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Mechanisms of villous atrophy in autoimmune enteropathy and coeliac disease

R. CICCOCIOPPO*, S. D'ALÒ†, A. DI SABATINO‡, R. PARRONI†, M. ROSSI*, C. DOGLIONI§, M. G. CIFONE† & G. R. CORAZZA‡ ‡*Gastroenterology Unit, IRCCS Policlinico S. Matteo, University of Pavia, Departments of †Experimental and *Internal Medicine, University of L'Aquila and §Pathology Unit, Hospital of Belluno, Italy*

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SUMMARY

Since in coeliac disease mucosal flattening has been suggested to result from an increased enterocyte apoptosis triggered by Fas/Fas ligand system and perforin cytolytic granules, we looked for a similar mechanism in autoimmune enteropathy. Moreover, we tried to assess whether enterocyte autoantibodies, which are the hallmark of autoimmune enteropathy, may be involved in triggering enterocyte apoptosis in this condition. Immunohistochemical staining with anti-Fas, -FasL and -perforin MoAb, and TUNEL technique were applied on endoscopic duodenal biopsies of two autoimmune enteropathy patients, two untreated coeliac patients and two biopsied controls. Cytotoxicity assays were carried out by incubating peripheral blood mononuclear cells from a healthy subject (effectors) with enterocytes primed with patient or control sera (targets). In autoimmune enteropathy a large number of enterocytes were apoptotic, as in coeliac disease, whereas neither Fas/Fas ligand or perforin expressions were up-regulated. On the other hand, antibody-dependent cellular cytotoxicity assay revealed the ability of sera from patients with autoimmune enteropathy to mediate enterocyte death through apoptosis. These results point to enterocyte autoantibody-dependent cellular cytotoxicity as the prevalent mechanism of increased enterocyte apoptosis in autoimmune enteropathy but not in coeliac disease.

Keywords ADCC apoptosis enterocyte antibodies Fas/FasL system perforin

INTRODUCTION

Autoimmune enteropathy is a condition characterized by villous atrophy, indistinguishable from that of coeliac disease but unresponsive to any dietary restriction, and by circulating enterocyte autoantibodies (EAA) [1]. Autoimmune enteropathy has been reported primarily in infancy [1], but it can, rarely, also affect adult patients [2,3].

In coeliac disease mucosal flattening has been suggested to result from an increased enterocyte apoptosis [4], triggered by the release of lymphocyte perforin cytolytic granules and by the Fas/Fas ligand (FasL) cognate interaction [5]. Although it is generally accepted that activated T cells play an essential role in the development of villous atrophy both in experimental models [6] and in coeliac disease [7] and that autoreactive T cell clones are also involved in the generation of autoimmune enteropathy [8], recent studies suggest a significant heterogeneity within this condition and patients with T cell activation deficiency have been reported [9,10]. In addition, the discovery of a 75-kDa autoimmune enteropathy-related autoantigen expressed in well differentiated enterocytes [11] supports the view that EAA may have a pathogenic role [12].

Correspondence: Prof Gino Roberto Corazza, Gastroenterology Unit, IRCCS Policlinico San Matteo, Piazzale Golgi 5, 27100 Pavia, Italy.
E-mail: gr.corazza@smatteo.pv.it

The present study aimed to investigate enterocyte apoptosis in two adult patients with villous atrophy due to autoimmune enteropathy and to assess the role of EAA in this process. Biopsies and sera from patients with coeliac disease were studied for comparison. Our results point to antibody-dependent cellular cytotoxicity (ADCC) as the prevalent mechanism of increased enterocyte apoptosis in autoimmune enteropathy but not in coeliac disease.

