro production of EmA by the duodenal mucosa of CD patients on a gluten-free diet stimulated with gliadin in standard conditions—has not always been confirmed (4). The present study was performed to investigate the clinical usefulness of the in vitro production of EmA in CD diagnosis in a large number of

consecutive patients with suspected CD. We also evaluated the reliability of the in vitro challenge in CD patients on a gluten-free diet (GFD).

Patients and Methods

A total of 234 patients were included in the study, one part to evaluate the diagnostic accuracy of serum and cultured EmA assays, the other to evaluate the reliability of the in vitro challenge.

Patients enrolled to evaluate EmA diagnostic accuracy in sera and supernatants

We enrolled 191 consecutive patients, including both children and adults, who had undergone intestinal biopsy for suspected diagnosis of CD at the Division of Internal Medicine of the University Hospital or at the Pediatric Division of the 'Di Cristina' Hospital, both in Palermo, between May 1998 and April 1999. The patients included presented one or more of the following symptoms: poor growth or weight loss (97 cases), anaemia (82 cases), chronic diarrhoea (82 cases), abdominal pain (71 cases) alternating bowel habits (61 cases), constipation (20 cases), vomiting (8 cases). Exclusion criteria were: (a) presence of clinical signs or laboratory data which could indicate a diagnosis other than CD as more probable (ESR >15, positivity of serum reactive C protein, presence of blood in stools, fever); (b) a previous serological or histological evaluation for suspected CD. According to the final diagnosis, these patients were divided into two groups: Group 1 comprised 91 newly-diagnosed CD patients (40 males; age range 7 months to 84 years, median 2 years) and Group 2 included 100 subjects with normal intestinal morphology (villi/crypts ratio >2.5–3 for children, >3 for adult patients) and diseases other than CD (44 males; age range 9 months to 76 years, median 21 years).

CD diagnosis in the Group 1 patients was based on the evidence of clinical symptoms and intestinal histology damage (inflammatory infiltration of the mucosa with enlarged crypts and/or intestinal villous atrophy) on a gluten-containing diet, disappearance of the symptoms and normalization of the intestinal histology on a gluten-free diet and reappearance of the symptoms on gluten-challenge. Table I specifies the number of paediatric and adult patients recruited and the final diagnoses of the control patients (Group 2).

Patients enrolled to evaluate the reliability of the in vitro challenge

Two other patient groups were formed to evaluate the reliability of the in vitro challenge in CD patients on a gluten-free diet. Group 3 included 21 patients on a gluten-free diet after 16–123 months (8 males; age range 3–51 years, median 5 years), with normal intestinal architecture; Group 4 included 22 patients who served as controls (12 males; age range 4–60 years, median 33 years), with gastroesophageal reflux disease-like symptoms (epigastric pain, regurgitation) who underwent esophago-gastroscopy. These patients were negative for serum EmA and anti-gliadin antibodies (both IgA and IgG) and had a normal intestinal histology and no family history of CD.

Table I. Number of paediatric and adult patients included in the study and final diagnoses of the subjects considered as controls

	No. of cases
Children	100
Coeliacs	60
Total controls	40
Multiple food intolerance	18
Irritable bowel syndrome	13
Intestinal giardiasis	9
Adults	91
Coeliacs	31
Total controls	60
Irritable bowel syndrome	57
Crohn disease	3

CD diagnosis in the Group 3 patients was based on the criteria of the ESPGHAN (6). The patients of Group 4 were found to have peptic esophagitis (18 cases) or gastric erosions/ulcers (4 cases).

The protocol was approved by the ethics committee of the 'Di Cristina' Hospital of Palermo and informed consent was obtained from adult patients or from the parents of the children involved in the study.

Serum anti-gliadin (AGA) and anti-endomysial (EmA) antibodies determination

Serum levels of both IgG and IgA AGA were determined as previously described (7), with a commercially available enzyme-linked immunosorbent technique (Alpha-Gliatest, Eurospital Pharma, Trieste, Italy). Results were expressed as a percentage of reference serum; 20% was the upper normal limit for IgG and 10% for IgA antibodies. These values are equal to the mean $\pm$ 2 *s* (standard deviation) of the values obtained in our laboratory in a large group of age-matched normal subjects.

