

**CROSS-REACTIVITY BETWEEN IGE-BINDING PROTEINS FROM ANISAKIS
SIMPLEX AND DERMATOPHAGOIDES PTERONYSSINUS .**

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Abbreviations: As: *Anisakis simplex*, Dp: *Dermatophagoides pteronyssinus*, tr: tropomyosin, hem: hemocyanin, mw: molecular weight, kD: kilodalton, SPTs: skin prick tests, IgE: immunoglobuline E, kU/l: kilounit/liter, PBS: phosphate-buffered saline, TBS-T: Tris-buffered saline containing 0.05% Tween 20.

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ABSTRACT

Background: An association was found between *Anisakis simplex* (As) and *Dermatophagoides pteronyssinus* (Dp) sensitization. One study recently has shown a cross-reactivity between As and Dp and tropomyosin (tr) has been suspected as one of the proteins responsible of this cross-reaction. The aim of our study was: 1) to confirm the cross-reactivity between Dp and As; 2) to determine the importance of tr in this cross reaction.

Methods: SDS-PAGE analysis of Dp and As (metabolic and somatic) extracts was carried out. Then an IgE immunoblotting test using serum from a patient who had specific IgE only to Dp and As and immunoblotting inhibition experiments using Dp extract and tr as inhibitors were performed.

Results: We found that patient's serum reacted: 1) against larval As antigens with a molecular weight (mw) of 25 kilodalton (kD) and a mw > 100 kD, 2) against various metabolic As antigens with a mw > 100 kD, a mw ranging approximately from 35 to 50 kD, and a mw around 20 kD, and 3) against Dp proteins with mw between 35 and 55 kD. Preincubation of patient's serum with Dp extract caused the disappearance of reactivity against antigens with a mw > 100 kD in both larval and metabolic As extracts and against proteins with mw ranging approximately from 35 to 50 kD in the metabolic As extract. Preincubation of patient's serum with As extract caused the disappearance of reactivity against antigens with mw between 35 and 55 kD in the Dp extract. Pre-incubation of patient's serum with tr did not induce any change in the immunoblotting profile.

Conclusions: The results show that 1) cross-reactive components between Dp and As are some proteins with a mw ranging approximately from 35 to 50 kD and with a mw > 100 kD, and 2) tr is not involved in cross-reactivity between As and Dp.

INTRODUCTION

Anisakis simplex (As), a nematode belonging to the Anisakidae family, has been found as a parasite in fish, crustaceans, cephalopods, and marine mammals. The ingestion of parasitized fish might determine development of anisakidosis in humans (1). In the last few years As has been recognized to be responsible for hypersensitivity reactions in humans (1).

The involvement of an IgE-mediated response to As has been proven by means of skin prick tests (SPTs), serum specific IgE detection, and IgE immunoblotting test (2, 3).

We have previously reported that As sensitization (defined as the presence of a positive As SPT) was significantly and independently associated with SPT reactivity to house-dust mites, *Alternaria tenuis*, codfish, and soybean (2). Similarly, Pascual and coworkers found a strong association of As sensitization with *Dermatophagoides pteronyssinus* (Dp) (4). These authors also found an association between As sensitization and sensitization to *Daphnia*, squid, and shrimp (4).

Cross-reaction may explain these associations. Indeed As shares some proteins with other antigens (e.g.: *Ascaris lumbricoides*, *Ascaris suum*, *Toxocara canis*, *Blattella germanica*, *Chironomus* spp, *Hysterothylacium aduncum* and the dust mites *Acarus siro*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, and Dp) (5-8). In particular Johansson and coworkers found that in two serum samples obtained from two farmers the cross-reactivity between As and the dust mites such as *Acarus siro*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, and Dp involves several allergens (8). The same authors (8) suspected that tropomyosin (tr), a cross-sensitizing invertebrate panallergen (9), might be an allergen responsible for the cross-reaction between As and Dp.

Therefore the aim of our study was 1) to confirm the previously reported cross-reactivity between Dp and As by IgE immunoblotting test (8) and than 2) to determine whether tr is a cross-reacting allergen in Dp and As by IgE immunoblotting test using the serum of a child who had both a positive SPT (2) and a high specific IgE level only against these two allergens.

METHODS

Serum samples

Serum sample was obtained from a child with asthma due to *Dp* confirmed by clinical history and presence of cutaneous (SPT equal to 4+) (2) and serum IgE to *Dp* [CAP System RAST FEIA (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) equal to 18.9 kU/l (kilounit/liter)] and with cutaneous (SPT equal to 4+) (2) and serum IgE to *As* [CAP System RAST FEIA (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) equal to 13.7 kU/l]. The patient was enrolled and studied during a previously study (2) and had no symptoms after eating fish.

Serum samples from two healthy children were used as negative control in immunoblotting experiments.

Antigens

The somatic *As* antigen preparation was obtained as follows. Two hundred milligrams of live third-stage larvae (supplied by Doctor Teresa Manfredi, Institute of Veterinary Pathology, University of Milan, Italy) extracted from the body cavity of fish were suspended in 2 ml of phosphate-buffered saline (PBS) and ruptured in a Potter homogenizer. After centrifugation for 30 min at 2700 g (4°C) the supernatant was dialyzed against PBS, filtered through a 0.45 µm filter and stored at -20°C until use.

