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SHORT REPORT

Contribution of immunofluorescence to identification and characterization of antineutrophil cytoplasmic antibodies in inflammatory bowel disease

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Abstract We evaluated the combined use of different fixatives for the identification of atypical perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) in patients with inflammatory bowel diseases (IBD) by indirect immunofluorescence (IIF). Sera from 59 ulcerative colitis (UC) and 37 Crohn's disease (CD) patients, and from 64 healthy controls were studied. The IIF on ethanol-, formalin-, and methanol-fixed neutrophils was used for the detection of ANCA. Enzyme linked immunosorbant assay (ELISA) was performed to identify the antigens recognized by ANCA. ANCAs were present in 35 of 59 (59.3%) UC patients and in 10 of 37 (27.02%) CD patients. Atypical p-ANCA positivity was strongly associated with UC disease (44.1% in UC vs. 8.1% in CD; $p=0.0002$). The combined application of different fixatives contributed to make easy the differentiation between typical p-ANCA and atypical p-ANCA. Atypical p-ANCA determination appears to be a useful parameter for the distinction between UC and CD.

Keywords Anti-neutrophil cytoplasmic antibodies · Atypical perinuclear anti-neutrophil cytoplasmic antibodies · Crohn's Disease · Inflammatory bowel diseases · Ulcerative Colitis

Introduction

Inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic, relapsing, and tissue destructive idiopathic inflammatory conditions. The etiology of these diseases has not yet been fully elucidated [1]. Autoimmune processes may play a role in their pathogenesis since several types of autoantibodies have been found in the diseases, such as antibodies to neutrophils (anti-neutrophil cytoplasmic antibodies [ANCA] and anti-Saccharomyces cerevisiae antibodies [ASCA]) [2, 3].

According to the consensus statement on testing and reporting of ANCA [4], reporting of indirect immunofluorescence results should distinguish among cytoplasmic (c-ANCA) and perinuclear (p-ANCA) patterns of ANCA. ANCA detected in UC are called p-ANCA, although they differ substantially from the typical p-ANCA. They are characterized by a broad inhomogeneous rim-like staining of the nuclear periphery [5]. The target antigens of atypical p-ANCA have not been yet identified. Considerable evidence supports the notion that these are not cytoplasmic antigens, like those for typical p-ANCA, but nuclear antigens, associated with the inner side of the neutrophils' nuclear membrane [5]. The goal of our study was to evaluate the interest of the use of different fixatives in the identification of atypical p-ANCA in patients with IBD by indirect immunofluorescence. Our study included investigation of the presence of antibodies against myeloperoxidase (MPO), proteinase 3 (PR3), lactoferrin (LF), elastase, cathepsin G (CatG), and bacterial permeability increasing (BPI) protein. We also evaluated the correlation between the presence of atypical p-ANCA and some clinical parameters of UC disease.

Methods

Ninety-six consecutive patients diagnosed as having IBD (59 UC, 37 CD) at the Department of Gastroenterology of

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Table 1 Clinical features of patients with Crohn's disease and ulcerative colitis

	Crohn's disease	Ulcerative colitis	Total
Patients	37	59	96
Sex (male/female)	24/13	31/28	55/41
Age (years) (mean [SD])	39.4 (13.8)	40.5 (12.4)	40.1 (13.2)
Disease location			
Ileum	13		
Colon	12		
Ileocolon	12		
Pancolitis		30	
Left-sided		22	
Proctitis		7	
Extraintestinal manifestations	16	13	29

Hedi Chaker University Hospital of Sfax (Table 1) between June 2005 and January 2009 were investigated. The study was approved by the local ethics committee, and all enrolled patients gave their informed consent to participate. The diagnosis of IBD was made using standard endoscopic and histological criteria, and clinical characteristics were given according to the Montreal classification [6]. Sixty-four healthy subjects were included as controls.

ANCA detection was performed by indirect immunofluorescence on ethanol-fixed neutrophil slides (Euroimmun®, Lübeck, Germany) at 1:10 sampling dilution, and using a fluorescein-labeled immunoglobulin G (IgG), according to the manufacturer's recommendations. ANCA positive sera in ethanol-fixed neutrophils were tested by indirect immunofuorescence using formalin and methanol fixed neutrophils (Euroimmun®, Lübeck, Germany). Interpretation of the immunofuorescence results was based on the behaviour of the specimens on ethanol-, formalin-, and methanol-fixed slides and included the following patterns: c-ANCA occurred as coarse, speckled cytoplasmic fluorescence with accentuation between the nuclear lobes on both ethanol- and formalin-fixed substrates. Typical p-ANCA yielded a finely rimmed, homogeneous fluorescence staining of the perinuclear cytoplasm with nuclear extension on ethanol- and methanol-fixed neutrophils and as granular cytoplasmic staining on formalin-fixed cells. Atypical p-ANCA were characterized by a broad, non-homogeneous rim-like staining of the nuclear periphery or a combined pattern characterized by diffuse cytoplasmic staining, not accentuated between the nuclear lobes, along with broad, non-homogeneous perinuclear fluorescence on ethanol- and methanol-fixed neutrophils and weak perinuclear staining or a lack of fluorescence on formalin-fixed neutrophils.

