

Endomysium antibodies in coeliac disease: an improved method

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Abstract

The ultra structural binding sites of endomysium antibodies have been studied on human umbilical cord tissue. The sensitivity and specificity of IgA endomysium antibodies were compared with recently described methods using basement membrane of smooth muscle of monkey oesophagus. Thirty adults affected by coeliac disease (10 in remission) and 75 healthy adult controls with normal intestinal mucosa (35 false antigliadin positive) were investigated. Sensitivity and correlation of endomysium antibodies with total villous atrophy in untreated coeliac disease patients were 100% on the human umbilical cord smooth muscles, and only 90% on the muscular layer of primate oesophagus. Indirect immunofluorescence was superior to peroxidase staining in detecting these IgA antibodies. The easy availability and enhanced testing sensitivity of the umbilical cord is an advance towards a better diagnostic tool for coeliac disease.

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Coeliac disease is a permanent intolerance to gliadin leading to intestinal villous flattening and crypt hyperplasia in susceptible subjects. Immune reactions to gliadin probably play a part in the pathogenesis of the disease although the immunological mechanisms are still poorly understood.^{1,2} Both a cell mediated response and enhanced intestinal secretion of immunoglobulins have been found in coeliac disease.³ Antigliadin (AGA), reticulin antibodies, and endomysium antibodies (EmA) are found in serum samples of patients with coeliac disease.⁴ AGAs are not specific to coeliac disease; high serum AGA titres are found in patients affected by other gastrointestinal disorders. Sensitive methods such as the enzyme linked immunosorbent assay (ELISA) permit detection of serum antibodies in most normal subjects; these, however, are present in titres below an arbitrary range specific to different laboratories.⁵

Chorzelski *et al* first described the EmAs directed against the membrane of smooth muscle bundles in primates.⁶ These, closely related to reticulin antibodies, were present in patients with dermatitis herpetiformis and coeliac disease.⁷ An almost 100% sensitivity and specificity has been reported for IgA class EmA in coeliac disease.^{8,9} EmA thus seem to be a new valuable screening test for gluten sensitive enteropathy.¹⁰ EmA are detected in rats, primate or human oesophagus, kidney, liver, stomach, and small and large intestinal tissue.¹¹ In commercially available kits EmA are detected on monkey oesophagus.

The aim of this study was to find an improved

method for determining EmA on easily obtained human tissue.

Patients and methods

COELIAC DISEASE

Serum samples from 30 patients (20 women and 10 men) with coeliac disease proved by biopsy according to the revised ESPGAN criteria,^{12,13} mean age 35 years (range 5-68), were examined. Twenty patients had flat intestinal mucosa; 10 were in remission while receiving a gluten free diet and had a histologically normal jejunal mucosa.

CONTROLS

A second group included 35 patients (20 women and 15 men) with positive serum samples for AGA. Subsequent histological examination of the jejunal mucosa showed no histological abnormalities. Mean age was 40 years (range 3-65).

The third group of serum samples was obtained from 40 non-coeliac disease patients (20 women and 20 men) without any gastrointestinal disorder and with AGA below the normal range (IgA < 5 and IgG < 15). Mean age was 37 years (range 5-65).

In all patients investigated, an IgA deficiency was excluded by serum testing.

IMMUNOHISTOCHEMICAL EXAMINATION

Immunohistochemical examination to determine the presence of EmA were carried out on 5 µm thin cryostat sections of human umbilical cord and on commercially available sections of monkey oesophagus (EmA Kit Biosystems, Genova, Italy).

The umbilical cord was cut and embedded immediately after excision in tissue tek OCT (Miles-Bayer, Milano, Italy) or was frozen at -70°C for several days and then embedded in OCT.

The umbilical cord sections were fixed for 10 minutes in acetone at -20°C and for 30 minutes in chloroform at room temperature. After washing in phosphate buffered saline (PBS) 0.05 M pH 7.4 (Sigma, Milano, Italy) sections were incubated with 1% bovine serum albumin (Sigma), and after that, with undiluted or 1:5, 1:10, 1:50, and 1:100 diluted patient serum samples. After washing in PBS, sections were incubated in a dark room with fluorescein isothiocyanate (FITC) conjugated antiserum samples against human IgA (Behring, Aquila, Italy) at a dilution of 1:10 in PBS. The slides were washed again, mounted in aqueous mounting medium

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(Merk, Roma, Italy), and examined by fluorescence microscopy.