IgA-class EmA values were determined with a commercially available indirect immunofluorescence technique on monkey oesophagus (Anti-endomisio, Eurospital Pharma, Trieste, Italy), as previously described (8). IgA EmA titres were semi-quantified as follows: 0 = not detectable, 1 = positive at dilutions between 1/5 and 1/20, 2 = positive between 1/40 and 1/80, 3 = positive at 1/100, 4 = positive at 1/200, 5 = positive at dilutions >1/200. Total immunoglobulins (IgA, IgM, IgG) were also measured by ELISA.

IgA anti-endomysial antibody determination after intestinal mucosa in vitro culture

This test was performed in accordance with the method described by Picarelli et al. (5), using a commercially available kit (Antiendomysium-biopsy, Eurospital, Trieste, Italy). Briefly, a specimen of small intestinal mucosa was

incubated immediately after biopsy in a culture medium for 48 h at 37 °C in the presence of the gliadin peptide corresponding to the 31–43 (Leu-Gly-Gln-Gln-Pro-Phe-Pro-Pro-Gln-Gln-Pro-Tyr) aminoacid sequence of -gliadin (1 mg/ml). After incubation, EmAs were detected in the culture supernatants, both undiluted and diluted 1:2, 1:4 and 1:8, and 40 μl of supernatant was applied to each section of monkey oesophagus for 45 min according to the same procedure followed for serum EmA determination. As for serum EmA, titres in the supernatants were also semi-quantified as follows: 0 = not detectable, 1 = positive without any dilution, 2 = positive at dilution 1:2, 3 = positive at 1:4, 4 = positive at 1:8, 5 = positive at dilutions >1:8.

These results were also blindly evaluated by two different observers with experience in immunofluorescence assay.

Intestinal biopsy and histology

In the children, biopsy specimens were obtained distally to the ligament of Treitz, with a multipurpose Crosby capsule positioned under fluoroscopy, as previously described (9); in the adults, biopsies were taken during gastroduodenoscopy. In all cases, at least four biopsy specimens, each weighing about 80 mg, were taken; two mucosa fragments were immediately incubated for in vitro study (one with and one without gliadin) and the others were studied for histology. Specimens adequate in size and orientation according to the criteria of Perera et al. (10) were embedded in paraffin and slides were stained with haematoxylin and eosin and graded by conventional histology as follows: Normal, corresponding to normal villi/crypts ratio and no evidence of enhanced inflammatory infiltrate; Grade I, corresponding to infiltrative/hyperplastic lesions with normal villi and enlarged crypts, associated with diffuse intraepithelial inflammatory infiltration (types 1 and 2, according to Marsh (11)); Grade II, corresponding to partial villous atrophy with enlarged, deep crypts; and Grade III, corresponding to total villous atrophy (type 3, according to Marsh (11)). The severity of the intestinal mucosa damage was semi-quantified as follows: 0 = normal mucosa; 1 = grade I lesions; 2 = grade II lesions; 3 = grade III lesions. Furthermore, the number of intraepithelial lymphocytes (IELs) per 100 villous epithelial cells was assessed as described by Ferguson et al. (12). (The highest normal value in our laboratory is equal to 25 IELs per 100 epithelial cells.)

In all cases histology was described by an examiner unaware of the clinical condition of the patients and of the laboratory test results.

Statistical Analysis

The levels of sensitivity and specificity of the methods examined were calculated by standard statistical methods (13). Sensitivity was defined as the ratio of the true positive to true positive plus false-negative values; specificity was defined as the ratio of true negative to true negative plus false-positive values. Spearman's *r* correlation coefficient was used to correlate serum EmA or culture EmA titres with the degree of intestinal histology damage. The Wilcoxon rank sum test was used to compare culture EmA titres without and with 31–43 peptide incubation in patients with newly diagnosed CD (Group 1). The Mann-Whitney U test was used to compare the number of IELs per 100 villous epithelial cells in CD patients on a gluten-free diet (Group 3). All data were analysed considering both the whole study group and separating children and adult patients. A P value <0.05 was considered to be significant.

