ROLE OF IMMUNOLOGICAL MARKERS IN CERTAIN GASTROENTEROLOGICAL DISORDERS [+]

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Abstract:

Ulcerative colitis (UC) and celiac disease (CD) are autoimmune diseases. Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) have been described in patients with inflammatory bowel disease, mainly ulcerative colitis. However, their role in pathogenesis and diagnostic value are still controversial.

The aim of this study was to assess the prevalence of certain perinuclear anti-neutrophil cytoplasmic antibodies (p.ANCA) including anti-lactoferrin, anti-lysozyme and anti-elastase antibodies among patients with ulcerative colitis and celiac disease. This study was conducted during the period from August 2012 to January 2013. Subjects enrolled in this study were 34 ulcerative colitis patients (age range of 14-60 years with a mean of 31.29 years) and 34 celiac disease patients (age range of 12-58 years with a mean of 28.59 years). All patients were attending two Iraqi hospitals in Baghdad and Babylon cities who were subjected to endoscopic examination under the supervision of a gastroenterologist, for their primary diagnosis. The primary diagnosis was supported by serological examination for IgA /IgG tissue transglutaminase (tTG) for patients with celiac disease. Another 34 healthy subjects who were matching the patients groups in their age range and mean, were also enrolled in this study as a negative control group. Male to female ratios were approximately similar in all study groups.

All subjects were serologically analyzed using ELISA for the frequency of positive titers of anti-lactoferrin, anti-lysozyme and anti-elastase antibodies which were 32.4%, 35.3% and 47% in ulcerative colitis patients and 5.9%, 8.9% and 11.8% for celiac disease patients, respectively. The titer of anti-lactoferrin and anti-lysozyme antibodies had exhibited a high convergent, however anti-elastase titer was the highest among the three p.ANCA, in this study for ulcerative colitis group. The titer of the three studied p.ANCAs in celiac disease patients were generally lower than their corresponding is in ulcerative colitis group. To the best of our knowledge, this study is first one that examined the prevalence of p.ANCAs in Iraqi celiac disease patients.

دور المعلمات المناعية في بعض اضطرابات الجهاز الهضمي

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المستخلص :

يعد **التهاب القولون القرحي** و **مرض الجوف** من الامراض ذاتية المناعة. لقد تم وصف الأضداد الذاتية السيتوبلازمية للخلايا العدلة النواة (P.ANCA) في المرضى المصابين بمرض المعي الالتهابي ، وبالدرجة الأولى التهاب

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القولون القرحي. مع ذلك يبقى دورها في نشوء المرض والأهمية التشخيصية مختلف عليه. تهدف هذه الدراسة لمعرفة مدى شيوع الأجسام المضادة للاكتوفرين (anti–LF) واللايسوزايم (anti–Lysozyme) والايلاستيز (anti–EL) والتي تنتمي إلى الأضداد السايتوبلازمية لضد العدلة، حول النواة لمرضى التهاب القولون القرحي ومرض الجوف . هدف هذه الدراسة كان لقياس انتشار بعض الأضداد الذاتية السيتوبلازمية للخلايا العدلة النواة (P.ANCA) التي تتضمن الأجسام المضادة للاكتوفرين (anti–LF) واللايسوزايم (anti–Lysozyme) والايلاستيز (anti–EL) ، للمرضى المصابين بالتهاب القولون القرحي و مرض الجوف . اجريت هذه الدراسة خلال الفترة من شهر اب 2012 ولغاية شهر كانون الثاني 2013. المرضى الذين تضمنتهم هذه الدراسة كانوا 34 مريضاً مصابين بالتهاب القولون القرحي (تراوحت اعمارهم من 14–60 سنه وبمتوسط اعمار 31.29 سنة) و34 مريضا مصاب بمرض الجوف تراوحت اعمارهم من 12–58 سنه وبمتوسط اعمار 28.59 سنة) . كل المرضى كانوا يراجعون مستشفيين عراقيين في مدينتي بغداد وبابل ، واخضعوا للتنظير الداخلي واجري التنظير بواسطة اطباء اختصاص بالجهاز الهضمي للتشخيص الاولي. التشخيص الاولي لمرضى الجوف كان مدعوم بالفحص المصلي لأضداد الترانسكلوتامنيز (anti–tTG, IgA or IgG). وكذلك 34 شخصا من الاصحاء كانوا مطابقين لأعمار مجاميع المرضى ، ادخلوا الدراسة كمجموعة سيطرة . نسبة الذكور للإناث كانت متشابهة لكل مجاميع الدراسة تقريباً.