Tissue sections, after incubation with five serum samples per group, were incubated with 1:15 diluted biotinylated antihuman IgA (Sigma) and, after washing in PBS, with avidin-biotin peroxidase (ABC Dako, Milano, Italy). After staining with aminoethylcarbazole and counter staining with haematoxylin, sections were mounted, and viewed by light microscope. The same procedure was used for staining formalin fixed umbilical sections. These sections were treated with 1% trypsin and endogene peroxidase was inhibited by incubation with 1% H₂O₂.

Fifteen sections of umbilical cord were fixed in acetone and than stored at -20°C for several days. The fluorescence staining procedure was continued as described before using five serum samples per group.

The determination of EmA on monkey oesophagus was performed according to the instructions of the kit used.

Positive and negative controls for EmA (Biosystems) were used for each batch and reproducibility of the two different tests was checked on separate days.

Results

A comparison was made between the binding specificities of EmA on commercial available monkey oesophagus or human umbilical cord specimens.

PATIENTS WITH COELIAC DISEASE

All 20 patients with active coeliac disease, flat jejunal mucosa, and positive for AGA were also positive for EmA. Their serum samples showed a honeycomb like fluorescence along the peritubular muscle layers of vessels on umbilical cord, a typical feature, staining the extracellular connective tissue (Figure). On monkey oesophagus the same reticular fluorescence pattern was seen around smooth muscle fibres in the muscularis mucosae. Only two patients with active disease were negative for EmA on monkey oesophagus but positive on human umbilical cord.

The 10 coeliac disease patients in remission with an histologically normal mucosa were all negative for EmA, both on umbilical cord and

monkey oesophagus. The honeycomb pattern was absent, whereas a weak cytoplasmatic fluorescence was present in the muscle cells.

CONTROL GROUP

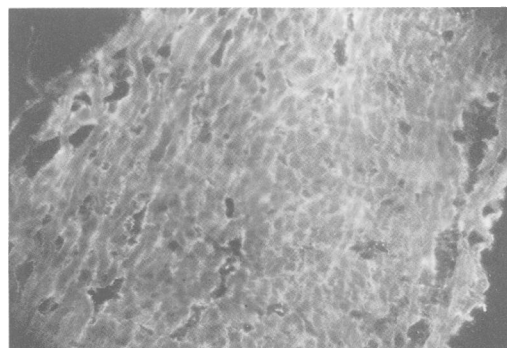
All patients with positive AGA but normal jejunal mucosa and without gastrointestinal symptoms were negative for EmA, as well as patients with normal mucosa and no AGA. No difference was seen between the two different methods. A weak, unspecific cytoplasmic fluorescence could be seen in the muscle cells of both tissues.

The appropriate dilution tested on frozen umbilical cord sections was between 1:5 and 1:10. Differences in staining pattern on the endomysium were seen between sections embedded in OCT and those previously frozen, the second being less intensive and poorly defined. Only weak reaction was seen on acetone fixed frozen specimens incubated with coeliac serum samples. Peroxidase stained frozen or formalin fixed sections showed not a typical honeycomb pattern but only a weak uniform staining in the muscle layer around the vessel, without any difference between coeliac disease patients and control group.

Discussion

Indirect immunofluorescence was used to determine EmA in serum samples of coeliac disease patients and controls on monkey oesophagus and human umbilical cord. EmA present in serum samples of subjects with active coeliac disease are primarily of the IgA class binding in an amorphous component adjacent to fine collagenous reticulin fibrils of the endomysial connective tissue. These fibrils connect neighbouring smooth muscle cells, smooth muscle bands, and elastic tissue.¹⁴

We found the specific honeycomb binding of EmA to the connective tissue surrounding muscle cells of vessels within the umbilical cord in all tested coeliac disease patients with flat mucosa. Binding was absent in remission patients and normal subjects with false positive or negative AGA. In two untreated coeliac disease cases EmA bound only to human tissue but not to monkey oesophagus, thus confirming that human tissue is a more sensitive material than that of primates. The higher specificity and sensitivity of EmA in adult coeliac disease patients and healthy controls on human umbilical cord are an important step towards detecting a simple and highly precise diagnostic tool for coeliac disease. Furthermore, this simple immunohistochemical method permits unlimited testing and characterisation of EmA in any immunology department. Umbilical cord is an easily and commonly available and inexpensive human tissue, which is a perfectly adequate substitute for the distal oesophagus of endangered species. In our experience, the method based on FITC conjugated antihuman IgA antibodies is superior to that of peroxidase staining because of its improved reading. The only disadvantage is the short stability of the fluorescence.



Immunofluorescent staining pattern of EmA from coeliac disease serum in cryostat section of human umbilical cord. Honeycomb staining around smooth muscle cells of vessels is seen. (Original magnification $\times 250$.)

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