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Occurrence of Nonceliac Gluten Sensitivity in Patients with Allergic Disease

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Key Words
Nonceliac gluten-sensitive enteropathy · Allergy · DQA1*05 · Inflammation · Anemia · Gluten-free diet

Abstract
Background: The aim of this study was to determine the occurrence of gluten sensitivity (GS) in a group of allergic patients and to assess the efficacy of a gluten-free diet (GFD) on the improvement of the symptomatology in those who were diagnosed with GS. ***Methods:*** 262 unrelated allergic patients with gastrointestinal symptoms of obscure origin were tested for GS condition by biopsy. All patients were also genotyped for the typical celiac DQ2 and DQ8 molecules and investigated for several hematological parameters such as antigliadin and antiendomysial antibodies. Patients displaying mucosal lesions were invited to follow a GFD. ***Results:*** Seventy-seven of the 262 allergic patients were positive to mucosal lesions, but negative to the antiAGA, anti-EMA and to DQ2 and DQ8 molecules. We found, instead, a prevalence of the DQA1*05 allele, whereas anemia of inflammatory origin represented the predominant complaint in our subjects. The positive patients, who, after the GS diagnosis, followed a GFD, exhibited control of symptoms as well as stabilization of the hematological parameters even if allergic manifestations were not abated. ***Conclusions:*** A nonceliac gluten-sensitive enteropathy (NCGSE) commonly occurs in allergic patients. Based on the high prevalence of NCGSE in allergy, it is recommended that biopsy should be part of the routine investigation of allergic disease to offer the benefits of treatment with a GFD to the patients.

Introduction

Gluten sensitivity (GS) consists of a pathological condition in which genetically susceptible individuals exhibit a wide and unexplained spectrum of symptoms and clinical signs. The lesion usually resolves when gluten is excluded from the diet. Celiac disease (CD) is the paradigm of GS. It is an autoimmune enteropathy caused by an abnormal immune response to dietary gluten and proteins derived from it.

The broad spectrum of gluten-sensitive intestinal mucosal changes that are characteristic, though not pathognomonic, of this condition, ranges from a complete disruption of the mucosal architecture with villous flattening and crypt hyperplasia to a slight increase in inflammatory lymphocytes infiltrated in both the epi-

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thelium and the lamina propria. Increased levels of serum antibodies, specific for various antigens, including gluten and the autoantigen tissue transglutaminase, are also present in celiac patients.

The CD presents a well-defined HLA class II association [1]. Indeed, in European Caucasian populations, more than 90% of celiacs carry the HLA-DQA1*05/DQB1*02 encoding the DQ2 heterodimer [2]. The α- and β-chains of this heterodimer are usually encoded in cis on a DR3 haplotype. However, they may also be encoded in trans with the DQA1*05 allele usually on the DR5 haplotype and the DQB1*02 allele usually on a DR7 haplotype. The DQ8 heterodimer (coded by DQA1*03/DQB1*0302) linked to the DR4 haplotype is commonly encoded by celiacs who do not carry the DQ2 heterodimer. However, it is also possible to find celiac patients carrying neither the DQ2 nor the DQ8 heterodimers, many of them have been reported to encode just one chain of the DQ2 heterodimer (that is either DQA1*05 or DQB1*02, but not both) underlying the primary importance of HLA-DQ molecules in the genetic susceptibility to CD [3]. The observation that CD is strongly associated with DQ2- or DQ8-encoded heterodimers or with a single molecule implies that CD4+ T cells play a pivotal role in the disease pathogenesis. Indeed, gluten-specific CD4+ T cells can be isolated from intestinal biopsies of CD patients but not from controls [4, 5]. The CD4+ cells are TCRαβ+ and typically belong to the Th1 phenotype, secreting large amounts of IFN-γ and TNF-α, in addition to other pro-inflammatory cytokines [6].

CD can be diagnosed in early childhood with classical symptoms, such as diarrhea and malabsorption, but it can also be diagnosed later in life, in adults, in which a wider spectrum of symptoms is shown. The 'classical' presentation of chronic diarrhea and malabsorption is currently a rarity. Due to early detection and increased awareness, CD now presents a myriad of 'atypical' signs and symptoms. Over the last 15 years, for this condition, the viewpoint on CD was transformed from the concept of a rare disease affecting primarily the children of Northern Europe with gastrointestinal symptoms, to an extraordinarily common disorder, GS, in people of all ages worldwide with symptoms affecting multiple organ systems [7].

