

Short communication

Periodate treatment of *Anisakis simplex* allergens

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Anaphylactic reactions after parasitized fish consumption are mediated by an IgE response. However, positive skin tests and specific IgE can also be found in many asymptomatic subjects who recognize a single medium-mol.-wt. antigen by IgE immunoblot. The study aimed to find out whether this unspecificity was due to the carbohydrate moieties of parasite antigens. Sixty-two patients with suspected parasite allergy, 51 blood donors, 18 bakers, and 38 atopic patients were studied by blotting. Parasite proteins were treated with periodate. Several selected sera were inhibited with a crude wheat extract and fungal amylase. Twelve patients (19%), eight donors (16%), six bakers (33%), and one atopic patient (3%) recognized a single medium-mol.-wt. band in blotting and should be considered false-positive. This band was periodate-sensitive, but specific IgE to this allergen could not be inhibited by a wheat extract nor by fungal amylase and was clinically irrelevant. Diagnosis of *Anisakis simplex* hypersensitivity by skin tests and/or specific IgE values should always be confirmed by specific IgE immunoblotting in order to detect the presence of clinically unrelated antibodies directed to periodate-sensitive allergens. These allergens are probably not a carbohydrate moiety of a parasite glycoprotein.

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Anisakis simplex is a fish parasite causing acute or chronic disease in man after raw or undercooked parasitized fish ingestion (1, 2). We recently described an entirely different disease caused in sensitized patients by ingestion of parasitized fish even if appropriately cooked. Most often, the symptoms appear from one to several hours after fish consumption, and the clinical picture is usually characterized by urticaria and/or angioedema. In some cases, the patients also develop anaphylaxis, sometimes severe enough to require treatment in emergency units. As in other allergic diseases, positive skin prick tests, as well as high specific IgE values to the parasite crude extract, are present in sensitized patients (3, 4).

However, soon after diagnosing our first cases, we found that positive prick tests and positive specific IgE values were also present in subjects without any suspected parasite hypersensitivity and even in normal controls. The presence of specific IgE directed to medium-mol.-wt. antigen/s was almost always detected in these subjects.

Material and methods

Patients

Sixty-two patients with suspected parasite allergy were included in the study. They had had at least one episode of urticaria and/or angioedema after fish consumption, and positive CAP and positive prick test to the parasite extract. Fifty-one blood donors were used as a normal population control. After finding that the serum of a baker gave a positive blot value, we studied 18 sera from bakers without any symptoms of urticaria or angioedema. Finally, 38 sera from atopic patients without suspected food hypersensitivity were also included.

Antigens

Crude *Anisakis* extract was obtained as described (4). Commercial fungal amylase and a crude wheat flour were extracted at 10% w/v in phosphate-buffered saline (PBS) for 1 h while shaking, centrifuged at 5000 g for 10 min, and sterilized by filtration.

Skin prick tests and specific IgE determinations

Prick tests with a 1/100 dilution of the parasite were performed in patients and blood donors, as previously described (4). Specific IgE values were obtained by the CAP System (Pharmacia, Uppsala, Sweden) with the corresponding Immunocaps.

Specific IgE detection by immunoblotting

After standard 12% acrylamide SDS-PAGE, *Anisakis* antigen was transferred to nitrocellulose by a diffusion method, as previously described (5). After blocking in 10 mM/l Tris, 0.15 mol/l NaCl, and 3% bovine serum albumin (BSA) (pH 7.4), nitrocellulose sheets were placed in a Mini-Protean II (Bio-Rad, Richmond, CA, USA) and incubated overnight with 600 μ l of 1:5 dilutions of the different sera in incubation buffer (10 mM Tris, 0.15 M saline, 3% BSA, 1% Tween 20, pH 7.4). The membranes were taken out of the device and, after three washes, incubated for 3 h with a 1/5000 dilution of a biotin-labeled monoclonal anti-IgE (Ingenasa, Madrid, Spain). After new washes, streptavidin-alkaline phosphatase was added at a 1/2000 dilution for 1 h and finally incubated with the substrate (4).

Oxidation of sugar residues

Carbohydrate structures were treated with periodate according to Vieths et al. (6). After transfer, the membrane was incubated with 0.1 mol/l NaIO₄ in 0.01 mol/l sodium acetate, pH 5, for 2 h in the dark. The membrane was then washed, and the blotting procedure followed as described above. The other membrane obtained from the same gel was incubated only in the acetate buffer and served as a control. Some selected sera were probed with the same treatment, only the concentration of periodate being changed (100–12.5 mM).

