least 2 or, even better, 3 or 4 days apart to optimize error reduction.

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Appendix

If $x_i$ is a random variate with $E(x_i) = \mu$, $Var(x_i) = \sigma^2$, and $Covar(x_i, x_{i+d}) = \sigma^2 \rho^d$ for $d = 0, 1, 2, \ldots$, then $\rho$ is called the autocorrelation. For this model, the variance of the sum and the difference of two variances are:

$$Var(x_i + x_{i+d}) = \sigma^2 + 2\sigma^2 \rho^d + \sigma^2 = 2\sigma^2(1 + \rho^d)$$

$$Var(x_i - x_{i+d}) = \sigma^2 - 2\sigma^2 \rho^d + \sigma^2 = 2\sigma^2(1 - \rho^d).$$

Thus it follows that the variance of the average of two measurements is:

$$Var((x_i + x_{i+d})/2) = 1/4 \cdot 2\sigma^2(1 + \rho^d) = \sigma^2(1 + \rho^d)/2$$

and the semivariance is:

$$1/2[Var(x_i - x_{i+d})] = 1/2 \cdot 2\sigma^2(1 - \rho^d) = \sigma^2(1 - \rho^d)$$

**References**

8. Maria Cristina Anania1

Celiac disease (CD) is a long-life intolerance to gliadin in genetically susceptible individuals (1–6). Despite contrary views (7), diagnosis is still based on the histologic findings of intestinal mucosal atrophy with crypt hyperplasia in individuals on a gluten-containing diet and a return to normal after a gluten-free diet (GFD). The presence of circulating anti-endomysial antibodies (EMAs) and their disappearance after GFD confirm the diagnosis (8–10). It has recently been shown that EMAs are produced by intestinal mucosa of CD patients. EMAs disappear in treated CD patients but are newly produced after in vitro exposure of intestinal biopsy samples to gliadin (11).

Culture of intestinal mucosa from treated CD patients in the presence of a peptic-tryptic (PT) digest of gliadin for 24 h frequently fails to produce detectable EMAs, but it has high specificity as well as high sensitivity in overt CD (12). Our aim was to increase the sensitivity of the in vitro culture by prolonging the duration to 48 h. We also tested a new culture method in batch to ease testing.

We enrolled 11 untreated, EMA-positive CD patients (5 males and 6 females; mean age, 24.7 years; age range, 17–42 years) and 29 treated CD patients (11 males and 18 females; mean age, 32.0 years; age range, 11–70 years) after at least 12 months of GFD (median length of GFD, 730 days; range, 342–4015 days) and two monthly, consecutive serum EMA tests that were negative. As disease-controls we studied 67 patients (20 males and 47 females, mean age, 35.3 years; age range, 20–50 years) with gastrointestinal diseases other than CD (42 with gastroesophageal reflux disease, 8 with ulcer disease, 3 with ulcerative colitis, 3 with Crohn disease, 2 with lymphoma, 1 with small bowel carcinoma, and 8 with Helicobacter pylori infection). All were EMA-negative.

Biopsy samples of duodenal mucosa were obtained from all of these patients for diagnostic purposes. All procedures were in accord with the ethical standards of the responsible institutional committee on human experimentation.
Gliadin was extracted from pure hexaploid bread wheat (*Triticum aestivum* variety San Pastore) and submitted to PT digestion as reported previously (4).

We obtained four duodenal biopsy samples (each ~60 mg) by endoscopy from each patient. One sample underwent morphometric analysis with hematoxylin-eosin staining; the revised Marsh histologic status was also evaluated (13, 14).

The culture medium, prepared with 13 mL of Trowell T8 medium, 4 mL of NCTC 135 medium, 3 mL of fetal calf serum, 2 mL of penicillin (10,000 kilounits/L) plus streptomycin (10,000 mg/L), 0.2 mL of L-glutamine (200 mmol/L), and 10 g/L gentamicin (Eurobio), was sterilized by filtration with a 0.22 µm pore size filter (Sigma Chemical).

Two specimens were divided into two parts and cultured using the two methods considered in the study.

**Classic method** (4). Two duodenal mucosal samples were placed on a stainless steel grid positioned over the central well of an organ culture dish, with the villous surface of the biopsy specimen uppermost, for 48 h at 37 °C, one in the presence and one in the absence of PT-gliadin (1 g/L).

