A Cytologic Assay for Diagnosis of Food Hypersensitivity in Patients With Irritable Bowel Syndrome

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BACKGROUND & AIMS: A percentage of patients with symptoms of irritable bowel syndrome (IBS) suffer from food hypersensitivity (FH) and improve on a food-elimination diet. No assays have satisfactory levels of sensitivity for identifying patients with FH. We evaluated the efficacy of an in vitro basophil activation assay in the diagnosis of FH in IBS-like patients.

METHODS: Blood samples were collected from 120 consecutive patients diagnosed with IBS according to Rome II criteria. We analyzed in vitro activation of basophils by food allergens (based on levels of CD63 expression), as well as total and food-specific immunoglobulin (IgE) levels in serum. Effects of elimination diets and double-blind food challenges were used as standards for FH diagnosis.

RESULTS: Twenty-four of the patients (20%) had FH (cow’s milk and/or wheat hypersensitivity); their symptom scores improved significantly when they were placed on an elimination diet. Patients with FH differed from other IBS patients in that they had a longer duration of clinical history, a history of FH as children, and an increased frequency of self-reported FH; they also had hypersensitivities to other antigens (eg, egg or soy). The basophil activation assay diagnosed FH with 86% sensitivity, 88% specificity, and 87% accuracy; this level of sensitivity was significantly higher than that of serum total IgE or food-specific IgE assays.

CONCLUSIONS: A cytometric assay that quantifies basophils after stimulation with food antigens based on cell-surface expression of CD63 had high levels of sensitivity, specificity, and accuracy in diagnosing FH. This assay might be used to diagnose FH in patients with IBS-like symptoms.

Keywords: Irritable Bowel Syndrome; Food Hypersensitivity; Flow-CAST; IgE; Diagnosis.

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder in which a disturbed brain–gut axis has been thought to have a mandatory role.1 However, many patients suffering from IBS report an association of symptoms with specific food ingestion, referred to as self-perceived food hypersensitivity (FH),2 and recent clinical studies imply that dietary factors might be more important in the pathogenesis of IBS than was earlier anticipated.3

Furthermore, approximately 20% of the population alter their diet owing to self-perceived FH, but the application of a double-blind, placebo-controlled, oral food challenge, considered as the gold standard for FH diagnosis, shows that questionnaire-based studies overestimate the prevalence of this disease.4

Because both IBS and FH with gastrointestinal symptoms often have the same clinical presentation, with patients suffering from mild to severe abdominal pain, abdominal discomfort, bloating, and alteration of bowel habits,5 differential diagnosis between these 2 conditions may be difficult and essentially based on elimination diets and double-blind, placebo-controlled challenges. Unfortunately, none of the available in vivo and in vitro allergy tests (ie, skin prick test and serum total immunoglobulin [Ig]E and specific IgE assays) has shown a good diagnostic reliability.6

More recently, the flow cytometric basophil activation test, based on the demonstration of altered membrane phenotypes on allergen-activated basophils, with up-regulation, surface expression, and cytofluorometric detection of CD63 protein, has been applied to allergy diagnosis.7 However, no studies have evaluated the diagnostic accuracy of the test in FH diagnosis in patients with IBS-like clinical symptoms.

In this study, we evaluated the diagnostic reliability of the flow cytometric allergen stimulation test to discriminate IBS from FH in a group of diagnosed IBS patients.

Patients and Methods

A total of 120 patients (97 women, 23 men; age range, 18–56 y; median age, 36 y), who had been consecutively referred as outpatients to the Department of Internal Medicine of the University of Palermo from January 2005 to December 2006 for IBS, completed this study.

The inclusion criteria were as follows: (1) age older than 17 years, (2) no previous referral to our clinic, and (3) diagnosis of IBS. Patients with a diagnosis of organic gastrointestinal disease were excluded.

IBS diagnosis was based on the Rome II criteria for functional gastrointestinal disorders8 (see Supplementary Materials and Methods). Furthermore, organic gastrointestinal disorders were excluded by an accurate work-up (described later).

After inclusion, the patients underwent a clinical evaluation that included a detailed family and personal clinical history and a physical examination. Afterward, 2 predesigned questionnaires were administered to all the patients: the first was re-

Abbreviations used in this paper: DBPC, double-blind, placebo-controlled; FH, food hypersensitivity; IBS, irritable bowel syndrome; Ig, immunoglobulin.
garding the type and severity of the symptoms, the second was regarding any possible self-perceived FH.