Table II. Number of patients positive for serum anti-gliadin (AGA IgG and IgA), serum anti-endomysial antibodies (serum EmA), EmA antibodies in the culture medium without gliadin-peptide incubation (culture EmA) and with gliadin-peptide incubation (culture EmA + gliadin), in patients with suspected coeliac disease, divided according to the final diagnosis: CD patients on gluten-containing diet (Group 1) and patients with gastrointestinal diseases other than celiac disease (Group 2)

	Group 1 ($n = 91$)	Group 2 ($n = 100$)
Serum AGA IgG	69/91 (76%)	25/100 (25%)
Serum AGA IgA	61/91 (67%)	10/100 (10%)
Serum EmA	80/91 (88%)	1/100 (1%)
Culture EmA	82/91 (90%)	0/100 (0%)
Culture EmA + gliadin	87/91 (96%)	0/100 (0%)

Results

EmA sensitivity and specificity in serum and supernatant assay

Table II gives the results of serum AGA and EmA and of intestinal mucosa EmA in vitro production in the 191 consecutive patients investigated for suspected CD (Group 1 plus Group 2), divided according to final diagnosis. As expected, the serum EmA assay had a higher diagnostic accuracy than the AGA assay: serum EmA were positive in 80/91 CD patients and in 1/100 gastrointestinal control (one patient with Crohn disease with serum EmA titre of 1:20). The false-negative results in CD (Group 1) were recorded in one patient with selective deficiency of serum IgA and in 10 patients with slightly abnormal intestinal mucosa architecture (villi/crypts ratio ranged between 3.1 and 2.5, with marked crypt hypertrophy) and with intestinal histology characterized by infiltration of lymphocytes and plasma cells in the lamina propria and in the mucosa (Fig. 1). Their intra-epithelial lymphocyte count ranged between 54 and 78 per 100 epithelial cells (infiltrative/hyperplastic lesion, Grade I according to the 'Methods' definition). Five of these patients were adults (age range 20–70 years) and six children (age range 2–13 years). They had one or more of the following symptoms: chronic diarrhoea (3 cases), anaemia (4 cases), poor growth (3 cases), abdominal pain (3 cases), and all patients were found to have the classical DQ2 haplotype. In all cases symptoms improved a few weeks after commencement of a gluten-free diet and disappeared during the following months. Furthermore, the intestinal biopsy per-