Many disorders occur in association with CD [8]; the great majority of these are merely chance associations. However, type 1 diabetes mellitus, thyroid disease, pulmonary and liver disorders, inflammatory bowel disease, rheumatic complaints as well as other conditions with a possible immunological etiology may occur among celiac patients more commonly than by chance alone. The association of CD with the allergy disease seems to be less probable because CD is considered to arise from an inappropriate Th1-mediated immune response to gluten [9], whereas the Th2-type lymphocytes are mostly involved in allergic reactions [10]. Thus, one would hypothesize that Th1- and Th2-type immunity are present in a well-diversified population of patients.

However, some reports have suggested that allergy manifestations are more frequent in patients with CD [11] and that asthma incidence has increased in patients who were diagnosed with CD during childhood [12]. Atopic disorders were frequently found in children [13] and adult patients with CD, and more often in their relatives than in normal control subjects [14]. In contrast, one single case-control study in children with CD denies the link between CD and allergy [15]. To evaluate the possible correlation of CD and allergy, we conducted a study to establish the occurrence of GS in a group of allergic patients with gastrointestinal symptoms. To this purpose, allergic patients exhibiting, besides the classical allergic symptoms, intestinal signs like abdominal pain and diarrhea of unknown origin were subjected to duodenal biopsy, to several serological tests such as antigliadin and antiendomysial antibodies, hemoglobin, iron, folate, ferritin and unconjugated bilirubin, and to the typical celiac HLA genotyping.

About 30% of the analyzed subjects were positive to mucosal lesions but negative to antigliadin antibody (AGA), antiendomysial antibody (EMA), and to DQ2 as well as DQ8 molecules. However, a consistent number of patients with mucosal lesions show anemia of chronic disease. The patients displaying mucosal lesions were also recommended to follow a gluten-free diet (GFD).

Significant improvement in symptoms and clinical signs was observed in response to a GFD, even in the absence of either serological or genetic peculiarities of CD, and we suggest that in our cohort there could be a gluten-responsive enteropathy, not meeting the conventional histological or serological criteria for true gluten-sensitive enteropathy and we propose to denominate it as a nonceliac gluten-sensitive enteropathy (NCGSE).

Subjects and Methods

Subjects

A group of 262 unrelated allergic patients (189 women and 73 men) with the classical symptoms such as asthma, rhinitis or contact dermatitis were assembled in the IMID unit. All patients also exhibited gastrointestinal manifestations such as stomach pain, diarrhea and weight loss. The allergic patients were classified into

three subgroups: subgroup A (32 subjects) positive to prick test versus aeroallergens such as pollen and *Dermatophagoides pteronyssinus* and/or *D. farinae*; subgroup B (140 subjects) positive to patch test versus contact allergens of the European standard series (GIRDCA); subgroup C (90 subjects) positive to both prick and patch test. The individuals positive to the patch test (either belonging to the B or to the C subgroup) reacted mainly to nickel and cobalt, and at a lower rate to thimerosal, fragrance mix, balsam of Peru and potassium dichromate. Patients positive to the nickel patch test were also subjected to the oral stimulation test and were progressively administered capsules from 1.25 mg $NiSO_4$ to 5 mg (Lofarma SpA, Milano, Italy). This study was approved by our Institutional Ethics Committee and informed consent was obtained from all subjects.

Duodenal Biopsy

All patients were tested by duodenal biopsy. A minimum of 3 biopsy specimens were taken from the second part of the duodenum by using standard forceps during upper gastrointestinal endoscopy. Formalin-fixed hematoxylin-eosin-stained biopsy specimens were analyzed under light microscopy. Intraepithelial lymphocytes (IEL) CD3+ CD8+ were counted after immunohistochemical staining.

Histopathological findings were staged according to the Marsh criteria [16] as revised by Rostami et al. [17]: 'infiltrative' lesions with intraepithelial lymphocytosis are defined as Marsh type I, 'infiltrative/hyperplastic' lesions in combination with cryptic hyperplasia are defined as Marsh II; 'partial (A), subtotal (B) and total (C) villous atrophy' as Marsh III. A cutoff of 25 IEL/100 epithelial cells was established to diagnose lymphocytic enteritis [18].