None of the enrolled patients were on any medication or were on an elimination diet at the time of the study. In fact, they were asked to suspend medications and/or diet at least 3 weeks before the beginning of the study protocol.

The study was conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the University Hospital of Palermo and all patients gave their written informed consent to participate.

**Healthy and Disease Control Groups**

Two control groups was selected. One was composed of 40 patients with various gastrointestinal diseases (28 women, 12 men; age range, 18 – 62 y; median age, 34 y): celiac disease (n = 16), active ileum-colon Crohn's disease (n = 15), and ulcerative colitis (n = 9), diagnosed according to standard serologic, endoscopic, and histologic criteria. These patients were selected at random from those diagnosed in our hospital during the period of the present study. The other group was composed of 40 healthy volunteers chosen from the students attending the University Hospital (28 women, 12 men; age range, 18 – 63 y; median age, 30 y).

**Work-Up for Irritable Bowel Syndrome Diagnosis**

All patients underwent first-step hematology and chemistry tests (including erythrocyte sedimentation rate; serum C-reactive protein level; blood cell counts; electrolytes; and thyroid, liver, and renal function); stool examination for occult blood, ova, and parasites; and a lactose-H2 breath test after oral load of 50 g of lactose. Only the patients showing symptoms after lactose load were excluded, the simple evidence of malabsorption was not considered a reason for exclusion. Particular care was taken to exclude a diagnosis of celiac disease because some patients had been on a diet with a reduced content of wheat owing to self-perceived wheat-intolerance (see Supplementary Materials and Methods). However, no patients were following a strict gluten-free diet. Patients also underwent sigmoidoscopy with biopsy if younger than age 40 or underwent a colonoscopy with biopsy if older than 40 years of age. Patients with negative results for all of the examinations described earlier and with a clinical history indicating IBS, according to the Rome II criteria, were considered to be suffering from IBS and were enrolled in the study. All medications and food restrictions were suspended at least 3 weeks before the beginning of the study.

**Study Protocol**

After enrollment in the study, the patients completed the Symptoms Severity and Food Hypersensitivity questionnaires (see Supplementary Materials and Methods), and underwent serum total and food-allergen–specific IgE determination, together with the flow cytometric basophil activation test. The diagnostic tests were performed by different physicians, unaware of the clinical history of the patients and the results of the other tests.

The study patients then were observed for a 4-week run-in period. After this they underwent an elimination diet without cow's milk and derivatives, wheat and derivatives, egg, tomato, and chocolate for 4 weeks. Patients self-reporting FH also were asked to avoid ingestion and/or contact with the food(s) causing symptoms. The patients wrote a dietary diary, and adherence to the elimination diet was evaluated by trained dieticians. Patients who specified a symptom/sign improvement after the elimination diet period underwent a double-blind, placebo-controlled (DBPC), oral food challenge first with cow's milk proteins and then with wheat proteins.

After FH had been excluded or confirmed, all IBS patients were invited to continue the follow-up evaluation with regular visits every 6 months for 2 years. During the follow-up visits the patients again underwent a physical examination, clinical history, and routine hematochemical assays, and when considered opportune some instrumental examinations were repeated.

**Symptoms Questionnaire**

Severity of the symptoms was assessed in the study both at the end of the run-in period and at the end of the elimination diet period. Symptoms were assessed using a questionnaire scoring system validated for use in IBS, including an IBS symptom severity score (range, 0 – 500). This is a system for scoring pain, distension, bowel dysfunction, and general well-being, with mild, moderate, and severe cases indicated by scores of 75 to 175, 175 to 300, and greater than 300, respectively. A reduction in score of 50 or more was regarded as a clinically significant improvement, whereas an increase in score of 50 or more was considered as a clinically significant worsening.

**Double-Blind, Placebo-Controlled Challenges**

DBPC for cow's milk was performed by administering capsules coded as A or B containing milk proteins (casein from bovine milk Sigma C7078, lactoalbumin Sigma L7252, lactoglobulin Sigma L2506; Sigma-Aldrich, St. Louis, MO) or xylose (Aldrich 245321; Sigma-Aldrich), respectively. DBPC for wheat proteins was performed with capsules coded as C or D containing wheat (Fluka, BCR563; Sigma-Aldrich) or xylose, respectively. Capsule A or B was given for 2 consecutive weeks, and then after 1 week of washout the patients received the other capsule for another 2 weeks. After 1 week of washout, capsule C or D was given for 2 consecutive weeks, then after another week of washout, the patients received the other capsule for 2 weeks. The challenges were stopped when a clinical reaction occurred (ie, the onset of abdominal discomfort or pain) associated with a change in stool frequency and/or appearance.