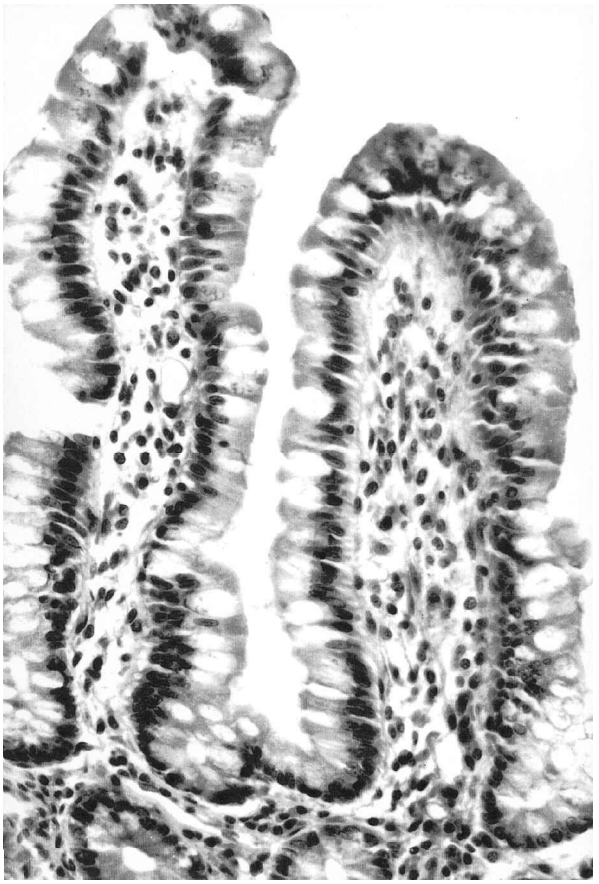

Fig. 1. Aspect of the intestinal mucosa in one of the coeliac disease patients with Grade I lesion histology (according to the Methods definition). Normal villi with lymphocyte infiltration (×50) can be seen. This histology aspect was typical for all the studied cases negative for serum EmA but positive for cultured EmA assay.

formed after 12 months on GFD showed a normal villi/crypts ratio (values ranged between 3.5 and 4.8), without any inflammatory infiltration in the lamina propria and in the mucosa (IEL ranged between 11 and 21 per 100 epithelial cells), while symptoms reappeared in all patients during a subsequent gluten challenge. In the CD patients (Group 1), the in vitro tests for EmA production without 31–43 peptide incubation showed positive supernatants in 82/91 cases (all the patients positive for serum EmA plus two of the patients with the infiltrative/hyperplastic pattern of the intestinal mucosa). After incubation of the intestinal biopsy samples with the 31–43 peptide, EmA were positive in 87/91 cases. In the latter experiment, apart from the subject with IgA deficiency, only three CD patients were negative for EmA in the culture medium. None of the subjects in Group 2 (patients with gastrointestinal diseases other than CD) showed EmA positivity in the supernatants of cultured intestinal mucosa, either with or without 31–43 peptide incubation. Table III gives the sensitivity and specificity of the tests in the whole study group and separately for children and adults. As regards serum EmA sensitivity, it must be underlined that there was a great difference between CD patients with slight intestinal mucosa damage (Grade I histology) and CD patients with partial or total intestinal villous atrophy (Grades II and III). In fact, serum EmA were positive only in 2/12 CD subjects with Grade I histology and in 78/79 CD patients with Grade II/III histology. Thus, serum EmA sensitivity was almost 100% in CD with severe intestinal lesions but only 17% in patients with mild mucosa damage. However, in the latter subgroup of CD patients, EmA assay in the medium of cultured intestinal mucosa with the addition of the 31–43 peptide was positive in 9/12 cases (Fig. 2). Furthermore, five of the subjects negative for serum EmA but positive for cultured EmA assay were also negative on serum anti-transglutaminase assay with a commercial ELISA.

Concordance between the two observers in the evaluation of EmA in culture medium results as 'positive' or 'negative' was 98%; EmA titre evaluation was also significantly concordant ($r = 0.96$; $P < 0.0001$).

Further evidence of in vitro EmA production and relationship with intestinal histology

In the 82/91 CD patients (Group 1) with EmA production in the in vitro intestinal culture system without 31–43 peptide incubation, EmA titres in the supernatants ranged from 1:1 (positivity only in the undiluted medium) to 1:8 with a median value of 1:1; after in vitro incubation with the 31–43 peptide the EmA titre increased over the baseline culture condition in 38/82 samples, whereas in the other 44 EmA titres were

Table III. Sensitivity and specificity of the serological tests and the determination of in vitro EmA production by intestinal mucosa in the diagnosis of coeliac disease. Consecutive patients with suspected CD were studied

	Serum AGA IgG	Serum AGA IgA	Serum EmA	Culture EmA without gliadin	Culture EmA with gliadin
All cases ($n = 191$)					
Sensitivity	76%	67%	88%	90%	96%
Specificity	75%	90%	99%	100%	100%
Children ($n = 100$)					
Sensitivity	88%	78%	87%	88%	95%
Specificity	75%	90%	100%	100%	100%
Adults					
Sensitivity	52%	45%	90%	94%	97%
Specificity	75%	90%	98%	100%	100%