كل الاشخاص اجريت لهم فحوصات مصلية باستخدام تقنية الأنزيم المرتبط الممتز المناعية (ELISA) لقياس تراكيز اضداد اللاكتوفرين (anti–LF) واللايسوزايم (anti–Lysozyme) والايلاستيز (anti–EL)، وكانت النتائج الموجبة (32.4%) ، (35.3%) و (47%) من مرضى التهاب القولون القرحي و(5.9%) ، (8.9%) و (11.8%) من مرضى الجوف ،على التوالي. واظهرت النتائج تقارب تراكيز اضداد اللاكتوفرين (anti–LF) واللايسوزايم (anti–Lysozyme) ، و كان اعلى تركيز مسجل تركيز ضد الايلاستيز (anti–EL) لمجموعه التهاب القولون القرحي. بينما لدى مجموعه مرض الجوف كانت التراكيز الثلاثة كانت هي الأوطأ لمرضى الجوف.

حسب معرفتنا هذه الدراسة هي الاولى التي تناولت قياس انتشار الأضداد الذاتية السيتوبلازمية للخلايا العدلة النواة للمرضى العراقيين المصابين بمرض الجوف.

Introduction:

Gastrointestinal (GI) disorders, including functional bowel diseases such as irritable bowel syndrome (IBS) and inflammatory bowel diseases such as Crohn′s disease and ulcerative colitis, afflict more than one in five Americans, particularly women. While some GI disorders may be controlled by diet and pharmaceutical medications, others are poorly moderated by conventional treatments. Symptoms of GI disorders often include cramping, abdominal pain, inflammation of the lining of the large and/or small intestine, chronic diarrhea, rectal bleeding and weight loss [1]. Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal system caused by strong genetic and non-genetic influences. The best known forms of IBD are ulcerative colitis (UC) and Crohn's disease. In the early history of Europe, the UC is mixed with infectious colitis or bacillary dysentery. The symptoms of illness are simply included diarrhea, rectal bleeding, abdominal pain, fever and multiple complication. Later on, UC was separated from that infectious causes and classified as inflammatory bowel disease [2].Ulcerative colitis affects the colon and the inflammation is limited to the mucosal layer [3].Characteristic histological findings are acute and chronic inflammation of the mucosa by polymorphnuclear (PMN) leukocyte and mononuclear cells, crypt abscesses, distortion of the mucosal glands and goblet cells depletion [4]. Celiac disease

(CD) is a syndrome characterized by damage of the small intestinal mucosa caused by the gliadin fraction of wheat gluten and similar alcohol-soluble proteins (prolamines) of barely, wheat and rye in genetically susceptible subjects. The presence of gluten in food of these subjects leads to self-continuous mucosal damage, whereas elimination of gluten results in full mucosal recovery. The clinical manifestations of celiac disease are changeable in nature and vary markedly with the age of the patient, the duration and extent of disease, and the presence of extra-intestinal pathological conditions. In addition, to these classical gastrointestinal forms, a variety of other clinical manifestations of the disease has been described, including atypical and asymptomatic forms. Therefore, diagnosis of celiac disease is extremely challenging and relies on a sensitive and specific algorithm that allows the identification of different manifestations of the disease. Serological tests developed previously provide a non-invasive tool to screen both individuals at risk for the disease and the general population. However, the current gold standard for the diagnosis of Celiac disease remains histological confirmation of the intestinal damage in serological positive individuals. The keystone treatment of Celiac disease patients is a lifelong elimination diet in which food products containing gluten are avoided [5]. Neutrophils are commonly found in blood circulation and attracted to the injury or inflammatory sites by pathogens. Moreover, dysregulation or abnormal function of neutrophils in disease stage will lead to inappropriate accumulation and tissue damage even with the absence of pathogens. Same as in intestine, the tissue neutrophils accumulate and damage the lining of the gut [6].The pathogenic importance of antineutrophil cytoplasmic antibodies (ANCA) associated with UC is controversial as both the antigen responsible for their production and how the antigen-antibody interaction occurs in *vivo* are unknown. A number of nuclear and cytoplasmic antigens have been proposed as a possible target for ANCA in UC [7].

Aims of study: Detection of anti-lactoferrin, anti-elastase and anti-lysozyme autoantibodies in ulcerative colitis patients, celiac disease and healthy controls.