**Batch method.** Two duodenal specimens were cultured in a batch of culture medium. Specimens, first washed in physiologic saline solution (9 g/L NaCl), were gently placed in a sterile tube containing 500 µL of medium. Specimens were left free in the medium and then cultured for 48 h at 37 °C, one in the presence and one in the absence of PT-gliadin (1 g/L). Oxygenation of the biopsy samples depended on the air column over the culture medium.

We collected 100 µL of the culture supernatants after 24 h of culture in both classic and batch conditions and stored it at −70 °C until use. The remaining supernatant, after 48 h of culture, was collected and stored at −70 °C until used. All operations were performed in a sterile environment.

An additional biopsy specimen from each of the 29 treated CD patients was divided into two parts and cultured in the presence of PT-gliadin for 168 h in both culture conditions. After 6, 24, 72, and 96 h, intestinal samples were removed, washed in physiologic saline solution, and again replaced in fresh medium containing PT-gliadin (1 g/L). At each step, the medium was completely changed. Supernatants were collected at 6, 24, 72, 96, and 168 h and stored at −70 °C until used. Detection of EMAs at the five time points was considered a sign of viability of the biopsy specimens.

EMAs were detected in sera diluted 1:5 and in undiluted culture supernatants by indirect immunofluorescence analysis on cryostat sections of monkey esophagus (Eurohospital, Trieste, Italy). The incubation times were 30 min for sera and 45 min for culture supernatants. Titration of EMA detectability was performed on dilutions up to 1:128. EMA-positive results were identified by reticulin-like staining of smooth-muscle bundles.

All tests were evaluated by two observers unaware of the patients' conditions. No patients were excluded from analysis. The 11 untreated CD patients had total or subtotal villous atrophy: 7 had a Marsh histologic status of class IIIC, and 4 had a histologic status of class IIb. All 29 treated CD patients and 67 disease-control patients had a normal mucosal architecture with no intraepithelial lymphocyte infiltration of the lamina propria.

After 24 h of culture, EMAs were observed in supernatants from 7 of 11 untreated CD patients (63.7%) irrespective of gliadin challenge and, after in vitro gliadin challenge, in 18 of the 29 treated CD patients (62.1%).

After 48 h of culture, EMAs were detected in supernatants from all 11 untreated CD patients, irrespective of gliadin challenge, and in supernatants of all 29 treated CD patients after in vitro challenge with PT-gliadin (Table 1).

After 24 h of culture, EMAs were detectable in supernatants diluted up to 1:8 in the classic method and up to 1:16 in batch conditions. After 48 h of culture, EMAs were detectable in both culture conditions in supernatants diluted up to 1:64.

No EMAs were detectable in supernatants of the untreated CD patients cultured with medium alone, irrespective of the time and the culture method used.

In disease-control patients, EMAs were not detectable in supernatants of biopsies cultured with and without PT-gliadin, neither after 24 h nor after 48 h in both culture conditions studied.

After 6 h of challenge with PT-gliadin, EMAs were not present, but they appeared after 24 h of challenge in 18 of 29 treated CD patients and after 72 h in all 29 culture supernatants, irrespective of the culture method used. After this time point, no EMAs were newly detectable in classic cultures, whereas they were detectable until 168 h of gliadin challenge in all culture supernatants of biopsy cultures in batch.

Our data confirm previous reports (11, 12, 15) of EMA detectability in culture supernatants from intestinal mucosa specimens of untreated CD patients and in those from treated CD patients after PT-gliadin challenge, but not in those from disease-controls other than CD. The in vitro culture system, because of its simplicity of use and the high specificity and sensitivity of EMA detection, can therefore be considered a useful diagnostic tool for CD.