PATIENTS AND METHODS

Patients and tissues

The two autoimmune enteropathy patients were adult women with severe malabsorption symptoms, total villous atrophy at histology unresponsive to a carefully monitored gluten-free diet and HLA-DQ2 positive. Small-bowel barium enema and abdominal computed-tomography scan excluded the presence of ulcerative jejunoileitis or intestinal lymphoma. Then, a gluten-free diet was combined with 3-methylprednisone treatment, which led to a substantial improvement either in clinical symptoms or in duodenal morphology. One of these two patients died because of a myocardial infarct after 18 months of steroid therapy, whereas the second patient is still alive. Both were positive for the presence of EAA [2] either IgA or IgG at high titres (1/20 and 1/40, respectively, for patient 1, and 1/320 and 1/640, respectively, for patient 2),

whereas both proved negative for IgA antigliadin and anti-endomysium. The two coeliac patients were adult women with total villous atrophy at histology and positivity for both IgA antigliadin and antiendomysium antibodies, shown subsequently to be responsive to gluten-free diet; and the two control subjects were adult consenting women undergoing upper gastrointestinal endoscopy for functional dyspepsia, with negativity for both IgA antigliadin and anti-endomysium antibodies.

Size-appropriate and well-orientated endoscopic duodenal biopsy specimens were obtained from all the previously described subjects and processed according to standard methods for histology and immunohistochemistry.

Immunohistochemistry

For the *in situ* detection of apoptotic enterocytes, the terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick end labelling (TUNEL) technique was applied [13], using the peroxidase ApopTag Kit (Oncor, Gaithesburg, MD, USA). As the positive control, the Apoptag control slide was used, whereas the negative control was performed on a seriate section by omission of the terminal deoxynucleotidil transferase enzyme.

To detect Fas and FasL expression, sections were incubated with mouse monoclonal antihuman antibodies (Fas antibody, Upstate Biotechnology, Lake Placid, NY, USA; 1 : 100 dilution and FasL antibody, F37720 Transduction Laboratories, Lexington, KY, USA; 1 : 150 dilution) after proteolytic digestion (trypsin 2% in $CaCl_2$ 0·1%, pH 7·8 and protease K 20 μg/ml, Sigma, St Louis, MO, USA, respectively) and endogenous peroxidase inactivation. As positive controls, a cytocentrifugate of human Jurkat cell line for Fas expression and a section of hyperplastic mesenteric lymphnode for FasL expression were used, respectively. As negative controls, sequential sections were incubated without the specific primary antibody.

To detect perforin expression, sections were pretreated in a microwave oven in EGTA solution (0·05 M) four times for 5 min each at 700 W and, after endogenous peroxidase blocking, incubated with a human monoclonal antiperforin antibody (clone KM 585, Kamiya Biomedical, Thousand Oaks, LA, USA; 1 : 1000 dilution). As the positive control, a section of lymph node involved by Kikuchi disease was used, whereas for the negative control a sequential section was incubated without the primary antibody.

Morphometric analysis

Immunohistochemical sections were examined in a blind fashion by an expert observer. Counts were performed at a constant magnification (×1000) by a differential count of at least 500 cells in the epithelium and in the lamina propria and the results expressed as a percentage of positive enterocytes, intra-epithelial lymphocytes or lamina propria mononuclear cells. In order to investigate a possible relationship between the degree of enterocyte apoptosis and that of villous atrophy, the percentage of $TUNEL^+$ enterocytes was correlated with the surface area to volume ratio of the duodenal mucosa morphometrically evaluated by the method of Dunnil and Whitehead [14], as standardized in our laboratory [15].

Cytotoxicity assays

For the isolation of enterocytes, a ring of 4 cm length of terminal ileum, obtained from a patient who underwent surgical resection for right colon cancer, was washed with sterile saline solution and placed in sterile medium (PBS, calcium and magnesium free, supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml and 5% FCS). The ring was then opened along the mesenteric side and the serosal and muscular layers gently separated from the mucosa and submucosa. The epithelial layer was removed with 1 mM EDTA (Sigma) and 1 mM DDT (Sigma). After continuous agitation for 1 h at 37°C, the single cell suspension was pelleted from the supernatant and washed once with 5 ml RPMI-1640 medium (GIBCO, Life Technologies Ltd, Paisley, UK) supplemented with antibiotics and 10% FCS. Finally, enterocytes were separated on a discontinuous (25% and 40%) Percoll density gradient (Pharmacia Fine Chemicals, Pharmacia Inc., Uppsala, Sweden). Freshly isolated enterocytes were resuspended in RPMI 1640 supplemented with FCS, antibiotics and glutamine and used as target cells in the cytotoxicity assays. Their viability was determined by trypan blue exclusion.