Subjects, Materials and Methods:

Subjects: A prospective study was conducted on the main following groups:

Patients group: A total of 68 patients were enrolled in this study during the period from August 2012 to January 2013. A structure interview using standard questionnaire was administered by interviewers with patients during their visit to Al-yarmook hospital in Baghdad-Iraq and Marjan medical city in Babylon-Iraq. In the unit of digestive system diseases ,each patients was subjected to endoscopy by a gastroenterologist including 34 with ulcerative colitis (17 males , 17 females) their ages ranged (14-60 years) and 34 with celiac disease (18 males ,16 females) their ages ranged (12-54 years). All CD patients were positive for anti-tTG (IgA+/-IgG isotype).

Control group: The control group consisted of 34 healthy subjects (18 males, 16 females) without any history of gastrointestinal or other diseases. The age ranged (12-58 years).

Specimen: Venous blood samples (5 ml) were collected in dry clean plain tubes from patients and controls. Serum was obtained from freshly drawn blood. Serum was quickly frozen at -20^{o}C and stored until the tests were processed.

Study Protocols: Anti-lactoferrin, anti-lysozyme and anti-elastase antibodies using ELISA was detected in all study groups (UC, CD and controls) whereas anti-tTG (IgA and IgG) antibodies was measured in CD patients.

Materials: five serological tests were performed in this study including anti-lactoferrin, anti-lysozyme , anti-elastase (IMMUCHEM-Belgium) , anti-tTG IgA and anti-tTG IgG antibodies (Aesku Diagnostics Mikroforum ring 2. 55234 Wendelsheim Germany) using Enzyme linked immune sorbent assay technique.

Statistical Analysis: The following statistical data analysis approaches were used in order to analyze and assess the results of the study:

1. Descriptive data analysis:

2. Inferential data analysis:

These were used to accept or reject the statistical hypotheses, which included the following:

a- One-way ANOVA for equality of Means in the three of different groups (ulcerative Colitis, Coelic disease, and control), with (LSD) Least significant difference test.
b- Odds ratio for findings the numbers of times exceeding the first factor (studied groups of ulcerative Colitis and Coelic disease) according to their outcomes individuals related to individuals of normal and elevated outcomes in the 2X2 association table.

Results:

Demographic characteristics of study groups: The age distributions and data of the patients were summarized in table (1).

Table (1) Demographic characteristics of study groups

Variable	Ulcerative colitis patients	Celiac disease Patients	Control	Total	C.S. P-value
No.	34	34	34	102	
Sex (male/female) ratios	1:1 (17/17)	1.12:1 (18/16)	1.12:1 (18/16)	1.08:1 (53/49)	C.C.=0.028 P=0.961 NS
(Age range) mean in years	(14_60) 31.29	(12-54) 28.59	(12-58) 35.53	(12-60) 31.8	C.C.=0.222 P=0.725 NS

NS: Non Sig. at P>0.05

The result had been indicated that there was a non-significant differences at P>0.05 for the observed frequency's distributions of the three samples, which were corresponding proportionally.

Table (2): Multiple Comparison by (LSD) among all pairs of groups in each concentration independently

Concentrations	Group (I)	Group (J)	Sig.	C.S. (*)
Anti-Lactoferrin	Ulcerative Colitis	Celiac disease	0.001	HS
		Control	0.000	HS
	Celiac disease	Control	0.052	NS
Anti-Lysozyme	Ulcerative Colitis	Celiac disease	0.001	HS
		Control	0.000	HS
	Celiac disease	Control	0.031	S
Anti-Elastase	Ulcerative Colitis	Celiac disease	0.000	HS
		Control	0.000	HS
	Celiac disease	Control	0.013	S

(*) HS: Highly Significant at P< 0.01 ; S : Significant at P< 0.05 ; NS : Non-Significant at P> 0.05
Group(I)=include ulcerative colitis and celiac disease ;Group(J)= include Healthy control and celiac disease

Distribution of samples according to different groups:

Table (3): Descriptive Statistics the studied parameters of different concentrations distributed among different groups