A recent study (12) reported a low sensitivity for in vitro gliadin challenge of duodenal biopsies from treated CD patients, cultured for only 24 h. We, too, obtained low sensitivity at 24 h but high sensitivity after prolonging duodenal biopsy cultures to 48 h. Furthermore, specimens

| Table 1. EMA detection in culture supernatants from patients with and without CD.† |
|-----------------|-----------------|-----------------|-----------------|
|                 | 24 h            | 48 h            |
|                 | Medium alone    | Medium + PT-gliadin | Medium alone    | Medium + PT-gliadin |
| Untreated CD (n = 11) | 7/11            | 7/11            | 11/11           | 11/11              |
| Treated CD (n = 29)   | 0/29            | 18/29           | 0/29            | 29/29              |
| Disease-controls (n = 67) | 0/67           | 0/67            | 0/67            | 0/67              |

† Production of EMAs by intestinal mucosa samples of CD patients on a gluten-containing diet or on a GFD, and in disease-controls other than CD. Biopsy specimens were cultured for 24 and 48 h in the presence or absence of PT digest of gliadin (1 g/L). The same results are observable in either batch or classic cultures.
cultured with the new batch method also allowed increased sensitivity with improved ease of testing. Moreover, in previous in vitro studies (11, 12), EMAs were reported in supernatants diluted up to 1:8, whereas we found EMAs in 1:64 dilutions when culture time was prolonged to 48 h, irrespective of the culture technique used. As in previous studies (11, 12), we found no false-positive results even at 48 h.

The long time of culture and the change of the culture method required us to test the viability of the specimens. When we used the production of new EMAs as a marker of survival of intestinal biopsies, our study showed viability in the batch culture for 168 h, whereas biopsies cultured with the classic method did not produce EMAs after 72 h. The appearance of EMAs in cultures supernatants from treated CD patients after 24, 72, 96, and 168 h of gliadin challenge and their absence after 6 h can be considered not as simple EMA release, but as evidence of new EMA production and, therefore, evidence of the viability of the intestinal specimens.

Our results for 48-h cultures confirm previous studies (11) and extend the utility of the organ culture system, especially in situations of patchy atrophy or mild gluten-sensitive enteropathy (7), in which patients may escape correct diagnosis when biopsy specimens undergo only morphologic examination.

The batch culture system is easy to set up and less expensive than culture in Petri dishes on steel grids. We conclude that EMA detection in culture supernatants of intestinal biopsy specimens is an effective and useful tool in the diagnostic armamentarium for CD.

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References


Measurement of Indocyanine Green Dye Is Improved by Use of Polyethylene Glycol to Reduce Plasma Turbidity, Maurie J. Luetkemeier and Jill A. Fattor (The Department of Exercise and Sport Science, University of Utah, 1850 East, 250 South, Room 241, Salt Lake City, UT 84112; * address correspondence to this author at: The Department of Exercise and Health Science, Alma College, 614 West Superior, Alma, MI 48801; fax 989-463-7018; † current address: Exercise Physiology Laboratory, Department of Integrative Biology, University of California, Berkeley, Berkeley, CA 94720)

Indocyanine Green (ICG; CardioGreen®; Akorn Inc.) is a sterile, water-soluble dye that is used clinically as a dilution indicator for studies involving the heart, liver, lungs, and circulation. When ICG is infused intravenously into the bloodstream, it rapidly binds to plasma proteins and thereby is confined to the vascular space (1, 2). ICG is removed exclusively by the liver at the rate of 18–24% per minute (2), so the elimination of ICG follows an exponential curve with a half-life of ~150–180 s (3).

The concentration of ICG is determined spectrophotometrically with undiluted plasma samples at 805 nm. Substances that alter the absorbance of the plasma interfere with the accuracy of the measurements. Background opacity or turbidity of plasma is predominantly linked to the non-albumin fraction of the plasma proteins and is manifested by variation in the background absorbance reading. The manufacturer of ICG states that ICG binds primarily (95%) to albumin, with lesser binding to α-globulins. Therefore, the accuracy of measuring ICG may be improved by first removing the non-albumin fraction of the plasma proteins.

Zweens and Franken (4) used polyethylene glycol (PEG) to precipitate the non-albumin fraction of the plasma proteins in a study involving another dilution indicator, Evan’s blue dye. Evan’s blue dye, like ICG, attaches primarily to albumin, but it is measured at a different wavelength. The authors showed that the variable background absorbance of plasma at 620 nm was virtually eliminated by mixing plasma samples with equal volumes of a 240 g/L solution of PEG (mean molecular weight, 3350). In addition, they reported nearly 100%