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized peripheral blood of a healthy subject by Lymphoprep gradient centrifugation (Nicamed, Oslo, Norway) and further purified by plastic adherence to remove monocytes. Cell recovery ranged between 85 and 95% and viability exceeded 95%. The resulting composition of PBMC population was 73% $CD3^+$, 12% $CD16^+$ $CD56^+$ $CD3^-$, 15% $CD19^+$ as assessed by flow-cytometric analysis on a FACS II analyser (FACScan; Becton Dickinson Co., San Jose, CA, USA). PBMCs, the ability of which to mediate ADCC was previously tested against anti-P815-coated P815 cells – a chemically induced natural killer resistant mouse mastocytoma line – were used as effector cells in the cytotoxicity assays.

Target enterocytes were subdivided into four aliquots. The first aliquot, subdivided into three fractions, was preincubated (1 h at 37°C) with sera from patients with autoimmune enteropathy, untreated coeliac disease or controls, all heat-inactivated prior to use in the assay, and then resuspended along with effector peripheral blood mononuclear cells in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM-HEPES, pH 7·4, at 37°C in a 5% CO_2 atmosphere for 4 h, to give an estimation of ADCC-mediated enterocyte apoptosis. The second aliquot was neither preincubated with sera nor resuspended with effector cells, to give an estimation of spontaneous enterocyte apoptosis. The third, subdivided into three fractions, was labelled with sodium ^{51}Cr (New England Nuclear, Boston, MA, USA) for 1 h and the level of spontaneous release of ^{51}Cr indicated a good cellular uptake (spontaneous release: mean 6386 cpm). Subsequently, the three fractions were incubated with sera from patients with autoimmune enteropathy, untreated coeliac disease or controls, all heat-inactivated, and then resuspended with effector PBMCs (4 h at 37°C) to give an estimation of ADCC-mediated enterocyte necrosis. The fourth was labelled with sodium ^{51}Cr but was neither preincubated with sera nor resuspended with effector cells to give an estimation of spontaneous enterocyte necrosis. In a subset of experiments, target enterocytes were preincubated with sera from patients with autoimmune enteropathy which were precleared by adding 20 μl/ml of agarose conjugate (Protein L-Agarose # sc-2336, Santa Cruz Biotechnology Inc., CA, USA) and incubated on a rocker platform at 4°C overnight.

In the co-culture experiments the effector PBMCs were added to give effector : target ratios of 100 : 1, 50 : 1, 25 : 1 and

12·5 : 1. After incubation, 100 μl medium from each well of labelled co-cultures was removed carefully and counted in a gamma counter. The percentage of specific ^{51}Cr release was calculated according to the following formula: 100 × (experimental release – spontaneous release)/(maximal release – spontaneous release), where maximal release was determined by incubating target cells in the presence of 1 N HCl (maximal release: mean 31 480 cpm). At the same time, an aliquot of unlabelled co-cultured cells was analysed for effector/target conjugate formation as described elsewhere [16]. Results were expressed as the percentage of total targets that formed conjugates. The percentage of conjugate-forming cells was determined by inverted contrast phase microscopy inspection of at least 200 cells. Finally, unlabelled enterocytes were also cytocentrifuged (Cytospin Shandon Southern, 500 r.p.m. for 10 min) and processed either by TUNEL technique to evaluate the percentage of apoptosis, or by haematoxilin to evaluate enterocytes with apoptotic morphology.

RESULTS

In normal and autoimmune enteropathy duodenal mucosa, Fas expression was confined to few enterocytes, whereas in untreated coeliac disease a large proportion of enterocytes either at the mucosal surface or along the crypts expressed Fas (Fig. 1). Figure 2 shows that, differently from controls and autoimmune enteropathy patients, there are many FasL$^+$ intra-epithelial lymphocytes in untreated coeliac mucosa.