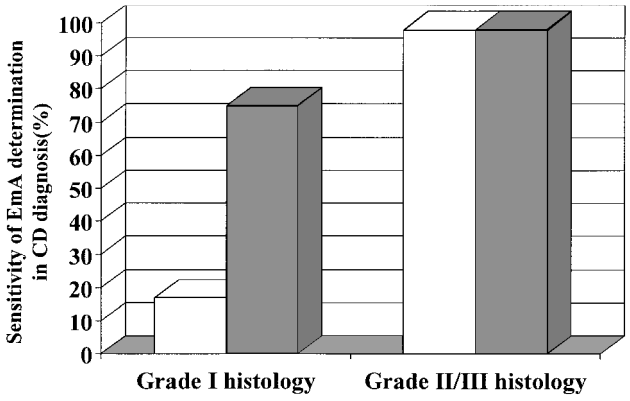

Fig. 2. Sensitivity of EmA determination in the serum and in the supernatant of cultured intestinal biopsies with addition of the 31–43 gliadin peptide in 91 coeliac disease subjects at diagnosis. The patients were divided according to severity of intestinal mucosa damage. Histology was graded as follows: Grade I, corresponding to infiltrative/hyperplastic lesions with normal vili and enlarged crypts, associated with diffuse intra-epithelial inflammatory infiltration; Grade II, corresponding to partial villous atrophy with enlarged, deep crypts and Grade III, corresponding to total villous atrophy. (□ Serum EmA; ■ cultured EmA).

unchanged: range titres 1:1 – >1:8, median 1:2 (Wilcoxon rank sum test: $z = 5.37$; $P < 0.0001$). No difference was observed between children and adult CD patients as regards the increase in EmA titre after incubation with the 31–43 peptide: there was a titre increase in 28/60 paediatric patients and in 10/31 adults.

When EmA titres of the cultured intestinal mucosa with the addition of the 31–43 peptide in the medium were analysed according to the severity of intestinal mucosa damage, we observed in CD patients on a gluten-containing diet (Group 1) a significant positive correlation between EmA in vitro production and the grade of histological mucosa damage ($r = 0.53$; $P < 0.0001$). Serum EmA titres of CD patients also positively correlated with severity of intestinal mucosa damage but had a lower r value than EmA titres of the medium culture: 0.42 ($P < 0.001$). The same trend was observed in both the paediatric and adult CD patients.

Reliability of the in vitro challenge in CD patients on a gluten-free diet

All but one of the 21 CD patients on GFD (Group 3) were negative for serum EmA, but this subject admitted to prolonged dietary lapses. EmA in the culture medium of intestinal mucosa without 31–43 peptide addition were positive only in this patient (at a titre of 1:1); he was an adult with normal intestinal mucosa architecture (villi/crypts ratio 3.5) but with intense inflammatory infiltration of the mucosa (IEL count = 54). When the intestinal mucosa of the CD patients on GFD was incubated for 48 h with 31–43 peptide, EmA production was observed in 10/21 culture media, at a titre of 1:1 in all cases. Table IV gives some clinical and histology characteristics in the patients with positive in vitro challenge and in those with negative challenge. The frequency of positive responses to this in vitro challenge was related to a shorter duration of the gluten-free diet and a higher IEL count. In contrast, none of the 22 controls (Group 4) was positive for EmA in the culture supernatants either with or without gliadin-peptide addition.

Discussion

Recent evidence that the intestinal mucosa of CD patients is the site of EmA production and that this could be stimulated in treated CD patients by in vitro challenge with gliadin (3) has opened interesting new perspectives both on the pathogenesis and the diagnosis of CD. However, studies published to date have included only a small number of CD patients (39 and 11 cases, respectively, in two papers by Picarelli et al. (3, 5), 36 in an Austrian study (4) and 7 in a paper by Biagi et al. (14)) and have not evaluated whether this new assay can improve diagnostic accuracy in patients with clinical symptoms suggesting CD. Furthermore, the reliability of the in vitro challenge is uncertain as, in contrast to the Italian studies (3, 5), Vogelsang et al. never detected EmA in vitro production after gliadin incubation in treated CD patients. Thus we designed the present study to evaluate the clinical usefulness of in vitro EmA production in CD diagnosis, enrolling consecutive patients with symptoms which led to a suspected CD diagnosis. The commercial kit allows this assay to be performed in a very simple manner, and it can readily be used in any laboratory where serum EmAs are routinely assayed.