Immunoblotting inhibition

To investigate whether cross-reactivities were responsible for some unexpected positive results, we incubated some sera (100 μ l + 500 μ l of incubation medium) overnight with 20 μ l of a 10% w/v extract of fungal amylase, *Anisakis*, or the crude wheat flour extract. The resultant mixtures were then placed in the lanes of the blotting device, and the basic procedure was followed without changes.

Statistical analysis

CAP and prick values were studied by Student's *t*-test. Fisher's exact test was used for comparison of blot results.

Results

Approximately half of the patients with suspected allergy to the parasite showed an intense recognition of multiple bands of different molecular weights by immunoblotting. Only one of the blood donors (CAP value of 65.9 kU/l) showed this pattern, and none of the bakers or atopics were considered "multiple-band" positive ($P < 0.001$, Fisher's exact test). On the contrary, the presence of a medium-mol.-wt. band or bands was found in 12 patients, eight blood donors, six bakers, and one atopic subject (Fig. 1). The "single-band" patients as a group had lower skin prick tests (mean 1.75 mm) than patients showing multiple bands (4.46 mm) ($P = 0.005$). Mean CAP values were also very different in both populations (10.6 vs 45.1 kU/l) ($P < 0.001$) (data not shown). However, it was noteworthy that 8/12 patients with suspected parasite allergy recognizing the single band had CAP results higher than 10 kU/l, suggesting that a high CAP value did not always indicate multiple band recognition.

In previous experiments, we had used the serum of a baker as a control. This serum gave a clear single band in immunoblotting, leading us to suspect that exposure to wheat flour or bread improvers was responsible for this finding. We studied the sera of 18 asthmatic bakers without any symptoms related to fish consumption. Six of them recognized this single band with different intensities. Only serum samples were available from the bakers and atopic patients; therefore, we had no results of skin prick tests from these groups. The 12 blot-negative bakers had a negative CAP value. On the contrary, 3/5 blot-positive bakers tested had positive results by CAP (51.8, 13.1, and 11.8 kU/l). One of the positive bakers was not tested by CAP due to an insufficient amount of sample, and the single positive atopic patient had a CAP value of 8.57 kU/l. All these facts led us to conclude that the crude parasite extract contained a major antigen causing unexpected positivities in the skin tests and CAP.

To determine whether carbohydrates were responsible for this phenomenon, we studied sera of truly sensitized and false-positive subjects after periodate treatment of the membrane. Periodate oxidation of transferred proteins resulted in a loss of recognition of this single band, while "multiple-band" patients still recognized several other allergens (Fig. 2). With different periodate-sensitive, periodate-resistant and negative sera, the lowest concentration of the oxidizing agent used (12.5 mM) showed the same results as at 100 mM, a fact that suggested that the loss of periodate-sensitive epitopes could be due to carbohydrate destruction.

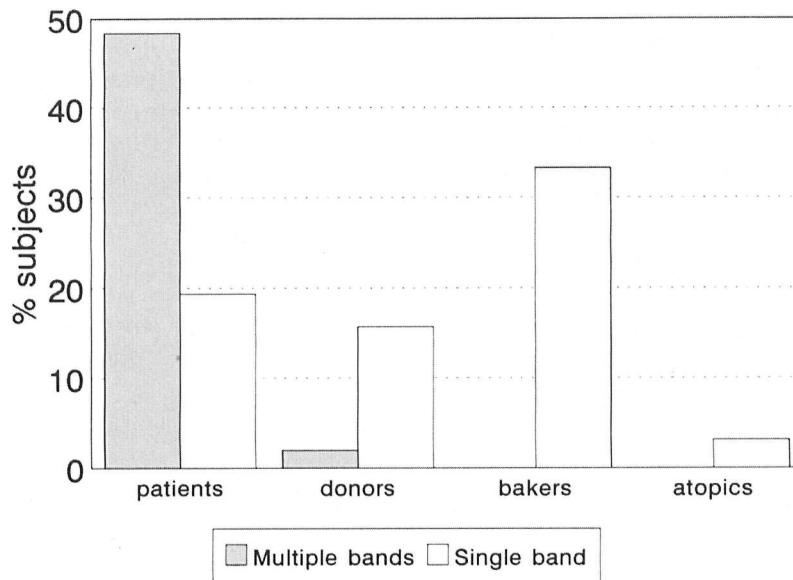


Fig. 1. Frequency of multiple and single-band IgE blottings among 1) patients with urticaria/angioedema/anaphylaxis and suspected parasite allergy ($n=62$), 2) blood donors ($n=51$), 3) bakers ($n=18$), 4) atopic patients ($n=38$).