Concentrations & Groups	No.	%	Mean Titer U/mL	Std. Dev.	95% C. I. for Mean L. B. - U. B.	Min. titer	Max. titer
Anti-Lactoferrin-Ulcerative colitis-Elevated	11	32.4	16.68	3.24	14.50- 18.86	11	20
Anti-Lactoferrin-Ulcerative colitis-Normal	23	67.6	3.87	1.71	3.13- 4.60	1.5	8
Anti-Lysozyme-Ulcerative colitis-Elevated	12	35.3	16.48	3.23	14.43- 18.53	13.5	26
Anti-Lysozyme- Ulcerative colitis- Normal	22	64.7	3.72	1.18	3.20- 4.24	2.3	7.5
Anti-Elastase - Ulcerative colitis - Elevated	16	47.0	18.63	4.14	16.42- 20.83	14	30
Anti-Elastase - Ulcerative colitis - Normal	18	53.0	7.56	1.25	6.94- 8.18	5	9
Anti-Lactoferrin- Celiac disease - Elevated	2	5.9	17.00	1.41	4.29- 29.71	16	18
Anti-Lactoferrin - Celiac disease - Normal	32	94.1	3.63	2.16	2.85- 4.41	1.5	9
Anti-Lysozyme - Celiac disease - Elevated	3	8.9	15.50	1.32	12.21- 18.79	14	16.5
Anti-Lysozyme - Celiac disease - Normal	31	91.1	3.55	0.72	3.29- 3.82	2.5	5
Anti-Elastase - Celiac disease - Elevated	4	11.8	16.13	1.03	14.48- 17.77	15	17
Anti-Elastase - Celiac disease-Normal	30	88.2	5.17	1.63	4.56- 5.77	2.5	8.5
Anti-Lactoferrin - Control –Normal	34	100	2.34	0.57	2.14- 2.53	1.5	3.5
Anti-Lysozyme - Control –Normal	34	100	2.32	0.46	2.16- 2.48	1.5	3.2
Anti-Elastase - Control –Normal	34	100	3.81	0.65	3.59- 4.04	2.5	5

L.B=Lower band , U.B= Upper band

The results showed a highly convergent concentration values between anti-lactoferrin and anti-lysozyme for ulcerative colitis. The highest elevated mean titer was reported with anti elastase in ulcerative colitis patients which were accounted for about half of the total number of patients. For CD patients, the mean titers for the three p.ANCAs lower than their corresponding ones.

Table (4): Odds ratio and their 95% confidence interval in the 2X2 association tables of times exceeding elevated individuals at ulcerative colitis group contrasted with celiac disease group

Concentrations of anti-	Sample's Contrasts	Odds Ratio	95% C.I. For Interval	
			L.B.	U.B.
Lactoferrin	Ulcerative colitis X Celiac disease	7.7	1.5	37.9
Lysozyme		5.6	1.4	22.4
Elastase		6.7	1.9	23.1

Table (4) shows that with concentration of anti-lactoferrin, ulcerative colitis group reported (7.7) times of elevated cases while only (1) elevated individual in celiac disease group, and reported (5.6) times of elevated cases of anti-lysozyme concentration while only (1) elevated individual in celiac disease group, and finally ulcerative colitis group reported (6.7) times of elevated cases against only (1) elevated individual in celiac disease group at the anti-elastase concentration. In addition to that, estimations by interval illustrated too highly width intervals at the three different antibodies concentrations.

Discussion:

The sex distribution of coeliac disease patients showed a slight male excess in the present study, but this was not significant statistically. This was incompatible to the results reported by [5, 8]. While the sex distribution of ulcerative colitis shown similar ratios. In UC population-based studies no significant differences was shown [9]. Some previous studies have shown male predominance by two folds [10]. In other study, ulcerative colitis is slightly more common in women than in men. Age of onset follows a bimodal pattern, with a peak at 15-25 years and a smaller one at 55-65 years, although the disease can occur in people of any age [11]. Other studies have also shown an increasing incidence of UC in children less than 18 years old [12]. In a Japanese nationwide survey, the peak age of onset was 20–29 years old for UC, whereas the median age of diagnosis in a Korean study was 35 years old [13]. The mean of ages in this study is (31.3) years for ulcerative colitis, which was compatible with the mean of age in other studies (31.3 and 32.1 years), and fewer than those reported by other studies (41.1 and 37.9 years) [14-15]. The mean ages in coeliac disease and control groups were (28.6) and (35.5) respectively, with no significant difference. This difference may be due to sampling biases and epidemiologic studies on selected populations which may provide healthier figures. Anti-neutrophil cytoplasmic antibodies (ANCAs) are a group of autoantibodies, mainly of the IgG type, against antigens in the cytoplasm of neutrophilic granulocytes (the most common type of white blood cell) and monocytes [16]. Autoantibodies against various “self” intra /or extracellular, cell surface-bound or soluble antigens are