Immunohistochemical and morphometric results are shown in Table 1. In comparison to biopsied controls, the percentage of TUNEL$^+$ enterocytes was remarkably increased in patients with both autoimmune enteropathy and untreated coeliac disease. This finding was accompanied by a proportional decrease in surface to volume ratio. However, in autoimmune enteropathy the percentages of Fas$^+$ enterocytes, FasL$^+$ intra-epithelial lymphocytes and lamina propria mononuclear cells, perforin$^+$ intra-epithelial lymphocytes and lamina propria mononuclear cells were markedly lower than in coeliac disease and did not differ consistently from those of biopsied controls. The immunohistochemical and morphometric results found in the two untreated coeliacs and in the two biopsied controls were representative of those previously found in larger samples of these groups [5,17].

The efficiency of isolated enterocytes to form conjugates with PBMCs was analysed under inverted phase contrast microscopy. The formation of stable conjugates was obvious at any effector/target ratio tested (>95%) when enterocytes were previously primed with autoimmune enteropathy sera. On the contrary, the number of bound targets was insignificant (<5%) when enterocytes were primed with coeliac or control sera. The results of ADCC assays are shown in Fig. 3. Analysis of enterocyte apoptosis indicates that, while a negligible percentage of TUNEL$^+$ enterocytes was detected after priming with coeliac or control sera, enterocytes primed with sera from the two autoimmune enteropathy patients showed a markedly higher effector/target ratio-dependent apoptosis. On the contrary, enterocytes primed with precleared autoimmune enteropathy sera showed a percentage of apoptosis (mean 3·6% at an effector/target ratio of 100 : 1, mean 2·6% at an effector target ratio of 50 : 1, mean 5·1% at an effector target ratio of 25 : 1, mean 4·8% at an effector target ratio of 12·5 : 1) like that of enterocytes primed with control sera (mean 3·8% at an effector/target ratio of 100 : 1, mean 2·4% at an effector target ratio of 50 : 1, mean 5·0% at an effector target ratio of 25 : 1, mean 4·7% at an effector target ratio of 12·5 : 1). With regard to the evaluation of spontaneous apoptosis, the percentage less than 5% observed, might be due to the short duration of the coculture. Analysis of specific ^{51}Cr release (insert of Fig. 3) showed that effector/target ratios as high as 100 : 1 were devoid of any significant enterocyte cytotoxic effect, thus indicating that enterocyte death induced by PBMCs, after priming with autoimmune enteropathy sera, was due to the triggering of an apoptotic death pathway. With regard to the evaluation of spontaneous enterocyte necrosis, a percentage lower than 1% was observed. In accordance with these results, the light microscopy observation of cytocentrifuged co-cultured cells revealed the appearance of the morphological features of apoptosis in a large number of enterocytes primed with sera from patients with autoimmune enteropathy (Fig. 4a), but not with coeliac disease (Fig. 4b).