All study patients were informed about the mucosal lesions and were recommended to follow a GFD.

Hematological Parameters

All patients were subjected to several hematological parameters determinded at the time of diagnosis, in particular hemoglobin and ferritin, iron, folate, and bilirubinemia serum were taken into account in our study. AntiEMA and antiAGA determinations were also reviewed. These serological tests were performed in various commercial laboratories. AGA and EMA were classified as positive if greater than the normal value for the laboratory of reference. The same serological values were determined in patients that followed the GFD for up to 6 months after the initial diagnosis of mucosal lesions.

HLA Typing

Samples of peripheral blood were taken from each individual and genomic DNA was extracted using standard methods. DQA1*05, DQA1*0201, DQA1*0301, DQB1*02 and DQB1*03 genotyping was performed by polymerase chain reaction (PCR) amplification, using sequence-specific primers (Expectram, ■■■). Primers designed on a conserved region of DRB gene were used as controls. The specific PCR products were analyzed on 2% agarose gels and visualized under a UV transilluminator after the ethidium bromide staining.

Data Analysis

The χ^2 test was used to compare the frequencies between the two study groups in 2 × 2 table.

The two-tailed t test was used to assess the statistically significance in the comparisons either of the visual analog scale (VAS) value or of the drug consumption, before and after the GFD. A $p < 0.05$ was considered to be significant.

Clinical Response to the GFD

Adherence of the patients to the diet was stated by clinical periodic interviews. To evaluate the efficacy of the GFD on the health state of the patients, they were assessed trough a VAS for symptoms. Patients were required to grade their mood and their perception of their overall health state related to symptoms at the start and at the end of the diet (after 6 months). The VAS scale has 11 points from 0 (best imaginable health state) to 10 (worst imaginable health state).

Patients were also required to list the number of the drugs consumed at the start and at the end of the diet. We reported the numbers of the drugs in a proportional scale from 1 (corresponding to the lowest number of drugs) to 10 (corresponding to the highest number of drugs).

Results

262 unrelated allergic patients (189 women and 73 men), 32 belonging to subgroup A, 140 to subgroup B, 90 to subgroup C (see Subjects and Methods) with symptoms of obscure origin such as stomach pain, diarrhea, weight loss and anorexia, were screened for mucosal lesions with a duodenal biopsy.

Based on the histological diagnosis, the allergic patients were divided into a negative group without mucosal lesions (n = 185; 131 women and 54 men) and a positive group with mucosal lesions (n = 77; 58 women and 19 men); all of them were equally distributed in the 3 allergic subgroups. Twenty-eight individuals of the positive group were classified as Marsh type I because they showed a normal histology with an increase in IEL. The remaining patients had type II or type III lesions because of the typical histological changes of the intestinal mucosa and an increase in IEL (table 1). Serological screening tests showed that all the patients were negative to AGA and EMA.

Based on the strong association of CD with the HLA-DQ2 and HLA-DQ8 heterodimers, we assessed the distribution of HLA DQA1*05, DQA1*0201, DQA1*0301, DQB1*02 and DQB1*03 alleles in the positive and negative allergic patients. The results are shown in table 2. The DR3 (DQA1*0501/DQB1*0201), DR5 (DQA1*0505/DQB1*0301), DR7 (DQA1*0201/DQB1*0202) and DR4 (DQA1*03/DQB1*0302) haplotypes were inferred from the alleles recognized by manual inspection, due to the strong linkage disequilibrium between alleles at these loci [19].

Table 1. Duodenal biopsy in allergic patients

Histology	A (n = 32)	B (n = 140)	C (n = 90)	Total (n = 262)
Negative	23 (71.8%)	100 (71.4%)	62 (68.8%)	185 (70.6%)
Positive	9 (28.1%)	40 (28.5%)	28 (31.3%)	77 (29.3%)
March classification of positive group				
Type I	5 (55.6%)	14 (35%)	9 (32.1%)	28 (36.4%)
Type II and IIII	4 (44.4%)	26 (65%)	19 (67.9%)	49 (63.6%)

Table 2. Haplotype and genotype analysis for DQA1*05, DQA1*0201, DQA1*0301, DQB1*02 and DQB1*03 genes in allergic patients