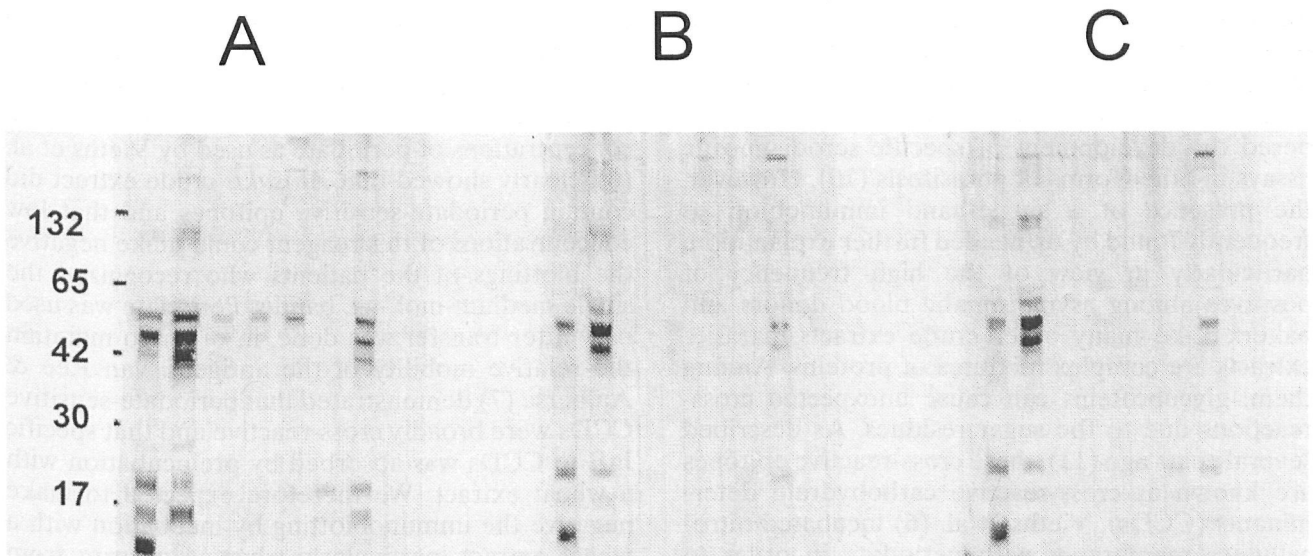


Fig. 2. Several sera with different blotting patterns were used to study effect of decreasing concentrations of periodate on blotted parasite proteins. A) untreated, B) 12.5 mM, C) 50 mM periodate.

However, periodate treatment of proteins can induce other effects such as conformational changes or oxidation of some amino acids (7).

To measure the influence of carbohydrates in the recognition of blotted parasite antigens, we inhibited different sera with a wheat extract. Fungal amylase was also used as an inhibitor to exclude that recognition of this single band was due to the presence of enzymes in the parasite extract which could be detected by enzyme-exposed subjects, such as the bakers. The results clearly showed that immunoblottings could be inhibited only by the

parasite extract (Fig. 3), and not by wheat carbohydrates or fungal amylase.

Discussion

A. simplex is a fish parasite causing chronic or acute infection in man. However, other parasites, such as *Pseudoterranova decipiens* and *Contracaecum osculatum*, have been found to cause disease in man (2). In addition, several other parasites, such as *Hysterothylacium*, *Capillaria gracilis*, and *Cucullanus hians*, have been found in fish in Spain (8). This