To obtain metabolic *As* antigens live third-stage larvae were washed three times and incubated (10 larvae/60ml) in MEM-EARLE's medium (Biochrom KG Seromed, Berlin, Germany), penicillin 100 IU/ml and streptomycin 100 µg/ml at 37°C in an atmosphere containing 5% CO₂. Every 48 hours for 8 days, conditioned medium was collected and replaced with fresh medium. The pooled aliquots were filtered through a 0.8 µm filter, lyophilized and stored at -20 °C until use.

The protein concentration of *As* preparations was determined by the Lowry method (10) using bovine serum albumin as standard.

For immunoblotting and immunoblotting inhibition experiments *Dp* extract was supplied by Lofarma (Milan, Italy), while hemocyanin (hem) and tr were purchased from Sigma-Aldrich (St. Louis, MO, USA).

SDS-PAGE, immunoblotting and immunoblotting inhibition analyses

Electrophoresis of As samples was carried out at 180 mA for 1 h in a 4-12% polyacrilamide precast Nupage Bis-Tris gel under reducing conditions according to manufacturer instructions (Novex, Invitrogen, Groningen, The Netherlands). The resolved proteins were stained with 0.1% Coomassie Brilliant Blue or transferred onto a nitrocellulose membrane (Protran BA 85, Schleicher and Schuell, Dassel, Germany) according to Towbin and coworkers (11). The membrane was saturated in TBS-T (Tris-buffered saline containing 0.05% Tween 20) and 5% dry defatted milk, before incubation with control or patient's serum diluted 1:15 in saturating buffer.

For immunoblotting inhibition experiments, 1 ml of diluted control serum or *As/Dp* IgE-positive patient was preincubated with *Dp* extract or metabolic/larval *As* extracts or hem and tr proteins (1 mg/ml).

Bound specific-IgE were detected by peroxidase-conjugated anti-human IgE serum, using an ECL western blotting kit (Amersham Pharmacia Biotech, Uppsala, Sweden) as substrate.

RESULTS

Larval and metabolic *As* extracts contained at least 30 different protein components with a molecular weight (mw) ranging between 200 and 5 kilodalton (kD) as shown by SDS-PAGE profile (Figure 1, lane 1). Immunoblotting experiments on larval and metabolic *As* extracts using *As/Dp* IgE-positive serum showed that some of them were expressing specific IgE-binding properties (Figure 1). In particular patient's serum reacted against larval *As* antigens with a mw of approximately 25 kD and > 100 kD. Normal serum showed no band. In addition a strong IgE-binding was observed to metabolic *As* antigen with 1) a mw greater than 100 kD, 2) a mw ranging approximately from 35 to 50 kD, and 3) a mw around 20 kD. Two aspecific bands at about 52 and 80 kD were equally recognized by control and patient's serum. Preincubation of *As* IgE-positive patient's serum with *Dp* extract caused the disappearance of any reactivity against antigens with a mw > 100 kD in both larval and metabolic *As* extracts and against proteins with mw ranging approximately from 35 to 50 kD present in the metabolic *As* extract. Furthermore metabolic and larval extracts are able to completely inhibit each other (Figure 1).

Immunoblotting inhibition experiments using hem or tr as inhibitors did not induce any change in the immunoblotting profile suggesting that in this case they are not involved in the cross reactivity between *As* and *Dp* extracts (Figure 1, lanes 7-8).

The cross-reactivity was confirmed by immunoblotting on *Dp* extract (Figure 2). *As* IgE-positive patient's serum, but not control serum, strongly bound proteins with mw ranging from about 35 to 55 kD. This band disappeared when serum was preincubated with larval or metabolic *As* extract.

DISCUSSION

House-dust mites are one of the most important cause of IgE mediated diseases (12, 13), while *As* seems to be an emergent allergen especially in Spain (1).

Several allergenic proteins from *Dp* (12, 14) and *As* (15) extracts have been identified.

The antigens in *As* are 1) excretion-secretion and surface antigens (metabolic antigens) and 2) body antigens (somatic antigens) (16).

A strong association between *As* sensitization and specific IgE against *Dp* allergens has been observed (2, 4) consequently cross-reactivity between *As* and *Dp* extracts has been hypotised.

Johansson and coworkers (8), using the sera of two patients, found that proteins with mw > 100 kD and around 36 kD are present both in *As* and *Dp* and suspected that tr (one known allergen of house dust mites with mw of 37 kD) (14) might be the same protein in *As* ad *Dp*.

Our study, using the serum of a child that presented symptoms to *Dp* and no symptoms after eating any fish despite was sensitized to both *Dp* and *As*, confirms the presence of a cross reactivity between larvae *As* extract and *Dp* as recently described (8). Moreover we showed also a cross reactivity between metabolic *As* antigen and *Dp*. According to our immunoblotting inhibition experiments, cross-reactive components between *Dp* and *As* are constituted by proteins showing a mw ranging approximately from 35 to 50 kD (metabolic antigens) and higher than 100 kD (metabolic and somatic antigens).