HLA class II	Negative (n = 185)	Positive (n = 77)
Haplotypes		
DR5 (DQA1*05/DQB1*03)[a]	83 (44.8%)	39 (50.6%)
DR3 (DQA1*05/DQB1*02)[a]	20 (10.8%)	10 (12.9%)
DR7 (DQA1*0201/DQB1*02)	18 (9.8%)	4 (5.2%)
DR4 (DQA1*0301/DQB1*03)	10 (5.4%)	2 (2.6%)
DR5/DR7[a]	14 (7.5%)	9 (11.6%)
DR5/DR4	6 (3.2%)	4 (5.2%)
DR7/DR4	3 (1.6%)	–
NEGATIVI	31 (16.7%)	9 (11.6%)
Shared HLA alleles		
DQA1*05[b]	123 (66.4%)	62 (80.5%)
DQB1*02	52 (28.1%)	23 (29.8%)

[a] No significant increase; [b] $p < 0.025$.

Table 3. Hematological characteristics of the allergic patients

Hematological parameters	Negative (n = 185)	Positive (n = 77)
Anemic*	14 (7.5%)	15 (19.4%)
Iron deficient	16 (8.6%)	9 (11.6%)
High ferritin	5 (2.7%)	4 (5.2%)
Folate deficient	11 (5.9%)	6 (7.8%)
Unconjugate hyperbilirubinemia	24 (12.9%)	13 (16.8%)

Anemia defined as hemoglobin <12 mg/dl in women and <13 mg/dl in men. Iron deficiency defined as serum ferritin <25 ng/ml. High ferritin defined as serum ferritin >200 ng/ml. Folate deficiency defined as serum level <5.4 ng/ml. Unconjugated hyperbilirubinemia defined as serum level >850 μmol/l). * $p > 0.005$.

The comparison shows that the frequency of DR5, DR3 and DR5/DR7 haplotypes increased in the positive group with respect to the negative group, but this increase was not significant. On the contrary, the frequency of the only DQA1*05 allele, given from DR3 and DR5 haplotypes, was significantly higher in the positive than in the negative group. The DQB1*02 allele distribution was similar in the two groups.

In order to investigate the clinical pictures of the positive patients in the allergic condition, we determined the most common hematological parameters. The most consistent variations with respect to the normal range were found in hemoglobin and in ferritin, folate as well as bilirubin serum levels.

As reported in table 3, we found a significant increase in the number of individuals (14 women and 1 man) with an anemic condition in the positive group with respect to the subjects (14 women) of the negative group. A non-significant increase could also be observed in the positive patients with respect to the negative ones for iron and folate deficiencies as well as for the high level of serum ferritin. When we examined the effect of the mucosal lesions severity on the anemic condition we found that the proportion of anemic individuals did not differ in relation to the rate of the IEL infiltration or to the villous atrophy rate (data not shown). In the same way the proportion of anemic subjects did not differ for the type of allergic condition. A closer inspection also shows that only 9 of the anemic positive patients (60%) and 10 of the anemic negative patients (71.4%) had a low serum ferritin level. Conversely, 40 and 29% of the positive and negative anemics, respectively, had a normal or high serum ferritin level. The combination of anemia with a normal or high serum ferritin level suggests anemia of chronic disease. A consistent number of subjects in the positive (16.8%) and negative (12.9%) groups show Gilbert's syndrome. This syndrome consists of a mild unconjugated hyperbilirubinemia that occurs in the absence of apparent liver disease or hemolysis, due to the disorder of the bilirubin conjugation. In our study group the amount of

patients with this condition is higher than in the normal population.

All patients with mucosal lesions were invited to follow a GFD for 6 months. Of 77 positive subjects, only 48 followed the diet carefully, hence we considered this group for the evaluation. The effect of the diet on the clinical severity of the patients was evaluated with the VAS for the symptoms and with the number of the drugs consumed by the patients. We compared the initial VAS values at the start of the diet with those after the treatment and the media of the VAS values got lower (7.4 ± 1.31 vs. 6.25 ± 1.52) with a statistically significant difference ($p = 0.0001$). We also compared the drug use before and after the diet and this also was significantly reduced (5.85 ± 1.42 vs. 3.55 ± 1.61; $p = 0.0001$).