Our study demonstrates that, compared with serum EmA determination, EmA determination in the medium of cultured

Table IV. Sex, age, duration of the gluten-free diet, villi/crypts ratio and intra-epithelial lymphocyte (IEL) count in 10 CD patients on gluten-free diet who were positive at the in vitro challenge and in 11 CD patients who were negative. All values are expressed as range and median

	Positive on in vitro challenge	Negative on in vitro challenge
Age (years)	3–51 (5)	4–42 (5)
Sex	4 M, 6 F	4 M, 7 F
Duration of the gluten-free diet (months)	16–48 (30)*	18–123 (48)*
Villi/crypts ratio	3.5–4 (3.6)	3.5–4.1 (3.6)
IEL count (×100 epithelial cells)	18–41 (31)**	13–23 (18)**

* $P < 0.005$ ($z = 2.8$).
** $P > 0.005$ ($z = 2.9$).

intestinal mucosa with the addition of the 31–43 gliadin-peptide in general does not significantly improve diagnostic accuracy in patients with suspected CD. However, if our results have confirmed (15, 16) that serum EmAs have a good sensitivity and specificity in CD diagnosis (88% and 99%, respectively, in the present whole study group), a very low sensitivity was found in patients with mild intestinal mucosa damage: 17% (2/12 patients). This concurs with previous reports indicating that the development of serum EmA positivity in CD depends on the severity of intestinal histopathology (17, 18). Furthermore, a recent study by Rostami et al. has shown that EmA sensitivity progressively increases from 31% to 100% when CD patients with progressively more severe intestinal mucosa damage are considered (19). In this respect, our results show that EmA assay on the medium of cultured intestinal mucosa is of great usefulness. In fact, this test correctly identified another 7 CD patients who were negative for serum EmA (the test was positive in 9/12 CD subjects with infiltrative/hyperplastic mucosa lesions). So, although if we consider the CD group studied as a whole the increase in sensitivity between serum EmA (88%) and cultured EmA (96%) assay is not statistically significant, it is certainly impressive in CD subjects with low-grade intestinal lesions (17% for serum EmA, 75% for cultured EmA).

The size of the CD population with mild intestinal lesions is unknown and this histology is probably frequent in subclinical forms of CD. In our experience, the infiltrative histology picture can be seen in approximately 10% of symptomatic gluten-sensitive patients; furthermore, it is observed in about 40% of the patients with dermatitis herpetiformis (22). Obviously, intestinal biopsy remains the gold standard for CD diagnosis, but mild-moderate intestinal histology lesions might not be correctly diagnosed by pathologists with little experience in CD and cultured EmA assay could reduce the frequency of false-negative results. We suggest that an intestinal mucosa culture with gliadin-peptide to assay EmA in the supernatants should be performed in all cases to help identify CD patients who have an infiltrative/hyperplastic picture of intestinal histology. In fact, this histology can also be found in patients with intestinal giardiasis or food intolerance (i.e. cow's milk protein or soy intolerance).

Unfortunately, in the present study, we did not perform the anti-transglutaminase (anti-tTG) antibodies serum assay in all our patients and, consequently, are unable to compare the diagnostic accuracy of cultured EmA determination with that of the serum anti-tTG, which is generally considered a more sensitive test than serum EmA (20, 21). However, in the subgroup of our patients with mild intestinal mucosa lesion, five subjects negative for serum EmA, but positive for cultured EmA assay, also underwent serum anti-tTG determination and were negative.