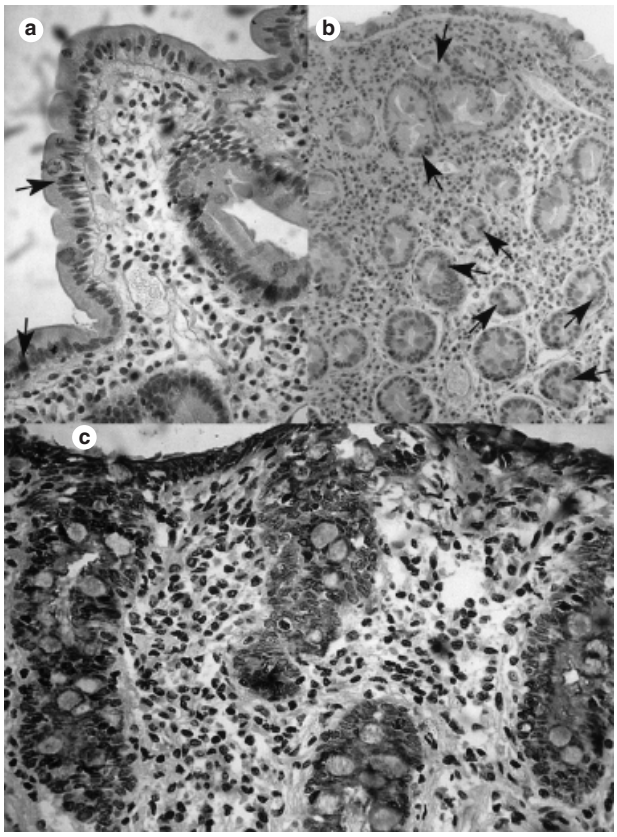

Fig. 1. Detection of Fas$^+$ enterocytes in normal (a), autoimmune enteropathy (b) and untreated coeliac mucosa (c). In normal and autoimmune enteropathy duodenal mucosa, few enterocytes are Fas$^+$ (arrows), whereas in untreated coeliac disease a large proportion of enterocytes either at the mucosal surface or along the crypts express Fas. Original magnification, ×400, ×200 and ×400, respectively.

DISCUSSION

Both coeliac disease and autoimmune enteropathy are characterized by hyperplastic villous atrophy [18], the only morphological difference being the normal proportion of γ/δ intra-epithelial lymphocytes in autoimmune enteropathy [18,19].

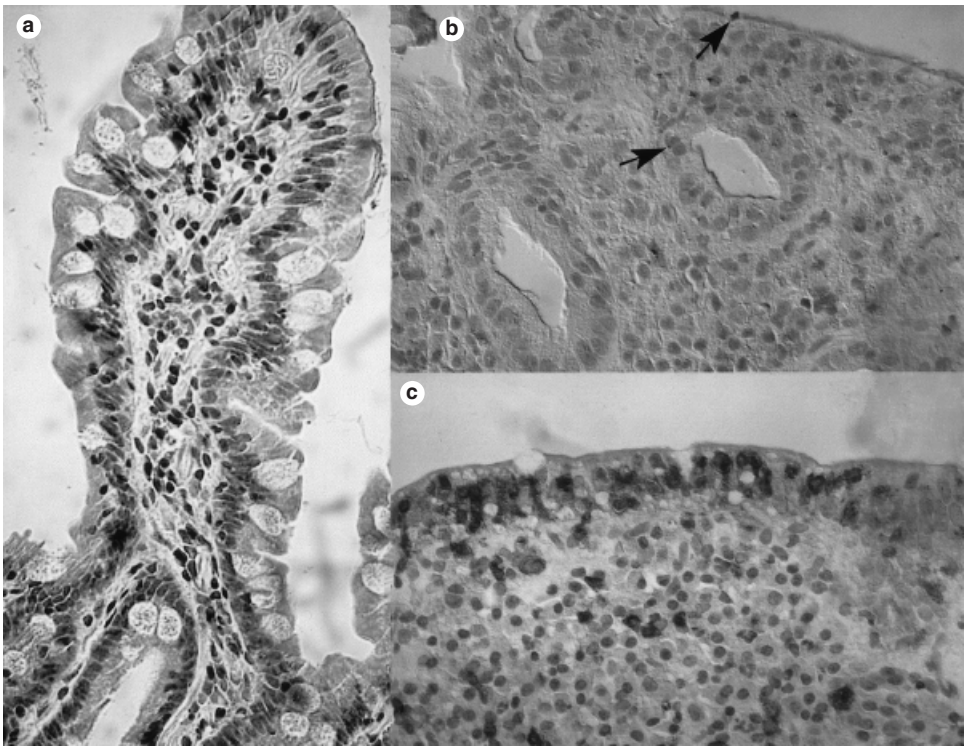

Fig. 2. Detection of FasL$^+$ intra-epithelial lymphocytes in normal (a), autoimmune enteropathy (b) and untreated coeliac mucosa (c). Differently from controls and autoimmune enteropathy patients, where only rare intra-epithelial lymphocytes are FasL$^+$ (arrows), there are many FasL$^+$ intra-epithelial lymphocytes in untreated coeliac mucosa. Original magnification, ×200, ×400 and ×400, respectively.