Figure 1 summarizes the study design.

**Serum Total Immunoglobulin E and Food Allergen-Specific Immunoglobulin E Antibodies**

Serum samples from all patients were collected and analyzed for serum total IgE and food allergen–specific IgE antibodies by using the Phadia CAP-system (Phadia, Uppsala, Sweden), according to the manufacturer’s instructions. The following 7 common food allergens were tested: egg, cow’s milk, soy, peanut, wheat, tomato, and fish. Levels of 0.35 kU/L or greater (level 1 on the specific IgE scale) were considered positive. Total IgE also was determined by the same method with a detection limit of 2 kU/L and an upper limit of 5000 kU/L. Normal limit for total IgE was 100 kU/L.
Flow Cytometric Allergen Stimulation Test

Patients were instructed to avoid systematically administered antiallergic drugs, such as corticosteroids, chromoglycic acid, indomethacin, or similar, for at least 7 days before blood sampling.

Flow cytometric allergen stimulation test was performed with Flow-CAST (Bühlmann Laboratories AG, Schönenbuch, Switzerland), according to the manufacturer’s instructions (see Supplementary Materials and Methods).

The manufacturer’s allergens used in all patients were \( \alpha \)-lactalbumin, \( \beta \)-lactoglobulin, casein, egg white, egg yolk, wheat, soybean, fish, tomato, plus other food allergens specifically suggested by each individual clinical history. As a positive control, we used a monoclonal Le27 anti-IgE antibody solution (Bühlmann Laboratories AG). As a negative control, only the stimulation buffer containing interleukin-3 was used.

To define a result as positive, we considered that the percentage of basophils activated after incubation with antigen should be at least 3-fold the percentage of basophils activated in the background tube. When the percentage of basophils activated spontaneously was less than 2.5%, we required an additional condition, namely that the percentage of basophils activated after contact with the antigen should be equal to or greater than 5%. These cut-off values were chosen on the basis of receiver operating characteristic curves plotted in our laboratory, enabling us to achieve the highest possible sensitivity with an optimal specificity.

The intra-assay variation of the test was 2.5% and was calculated from 4 blood samples taken from 2 healthy controls and 2 patients suffering from food allergy stimulated with stimulation buffer and anti-FceRI Ab and consecutively analyzed 10 times by flow cytometry. The interassay variation of the test was 6.8% and was calculated from the blood samples of 3 healthy controls and 3 patients suffering from food allergy stimulated with Stimulation Buffer and anti-FceRI Ab, analyzed 10 times by flow cytometry and retested after 2 weeks.

The reproducibility of the test was evaluated in 20 patients (14 suffering from IBS unrelated to FH, and 6 with IBS related to FH), and all determinations were performed by 3 expert operators (I.G., S.M.L.C.). The correlation coefficient was excellent, with a K value of 0.92 (\( P < .001 \)).

Statistical Analysis

Frequency analysis was performed using the chi-square test or the Fisher exact test. Means comparison was performed by the Student \( t \) test or the Mann–Whitney test where appropriate.

The Wilcoxon rank-sum test was used to compare the symptoms score before and after the diet treatment. Tables were constructed for frequency and percentage. The sensitivity and specificity of the immunologic assays and their positive and negative predictive values, along with their 95% confidence intervals, were calculated by standard statistical methods.

Table 1. Number and Percentage of IBS Patients With Scores Improved, Unchanged, or Worsened During a Four-Week Period of Elimination Diet

<table>
<thead>
<tr>
<th></th>
<th>Patients improved</th>
<th>Patients unchanged</th>
<th>Patients worsened</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number and percentage</td>
<td>N = 44 (36%)</td>
<td>N = 50 (42%)</td>
<td>N = 26 (22%)</td>
</tr>
<tr>
<td>Score at baseline</td>
<td>300 ± 70 ( a )</td>
<td>320 ± 85</td>
<td>305 ± 65</td>
</tr>
<tr>
<td>Score during the diet</td>
<td>160 ± 55 ( a )</td>
<td>295 ± 80</td>
<td>310 ± 70</td>
</tr>
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NOTE. Symptom severity scores before and after elimination diet also are shown. Cow’s milk and derivatives, wheat, egg, tomato, and chocolate were excluded in all subjects. The patients with self-reported food hypersensitivity also avoided food-causing symptoms.