In the present study, all our patients with infiltrative/hyperplastic lesions—Marsh's types 1 or 2 (11)—were symptomatic on a gluten-containing diet, and were cured and had completely normal intestinal histology on a gluten-free diet. One of them, a woman, was the mother of a CD child who on CD diagnosis showed serum EmA positivity (titre >1:400) and total villous atrophy of the intestinal mucosa. In addition, all patients showed the classical CD haplotype, DQ2.

Our results also confirm that the intestinal mucosa is the primary site of EmA production (3) and we hypothesize that in a quota of CD patients with minimal intestinal lesions EmA production by the intestinal mucosa would be low and not sufficient to give serum EmA positivity. This hypothesis is also in keeping with the results of Biagi et al., who detected EmA in the organ culture supernatants (without gliadin stimulation) of CD patients with negative serum EmA and partial villous atrophy (14). In fact, it has been demonstrated that in CD patients only the intestinal mucosa-associated lymphocytes, and not the peripheral blood ones, can produce anti-transglutaminase antibodies (23).

As regards the possibility that the in vitro challenge in CD patients on a gluten-free diet may determine EmA production in the culture medium, the problem arises of the antigen used in the in vitro system. In fact, Arentz-Hansen et al. recently demonstrated that only the α9(57–68) and α(62–75) peptides could stimulate in vitro T-cell response of intestinal lymphocytes in adult Norwegian CD patients. However, the specificity of these peptides might not be absolute, as in fact they failed to stimulate some intestinal T-cell lines from Dutch patients (24). Furthermore, previous studies have identified other gliadin peptides active on T lymphocytes in CD subjects (25, 26). Finally, we underline that our clinical study is based on B-cell mediated response (humoral immunity—antibodies production) in the intestinal biopsies of CD patients and the exact relationship between T- and B-mucosa lymphocytes in CD pathogenesis is still far from clear. In this study, we used the α-gliadin 31–43 peptide as the antigen, a sequence which has been previously demonstrated as being able to stimulate in vitro EmA production (5). However, our data showed that only half of the cases were positive at the in vitro challenge. It is interesting that a significantly higher inflammatory infiltration was found in the intestinal mucosa of the subjects who were positive at the in vitro challenge than in the mucosa of those who were negative; this could indicate that the presence of a threshold number of memory lymphocytes in the biopsy samples is necessary to produce EmA in vitro. This hypothesis is also supported by the shorter duration of the gluten-free diet observed in the patients who had a positive in vitro challenge than in those who were negative on challenge. Furthermore, the real active role of the lymphocyte population in the cultured biopsy samples is also confirmed in patients with active CD by the increase of EmA titre in about half of the samples cultured first without the 31–43 peptide and then with the addition of this peptide. It is difficult to compare our results with those of Vogelsang et al., who did not report EmA production during in vitro challenge in CD on a gluten-free diet (4), as they used three kinds of gliadin different from the

31–43 peptide which we used. However, it is important to underline that our experiments lasted 48 h, whereas those of the Austrian group lasted only 24 h. Similarly, Biagi et al. did not evidence EmA production after in vitro gliadin challenge, but also in this case the experiments lasted 24 h (14). Other previous data would seem to indicate that this difference is relevant, since the EmA titre in the culture medium after 48 h was higher than that recorded after 24 h (3). Furthermore, experiments lasting up to 72 h showed a positive in vitro challenge in 11/11 CD patients on a gluten-free diet (5). Other studies considering both the entity of the inflammatory infiltration in the biopsy sample and the time of incubation of the cultured mucosa must be performed on a great number of CD patients on a gluten-free diet before the determination of EmA production after in vitro challenge can be considered a reliable test in confirming CD diagnosis.

In conclusion, our study has shown that EmA assay in the medium of cultured intestinal biopsy with the addition of α-gliadin 31–43 can identify gluten-sensitive enteropathies characterized by an infiltrative/hyperplastic histological pattern and often associated with negative serum EmA. We suggest that EmA detection should be performed in cultured biopsy samples from all patients with negative serology but with symptoms consistent with a diagnosis of CD.

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