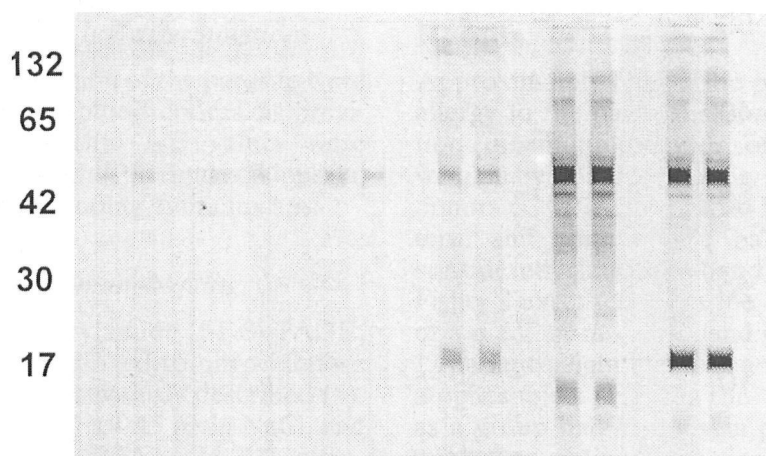


Fig. 3. Six different sera (in triplicate) were studied: left, uninhibited; middle, inhibited by fungal amylase; right, inhibited by *Anisakis* extract. Only parasite extract could induce inhibition in IgE immunoblotting.

fact and other types of cross-reactions with other parasites could explain the presence of positive skin tests and CAP values for the patients with urticaria and negative or anomalous IgE immunoblots (nearly 50% of the suspected cases). Phosphorylcholine and carbohydrate determinants cause much of the cross-reactivity among nematodes (9), and these cross-reactivities have hampered the development of specific serodiagnostic assays in other forms of parasitosis (10). However, the presence of a single-band immunoblot, so frequently found by us, needed further explanation, particularly in view of the high frequency of positives among asymptomatic blood donors and bakers. Like many other crude extracts, parasite extracts are complex mixtures of proteins. Among them, glycoproteins can cause unexpected cross-reactions due to the sugar residues. As described several years ago (11), these cross-reactive epitopes are known as cross-reactive carbohydrate determinants (CCDs). Vieths et al. (6) incubated nitrocellulose membranes with periodate in order to distinguish specific IgE directed to carbohydrates from other antibodies that recognize periodate-resistant epitopes. van Ree & Aalberse (7) have recently demonstrated that carbohydrates present in a mussel extract also show cross-reactivity with other vegetables such as wheat, potato, peanut, etc.

We aimed to establish whether or not the sera of patients and controls having positive skin tests and CAP values to *A. simplex* and showing a single band in the immunoblotting contained specific IgE to CCDs present in the crude parasite extract. This hypothesis was proposed to explain the high amount of positive prick and CAP results found in normal asymptomatic subjects, as well as among patients with urticaria/angioedema without a clear relationship to fish consumption. In fact, an evident

association between symptoms and fish ingestion was established only in those patients showing intense recognition of multiple bands in blotting. If these sera in fact reacted to CCDs epitopes, this unspecific reaction could then be easily absorbed or eliminated by deglycosylation or periodate treatment, making prick tests and CAP more reliable diagnostic tools. Our treatment, using the same concentrations of periodate as used by Vieths et al. (6), clearly showed that *Anisakis* crude extract did contain periodate-sensitive epitopes and that low concentrations of this reagent could make negative the blottings of the patients who recognized the single medium-mol.-wt. band/s. Periodate was used only after transfer was done, in order to maintain the relative mobility of the antigens. van Ree & Aalberse (7) demonstrated that periodate-sensitive CCDs were broadly cross-reactive and that specific IgE to CCDs was absorbed by preincubation with a wheat extract. We therefore expected to make negative the immunoblotting by incubation with a wheat extract, particularly when using sera from bakers. However, the inhibition performed with a wheat extract and fungal amylase clearly showed that these sera contained IgE antibodies not directed to a wheat-cross-reactive determinant, nor to an enzyme usually used by bakers. We have no explanation of the low number of false-positive results obtained in atopic patients. Obviously, the false-positive subjects would never be diagnosed as parasite-allergic by immunoblotting, in view of their characteristic pattern, but the presence of such reactivity could induce a positive result with any other *in vivo* or *in vitro* method. This fact is of particular importance, as we are now trying to estimate the prevalence of the sensitization to this parasite in Spain. It involves the study of many normal subjects. Perhaps the use of periodate-treated antigen could

reduce the unspecificity of the skin tests. Meanwhile, in our opinion, any positive skin prick test or CAP value to *Anisakis* should be confirmed by immunoblotting in order to demonstrate the presence of multiple positive bands to the parasite extract.

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