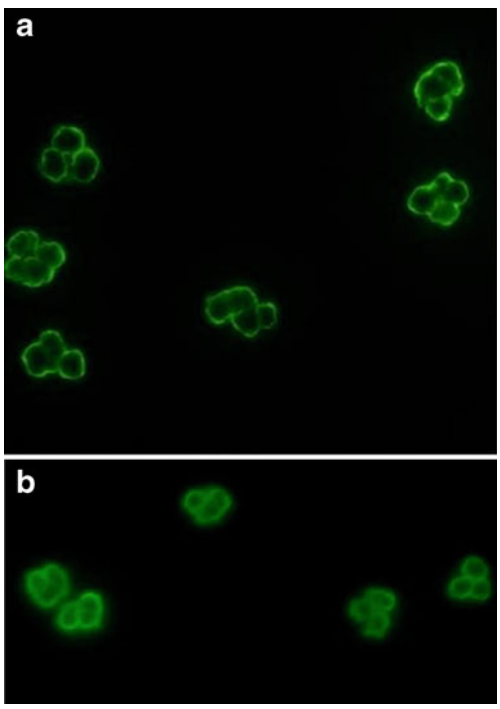

Fig. 1 Immunofluorescence staining patterns produced by ANCA assays, on ethanol-fixed neutrophils, for two different patients. (**a**) Atyical p-ANCA pattern on ethanol-fixed neutrophils. (**b**) Typical p-ANCA pattern on ethanol-fixed neutrophils

Table 2 Prevalence of anti-neutrophil cytoplasmic antibodies (ANCA) in patients with inflammatory bowel disease

	Ulcerative colitis (n=59)	Crohn's disease (n=37)	Healthy controls (n=64)	p value*
Total ANCA	35 (59.3%)	10 (27%)	0 (0%)	0.002
c-ANCA	7 (11.9%)	5 (13.5%)	0 (0%)	0.8
p-ANCA	2 (3.4%)	2 (5.4%)	0 (0%)	0.6
Atypical p-ANCA	26 (44.1%)	3 (8.1%)	0 (0%)	0.0002

*p value for comparison between ulcerative colitis and Crohn's disease

Serum samples were tested separately for the presence of immunoglobulin G (IgG) antibodies against PR3, LF, MPO, elastase, CatG, and BPI protein by ELISA (Euroimmun®, Lübeck, Germany) according to the manufacturer's protocol.

All statistical analyses were performed using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). Comparison of different groups was made using chi square test. The Student t test was used for comparison of means. The threshold for statistical significance was set at $p<0.05$.

Results

ANCA patterns (Fig. 1) were present in 35 of 59 patients (59.3%) with UC and in 10 of 37 patients (27%) with CD (Table 2). The ANCA patterns were mainly atypical p-ANCA for UC (44.1%), whereas for CD patients the prevalence of atypical p-ANCA (8.1%) was slightly higher than p-ANCA (5.4). The occurrence of c-ANCA was similar in both UC and CD (11.9% and 13.5%, respectively). None of the healthy controls had p-ANCA or c-ANCA.

Atypical p-ANCA positivity was significantly associated with UC (44.1%) compared to patients with CD ($\chi^2=13.9$, $p=0.0002$, $OR=8.9$).

We assessed the presence of IgG antibodies reacting with different cytoplasmic components of the neutrophil. In UC patients with atypical p-ANCA positive sera, one patient showed reactivity with BPI, another with elastase, and two with LF. In CD patients with atypical p-ANCA positive sera, only one showed reactivity with Cat G. We did not find anti-PR3 or anti-MPO in any patient. Atypical p-ANCA detection did not correlate with sex or disease location, but correlated with later age of onset ($p=0.02$). Occurrence of extraintestinal manifestations of UC did not affect antibody prevalence in the sera.

Discussion

Differentiating between atypical and typical p-ANCA on ethanol-fixed neutrophils by indirect immunofluorescence remains challenging. Simultaneous reactivity of atypical p-ANCA on formalin- and ethanol-fixed neutrophils and the feasibility of the new microscopic criteria suggested by Terjung et al. [5] have not been systemically studied in IBD [7, 8]. ANCA systems that replace formalin-fixed neutrophils with an enzyme (DNase I) digestion step during indirect immunofluorescence have also been developed but are rarely used [7, 8]. Sera reactivities displaying the p-ANCA pattern and associated with negative anti-MPO ELISA, negative pattern using formalin fixation, and perinuclear pattern using methanol fixation used by Desplat-Jégo et al. [2] determined the atypical p-ANCA pattern associated with IBD.

In the present study, we attempted to differentiate between p-ANCA and atypical p-ANCA patterns in IBD patients by using indirect immunofluorescence microscopy and various fixatives. The most frequent pattern in UC was atypical p-ANCA and we found significant differences in the occurrence of atypical p-ANCA in patients with UC (44.1%) and CD (8.1%). Similar results were found by other authors [9]. The atypical p-ANCA test alone was able to differentiate between UC cases and CD cases with a high specificity (97%) and positive predictive value (89.7%). However, the overall sensitivity was low. The use of this marker alone is insufficient for screening purposes for patients suspected to have IBD. However, the high specificity and the high positive predictive value support its use in the confirmation of a diagnosis.

For atypical p-ANCA, the antigenic targets have not been yet identified. Sugi et al. [10] reported that both anti-LF and anti-MPO antibodies showed high prevalence in Japanese IBD patients. Consistent with our results, Radice et al. [11] demonstrated that the specificity of p-ANCA was not directed against either PR3, LF, MPO, elastase, or BPI.

Our data lead us to conclude that we have to rely on the indirect immunofluorescence results in characterization of atypical p-ANCA.The occurrence of atypical p-ANCA in UC is associated with a characteristic clinical appearance and represents a distinct subgroup which is characterized by specific HLA markers [12]. In our study, the atypical p-ANCA in UC patients is associated with later age of onset. However, we did not observe any correlation between ANCA positivity and the other clinical features of the disease including disease location, extraintestinal manifestations, and sex. In conclusion, our results suggest that performing the ANCA-indirect immunoflourescence test, with the use of different fixatives, can help to differentiate among the different types of ANCA, particularly p-ANCA and atypical p-ANCA, and the determination of atypical p-ANCA may be useful to differentiate UC from CD.

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