\( a P < .0001 \), Wilcoxon rank-sum test.
worsened symptoms on an elimination diet, a diagnosis of IBS unrelated to FH was made. The other 44 patients showing a significant reduction in symptom score on an elimination diet underwent DBPC challenges.

Nineteen patients were positive for both cow’s milk and wheat protein challenges, 3 were positive exclusively for cow’s milk challenge, and 2 were positive exclusively for wheat challenge. In total, 24 patients were positive for DBPC food challenges. All DBPC-positive patients, the symptoms (abdominal pain, bloating, diarrhea, constipation, and so forth) reappeared after a median period of 3 days (range, 1–8 d) after commencing the challenge with cow’s milk or wheat proteins. Twelve of these 24 patients did not complete the challenge period with the active food owing to the severity of symptoms. None of these patients reacted on placebo administration.

The 24 patients with positive DBPC challenges to cow’s milk and/or wheat proteins fulfilled the Rome II criteria for IBS diagnosis and were diagnosed with FH.

The other 20 patients, who improved on elimination diet, did not react to the DBPC challenges; these 20 subjects underwent open challenges also with egg, tomato, and chocolate and did not react. Consequently, they were diagnosed with IBS not related to FH. Figure 2 summarizes the results, according to the study design.

According to the results of the elimination diet and the subsequent DBPC challenges, the patients then were divided into 2 subgroups: group A (IBS not related to FH), including the subjects with scores unchanged or worsened on elimination diet and the patients with negative DBPC challenge for cow’s milk and wheat proteins (n = 96; 80% of the total patients included); and group B (FH), including the subjects with scores improved on elimination diet and positive to DBPC challenge with cow’s milk and/or wheat proteins (n = 24; 20% of the total patient number).

Table 2 shows the demographic and clinical characteristics of these patient groups. IBS severity score at entry to the study was identical in both groups. However, the duration of IBS symptoms was significantly higher in patients with FH (P < .001). Furthermore, the FH patients showed a higher frequency of self-perceived food intolerance (chi-square = 7; P = .01) and of history of food allergy during infancy (chi-square = 5.9; P = .02).

Table 3 shows the results of the immunologic assays in the IBS patients and in the healthy and disease controls. According to these results, Table 4 shows the sensitivity, specificity, and diagnostic accuracy of the immunologic assays in the diagnosis of FH in patients with a clinical presentation of IBS. The in vitro basophil stimulation assay showed a higher sensitivity than serum total IgE and serum food-specific IgE in the diagnosis of cow’s milk hypersensitivity (P < .0001 and .01, respectively) and in the diagnosis of wheat protein hypersensitivity (P < .0001 and .01, respectively). Diagnostic accuracy also was higher for in vitro basophil stimulation assay: P less than .01 versus serum total IgE and P less than .05 versus serum food-specific IgE, both for cow’s milk hypersensitivity and for wheat protein hypersensitivity diagnoses. Specificity of the in vitro basophil stimulation assay was 86% in the IBS subjects, but no false positives were found in the healthy controls, whereas in the group of the disease controls 4 patients with Crohn’s disease and 5 patients with celiac disease were false positive for the in vitro basophil stimulation assay.
The patients then underwent a 2-year follow-up period, during which they were reassessed at 6-month intervals. FH patients continued to follow an elimination diet with the exclusion of the foods causing the IBS symptoms and all reported the persistent disappearance of or a consistent improvement in the symptoms. Symptom score at entry to the study was 350, at 40, at 6 months was 180, at 6 months was 350. In most of these subjects IBS symptoms persisted periodically, despite several treatments. None of them were found to be suffering from an organic cause of symptoms.