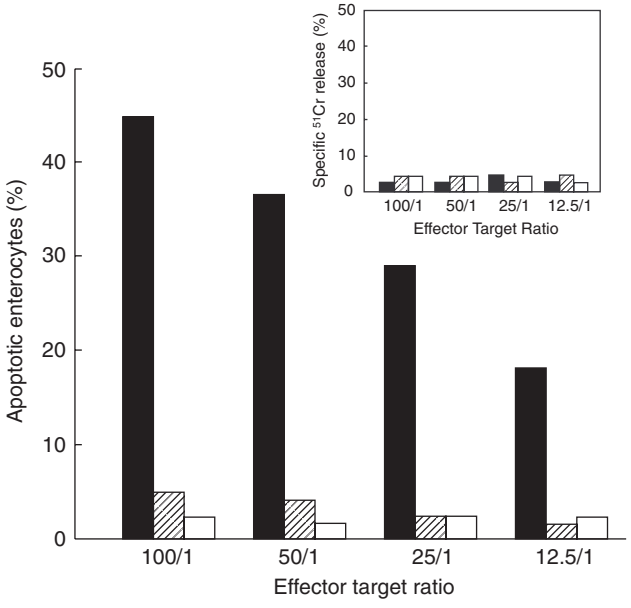

Fig. 3. Percentages of apoptotic enterocytes (target) after 4 h co-culture with peripheral blood mononuclear cells of a healthy donor (effector) at four different effector/target ratios. Enterocytes were previously incubated for 1 h with autoimmune enteropathy, untreated coeliac or biopsied control sera. The insert represents the percentages of specific ^{51}Cr release by co-cultured labelled enterocytes, primed with autoimmune enteropathy, untreated coeliac or biopsied control sera. Each column indicates the mean percentage of two patients for each group. ■, Autoimmune eneropathy; ▨, untreated coeliac disease; □, biopsied controls.

Since an increased enterocyte apoptosis has been held to be responsible for villous atrophy in coeliac disease [4], we looked for a similar mechanism in autoimmune enteropathy. Our results are consistent with a strict relation between villous flattening and epithelial cell apoptosis in this condition, although unlike coeliac disease [5] apoptosis does not seem to be associated to an overexpression of perforin and/or Fas/FasL levels. Since the hallmark of autoimmune enteropathy is the presence of EAA [1], it is conceivable that humoral autoimmunity may be involved in some way in triggering enterocyte apoptosis in this condition. Up to now the pathogenic importance of EAA has been seriously questioned on the basis of their delayed appearance with respect to the onset of autoimmune enteropathy [20,21]. However, the correlation between EAA titres and disease activity [22] and prognosis [23] makes the assumption that they represent a mere disease-related epiphenomenon less tenable. If this were the case, it would be very difficult to explain their absence as a consequence of enterocyte damage in coeliac disease. Moreover, in a very well-studied adolescent boy, IgG EAA did not bind to enterocytes in the patient's diseased mucosa, but bound strikingly to mature enterocytes in the patient's regenerated villi after cyclophosphamide therapy [24].

Since it is known that ADCC may induce either necrosis or apoptosis [25] depending on the target cell types [26], we looked for a serum-factor able to trigger ADCC-mediated enterocyte apoptosis in coeliac disease and autoimmune enteropathy. To this purpose, cytotoxicity assays with effector PBMCs and target enterocytes primed with patient or control sera, previously heat-

Table 1. Percentage of positive cells and surface to volume ratio in autoimmune enteropathy, untreated coeliac disease and biopsied controls

	Autoimmune enteropathy		Coeliac disease		Biopsied controls	
	Case 1	Case 2	Case 1	Case 2	Case 1	Case 2
TUNEL$^+$ enterocytes	59·3	48·2	41·2	38·2	3·2	2·8
Fas$^+$ enterocytes	3·7	4·5	81·3	90·7	2·3	2·8
FasL$^+$ intra-epithelial lymphocytes	3·2	2·6	87·9	84·7	1·5	2·5
Perforin$^+$ intra-epithelial lymphocytes	2·4	2·8	32·1	28·0	0·8	0·4
FasL$^+$ lamina propria mononuclear cells	3·9	3·1	24·6	29·1	1·9	2·8
Perforin$^+$ lamina propria mononuclear cells	1·7	2·3	19·0	22·3	1·2	1·5
Surface to volume ratio	6·9	8·5	7·1	8·8	33·4	37·2