frequently present in the sera of patients with autoimmune diseases [17]. Neutrophil apoptosis, or programmed cell death, is vital in controlling the duration of the early inflammatory response, thus restricting damage to tissues by the neutrophils. Anti-neutrophil cytoplasmic antibodies may develope either via ineffective apoptosis or ineffective removal of apoptotic cell fragments, leading to the exposure of the immune system to molecules normally sequestered inside the cells. This theory solves the paradox of how it could be possible for antibodies to be raised against the intracellular antigenic targets of ANCA [18]. Immune reactions that compromise the integrity of the intestinal epithelial barrier may contribute to ulcerative colitis. Serum and mucosal autoantibodies against intestinal epithelial cells may be involved. The presence of (ANCA) is a well-known feature of inflammatory bowel disease (IBD) [19]. The positive rates of ANCA, varied among UC groups with the various countries in which the studies have been carried out, ranging from 3% in an Indian study, up to 82.6% in a German study reviewed in [20]. Anti-neutrophil cytoplasmic antibodies were originally shown to divide into two main classes, c-ANCA and p-ANCA, based on the pattern of staining on ethanol-fixed neutrophils and the main target antigen. Anti-neutrophil cytoplasmic antibodies is most commonly associated with ulcerative colitis. Specifically, perinuclear ANCA (p-ANCA), found on the inside of the nuclear membrane, is highly associated with ulcerative colitis [21]. The role of p-ANCA in the pathogenesis of UC is not clear. Several nuclear and cytoplasmic antigens have been proposed as the target epitopes for p-ANCA in UC, but none have been proven to be the causing antigen [22].Lactoferrin, bactericidal/permeability-increasing protein, cathepsin G, elastase and lysozyme have all been reported as target antigens for ANCA in IBD [23]. Although ANCA are not very useful for distinction between UC and CD, they can be used as a diagnostic marker to distinguish IBD from other colitides and diarrhoeal illnesses [24].The presence of ANCA in high titers in a patient with coeliac disease reinforces the association of coeliac disease with other autoimmune diseases. Any direct link between ANCA and coeliac disease and a etiological role of ANCA in vasculitis remain to be seen [23]. Moreover, many other autoimmune diseases were also associated with coeliac disease and this may be due to correlation between coeliac disease and autoimmune disorders. These observations were consistent with some previous studies [25, 26]. The present results confirmed results of previous studies which showed that ANCA were frequent in UC and that P-ANCA is predominant in patient IBD [27]. The present study showed low prevalence of anti-lactoferrin, anti-lysozyme and anti-elastase antibodies among patients with UC. These results agreed with a similar pattern found in other study of which tested UC sera with ELISA for antibodies against different neutrophil autoantigens and its result [29]. This study also agreed with other study which stated that the frequency of ANCA by fixed-neutrophil ELISA was only 35% in Japanese patients with UC [31]. In UC, autoantibodies against granulocyte proteins can be detected, frequently simultaneously in one patient. Anti-LF antibodies have been found at high frequency in ulcerative colitis and sclerosing cholangitis [30]. In 2005, the study of Locht *et al.*, showed (53%) of patients with UC were positive in ELISA for antibodies against LF, while of (53%) ELISA positive sera, 60% were also positive in indirect immunofluorescence [31]. Previous study was reported that (0-46%) of UC patients were positive for anti-elastase antigens [32]. Other study had reported that (9-50%) of UC patients were positive for anti-lactoferrin antigens [33]. On the other hand, Kossa and his coworkers and Nassberger *et al*. tests demonstrated that (0-53%) of sera UC patients were positive with anti-lysozyme antigens [34, 35]. These result agreed with The current study which revealed that the (32.4%), (35.3%) and (47%) of ulcerative colitis patients were seropositive for anti-lactoferrin, anti-lysozyme and anti-elastase antibodies, respectively. Lerner *et al* observed the absence of ANCA in 35 celiac disease patients [36], These result disagreed with the current study which revealed that

(5.9%), (8.9%) and (11.8%) of celiac disease patients were seropositive for anti-lactoferrin , anti-lysozyme and anti-elastase antibodies, respectively. While other study agreed with our study which showed 4 of 51 celiac disease patients (7.7%) seropositive for ANCA [37].The chronic activation of PMN's in several forms of ongoing inflammation may explain why other lysosomal components can become autoantigenic targets for p-ANCA. The azurophil granule enzyme elastase (EL), which shows considerable sequence homology with proteinase 3 and cathepsin G and exhibits elastinolytic properties like proteinase 3 , is an important autoantigen in drug induced lupus and drug-induced vasculitis along with MPO [38]. In combination with PR3, elastase is known to stimulate endothelial tissue factor, which in turn could be relevant to the development of vascular inflammation [39].No previous studies to the best of our knowldge had discussed the presence of the anti-lactoferrin, anti-lysozyme and anti-elastase autoantibodies in patients with coeliac disease. This study show significant differences in concentrations of these autoantibodies in UC compare to CD and healthy control groups. The reason for the difference between the present results and the other studies results might be due to the difference in the clinical demographic data, patients might have a different disease treatment, status, severity and associated disease and may be due to difference of sample size.

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