**Discussion**

IBS is a highly prevalent disorder, characterized by recurrent abdominal pain and altered bowel habits, which is associated with a marked reduction in quality of life and causes a considerable financial burden. The origin and development of IBS are unclear and genetic, neurobiological and psychosocial factors seem to be involved in the pathogenesis of the disease. Furthermore, IBS appears to result from an interplay between susceptibility genes and impaired gut barrier functions, immunologic dysregulation, as well as bacterial and viral infections and other environmental factors. However, given the high frequency with which patients report an association of symptoms/signs appearing or worsening with food(s) ingestion, it is relevant to systematically explore the evidence for an association of FH with the clinical manifestations of IBS.

In this study, we investigated the diagnostic reliability of the flow cytometric allergen stimulation test to discriminate IBS from FH in a group of diagnosed IBS patients, some of whom reported a self-perceived FH. To make the diagnosis of true FH, all of the patients, whether reporting or not reporting a self-perceived FH, underwent an elimination diet and then patients reporting a symptom/sign improvement after the elimination diet period underwent a double-blind, placebo-controlled oral food challenge with cow’s milk proteins and then with wheat proteins.

Our results showed that approximately 20% of the IBS patients were suffering from multiple FH and the elimination diet...
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Improvement can be obtained only by following a very re-

FH were suffering from multiple FH and in these cases a clinical

period of elimination of these foods from the diet was never

daily by almost everyone in Western countries, and a prolonged

IBS symptoms were wheat and cow’s milk, which are consumed

indicate the foods causing the symptoms. This probably is

In fact, in our study only 50% of the FH patients were able to

elimination diet and reacted to the DBPC challenges. However,

only 12 of the 32 patients self-reporting FH improved on

estimated the real disease prevalence in IBS patients. In fact,

might not be clearly evident because the reaction often is

delayed or very delayed after ingestion, thus causing underdi-

the development or worsening of gastrointestinal symptoms

confirmed that the relationship between food(s) ingestion and

technique to analyze allergen-specific, IgE-mediated in vitro

activation of basophils. Basophils, on encountering specific

allergens recognized by surface receptor FceRI-bound IgE, up-

regulate the expression of different markers (eg, CD63 and

CD203c), which can be detected by flow cytometry using spe-

cific monoclonal antibodies. At present, the most commonly

used marker in basophil activation studies is CD63. In resting

basophils, CD63 is expressed weakly on the surface membrane

both in normal subjects as well as in patients with allergies. In

contrast, CD63 is expressed with a high density on activated

basophils and mirrors histamine release.

During recent years, the technique has proved to be an easily

accessible method that allows the simultaneous testing of sev-

eral putative allergens with a minimum amount of blood. In

previous studies in vitro basophil activation has been shown to

be useful in detecting classic IgE-mediated allergic disorders to

various allergens, such as aeroallergens (cypress pollen, house

dust mites), foods, hymenoptera venoms, natural rubber latex,

B-lactam antibiotics, and muscle relaxants. On the con-

trary, in our study we showed the high sensitivity and diagnos-

tic accuracy of the detection of basophil activation in the

diagnosis of FH in IBS patients, compared with a much lower

sensitivity of the other IgE-mediated assays.

The clinical evidence of delayed reaction in our patients

could seem absolutely independent from an IgE-mediated

mechanism. However, because serum IgE participates in the

activation of the basophils and in the expression of the CD63

marker on their surface, the complex pathogenetic mechanism

of the IBS-like gastrointestinal manifestation of FH should be

reviewed and a possible role for IgE not totally excluded.

Finally, it is noteworthy that we found a high frequency of

false-positive results when patients with chronic intestinal in-

flammatory diseases (eg, celiac disease, Crohn’s disease) were

tested. This could be owing to the impaired intestinal perme-

ability, which could favor a nonspecific basophil activation;

however, the hypothesis of concomitant FH in these diseases

cannot be excluded completely.

In our study, cytometric detection of basophil CD63 surface

expression after stimulation with several antigens (cow’s milk,

egg white, egg yolk, wheat, soybean, fish, and tomato, and so

forth) showed a good sensitivity, specificity, and diagnostic

accuracy.

In conclusion, we suggest that in patients with IBS-like

symptoms this method is feasible for making a differential

diagnosis of true FH. For these patients, this test might sup-

plement or better replace routine allergy diagnostic tests such

as the skin prick test and serum total and allergen-specific IgE

assay.
Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of CGH at www.cghjournal.org, and at doi:10.1016/j.cgh.2009.11.010.

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Reprint requests
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Conflicts of interest
The authors disclose no conflicts.