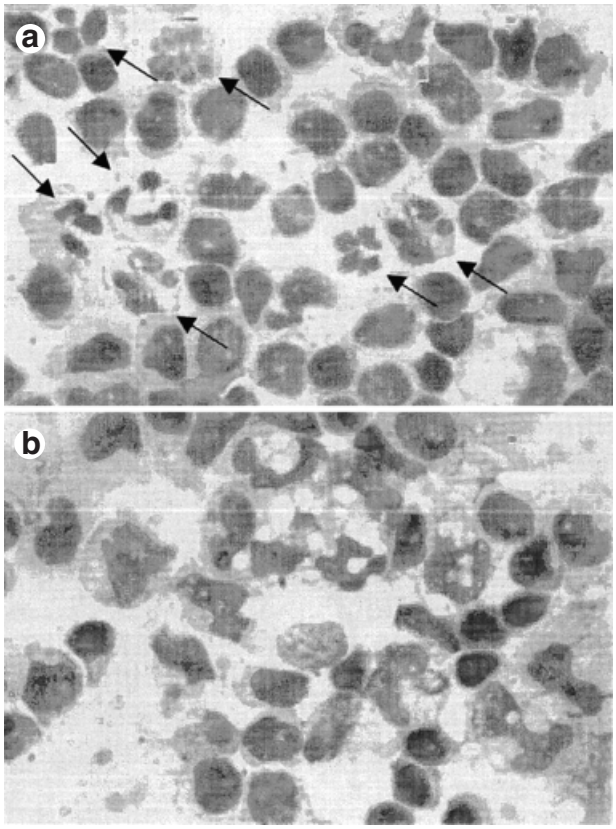

Fig. 4. Light microscopic detection of isolated human enterocytes (target) primed with autoimmune enteropathy (a) or control serum (b) co-cultured for 4 h with peripheral blood mononuclear cells (effector) at 50 : 1 effector/target ratio. Enterocytes show features of apoptosis characterized by nuclear fragmentation and cellular swelling (arrows) only when primed with autoimmune enteropathy serum (a). Original magnification, ×1000.

inactivated, were performed. Our results are consistent with a relevant and effector/target ratio-dependent apoptosis when enterocytes were primed with autoimmune enteropathy sera but not with precleared autoimmune enteropathy, coeliac or control sera. Furthermore, the occurrence of stable effector/target conjugate formation only when target enterocytes were previously incubated with autoimmune enteropathy sera revealed the specific antibody dependence in the target recognition. In addition, the insignificant ^{51}Cr release together with the clear appearance of nuclear apoptotic features confirm that apoptosis rather than necrosis is the pathway of ADCC-mediated enterocyte death in autoimmune enteropathy.

Although the identification of the serum factor responsible for ADCC-mediated enterocyte apoptosis in autoimmune enteropathy was not addressed specifically in the present study, it is more than likely that in our cytotoxicity assay antigen specificity was conferred by EAA which drive cytotoxic T lymphocytes and/or NK cells, engaged via their surface Fc receptors, to EAA-coated enterocytes [27]. In this context the lack of complete EAA disappearance despite significant improvement of the intestinal lesions in our two patients after steroid treatment [2] does not lessen their putative pathogenic relevance since steroids may have suppressed the effector mechanism of ADCC rather than the production of EAA *per se*.

The conceptual importance of our results lies in the demonstration that ADCC may cause villous atrophy of the small intestine, although we should remember that ADCC had been postulated as the main mechanism for autoimmune enteropathy since its first description [24]. Moreover, on clinical grounds our results offer the rationale for new and possibly safer therapeutic strategies, since high doses of intravenous immune globulins have been shown to block Fc receptors on cellular effectors of antibody-dependent cytotoxicity [28].

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