Discussion

GS is a widespread condition among humans. For example, in CD patients, an inflammatory response to dietary gluten meal leads to enteropathy, malabsorption, circulating antibodies against gluten and tissue transglutaminase as well as clinical symptoms such as diarrhea. The present study was conducted in order to estimate the prevalence of GS in a large group of allergic patients exhibiting symptoms and clinical signs of unknown origin. We found a high prevalence of patients (about 30%) showing duodenal lesions that occur in the proximal small intestine with histological changes of villous atrophy, crypt hyperplasia and/or increased intraepithelial lymphocytosis among allergic patients. At the time of diagnosis, not even a single patient was found to be serologically positive to AGA and EMA. When all allergic patients were typed for the presence of the typical celiac HLA genotype, we found that only about 30% of mucosal lesion-positive patients exhibited DQ2 and DQ8 molecules with respect to 23% of mucosal lesion-negative subjects. However, we observed that in the majority of positive patients without DQ2 molecule, one of the two DQ2 alleles, that is DQA1*0501, is generally present and DQB*0201 rarely is (table 2). Consistent with this we propose that the presence of only the α- or the β-chain of the DQ2 heterodimer, encoded by DQB*0201 or DQA1*0501 allele, could impede the progression from a mucosal lesion to a more severe condition with the production of autoantibodies in these subjects.

Since we observed a significant improvement in health state of the positive patients either considering the symptoms or the drugs used, when they followed carefully a GFD, we suggest that a NCGSE occurs in our allergic patients, not encountering the conventional histological or serological criteria for true gluten-sensitive enteropathy. Anemia represents the presenting complaint in the allergic patients with the diagnosis of NCGSE. The link between GS and anemia is well documented. We also observed that more women than men were anemic. This is consistent with a study that found anemia as a presenting complaint leading to the diagnosis of CD much more frequently occurring in women than in men [20]. The majority of studies have focused on micronutrient deficiencies as the cause of anemia in individuals with CD; in our study group, this hypothesis appears inadequate. In fact, in our analysis only 60% of the anemic NCGSE patients had anemia with iron deficiency. The remaining 40% presented a normal or high level of serum ferritin, which suggests an anemia of chronic disease. In addition, the frequency of anemia was similar among individuals irrespective of the degree of villous atrophy. Thus, another etiological factor, besides malabsorption, such as inflammation, would be necessary to explain this observation. Pro-inflammatory cytokines play an essential role in the inflammation. Such cytokines, in particular IFN-γ and IL6, are powerful mediators of hypoferremia in inflammation inducing the synthesis of the iron-regulatory hormone hepcidin [21]. Increased hepcidin synthesis, in turn, is responsible for increased ferroportin degradation and the inhibition of iron release from macrophages and enterocytes, leading to the well-known abnormalities in iron homeostasis associated with the anemia of chronic disease [22]. Taking into account that malabsorption by itself does not explain all clinical pictures of these patients, we found, in addition, a low prevalence of folate deficiency. A previous study found that 81% of a cohort of 16 children diagnosed with CD had low serum folate [23]. In our study group, we also observed a high prevalence of individuals with Gilbert's syndrome, a benign disorder of the bilirubin conjugation, which affects 7–10% of the average population. The high prevalence could be due to mechanisms of liver injury determined by inflammation. Thus, in our study, the inflammation that is of both intestinal and systemic origin could justify the clinical picture of our patients rather than malabsorption. Taken together, the data presented in this study demonstrate that the inflammation process seems to play an important role in the appearance of NCGSE in genetically predisposed people (DQA1*05 allele). Based on the high occurrence of NCGSE in the allergic patients, the screening of this disease should be included in the routine investigations because of the benefits of the treatment with

a GFD in these patients. The benefits could be the control of symptoms, the stabilization of the hematological parameters and the prevention of complications. Our target cohort was made up of 262 allergic patients with typical gastrointestinal symptoms; however, we cannot rule out that NCGSE could also be detected in individuals with extragastrointestinal symptoms or be asymptomatic.

Future analysis should concentrate on the extraintestinal signs of GS as they could offer the key to go deeply into the molecular basis of the GS. On the other hand, it is now clear that the spectrum of GS is wider than previously recognized; hence, we strongly suggest that allergic patients, responding to a GFD, even if they do not show signs of intestinal damage, be incorporated into the NCGSE group.

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