Moreover interestingly we found that hem and tr, known to be involved in cross-reactivity between mites and molluscs (17, 18), are not able, unlike *Dp* extract, to change the immunoblotting profile against *As* IgE-binding proteins, suggesting that they do not appear responsible of the cross-reactivity phenomenon between *As* and *Dp* extracts.

Our patient presented symptoms to *Dp* and no symptoms after eating any fish despite has been sensitized to both *Dp* and *As*. A similar feature may be present in other allergic situations (e.g.:

some subjects with latex allergy who have hypersensitivity to proteins present both in latex and fruits have symptoms to latex but not fruits) (19).

The presence of a cross-reactivity between *Dp* and *As* might prelude the appearance of a clinical cross-reaction especially in patients who usually eat fish.

Finally only follow-up studies of children with allergy to *Dp* and specific IgE to *As* can better define the clinical importance of the finding of this study.

In conclusion, our data suggest that 1) the components responsible for cross-reactivity between *Dp* and *As* are some proteins with a mw ranging approximately from 35 to 50 kD (metabolic antigens) and higher than 100 kD (metabolic and somatic antigens), and that 2) tr is not responsible for a cross-reactivity between *As* and *Dp*.

REFERENCES

1. Audicana M, Garcia M, del Pozo MD, Diez J, Munoz D, Fernandez E, et al. Clinical manifestations of allergy to *Anisakis simplex*. *Allergy* 2000;55(S59):28-33.
2. Bernardini R, Lombardi E, Novembre E, Ingargiola A, Pucci N, Favilli T, Vierucci A. Predictors of *Anisakis simplex* symptoms. *Allergy* 2000;55:979-80.
3. Moneo I, Curiel G, Fernandez de Corres L, Garcia M, del Pozo MD. Laboratory diagnosis of hypersensitivity to *Anisakis simplex*: a review. *Allergy* 2000;55(S59):34-8.
4. Pascual C, Crespo JF, Ortega N, Ornia N, San-Martin MS, Martin-Esteban M. High prevalence of sensitization to *Anisakis simplex* (AS) in patients with increased levels of total IgE [Abstract]. *J Allergy Clin Immunol* 1997;97:233.
5. Kennedy MW, Tierney J, Puseng Ye, McMonagle F, McIntosh A, McLaughlin D, et al. The secreted and somatic antigens of the third stage larvae of *Anisakis simplex* and antigenic relationship with *Ascaris suum*, *Ascaris lumbricoides*, and *Toxocara canis*. *Mol Biochem Parasitol* 1988;31:35-46.
6. Pascual CY, Crespo JF, San Martin S, Ornia N, Ortega N, Caballero T, et al. Cross-reactivity between IgE-binding proteins from *Anisakis*, German cockroach, and chironomids. *Allergy* 1997;52:514-20.
7. Fernandez-Caldas E, Quirce S, Maranon F, Gomez MLD, Botella HG, Roman RL. Allergenic cross-reactivity between third stage larvae of *Hysterothylacium aduncum* and *Anisakis simplex*. *J Allergy Clin Immunol* 1998;101:554-5.
8. Johansson E, Aponno M, Lundberg M, van Hage-Hamsten M. Allergenic cross-reactivity between the nematode *Anisakis simplex* and the dust mites *Acarus siro*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, and *Dermatophagoides pteronyssinus*. *Allergy* 2001;56:660-6.

9. Reese G, Ayuso R, Lehrer SB. Tropomyosin: an invertebrate panallergen. *Int Arch Allergy Immunol* 1999;119:247-58.
10. Lowry OH, Rosenbrough NJ, Farr AL, Randall RS. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
11. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci* 1979;76:4350-4.
12. Thomas WR, Smith W. House-dust-mite allergens. *Allergy* 1998;53:821-32.
13. Solomon WR, Platts Mills TAE. Aerobiology and Inhalant Allergens. In: *Allergy Principles & Practice*, Vol I, eds Middleton Jr et al, Mosby -Year Book, Inc. St. Louis, Missouri, 1998, pag 367.
14. Sidenius KE, Hallas TE, Poulsen LK, Mosbech H. Allergen cross-reactivity between house-dust mites and other invertebrates. *Allergy* 2001;56:723-33.
15. Moneo I, Caballero ML, Gomez F, Ortega E, Alonso MJ. Isolation and characterization of a major allergen from the fish parasite *Anisakis simplex*. *J Allergy Clin Immunol* 2000;106:177-82.
16. Ubeira FM, Iglesias R. Monoclonal antibodies in the study of *Anisakis simplex*. *Allergy* 2000;55(Suppl 59):18-27.
17. Mistrello G, Falagiani P, Riva G et al. Cross-reactions between shellfish and house-dust. *Allergy* 1992;47suppl:287.
18. Witteman AM, Akkerdaas JH, van Leeuwen J et al. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. *Int Arch Allergy Immunol* 1994;105:56-61.
19. Lavaud F, Cossart C, Reiter V, Bernard J, Deltour G, Holmquist I. Latex allergy in patient with allergy to fruit. *Lancet* 1992;339:492.

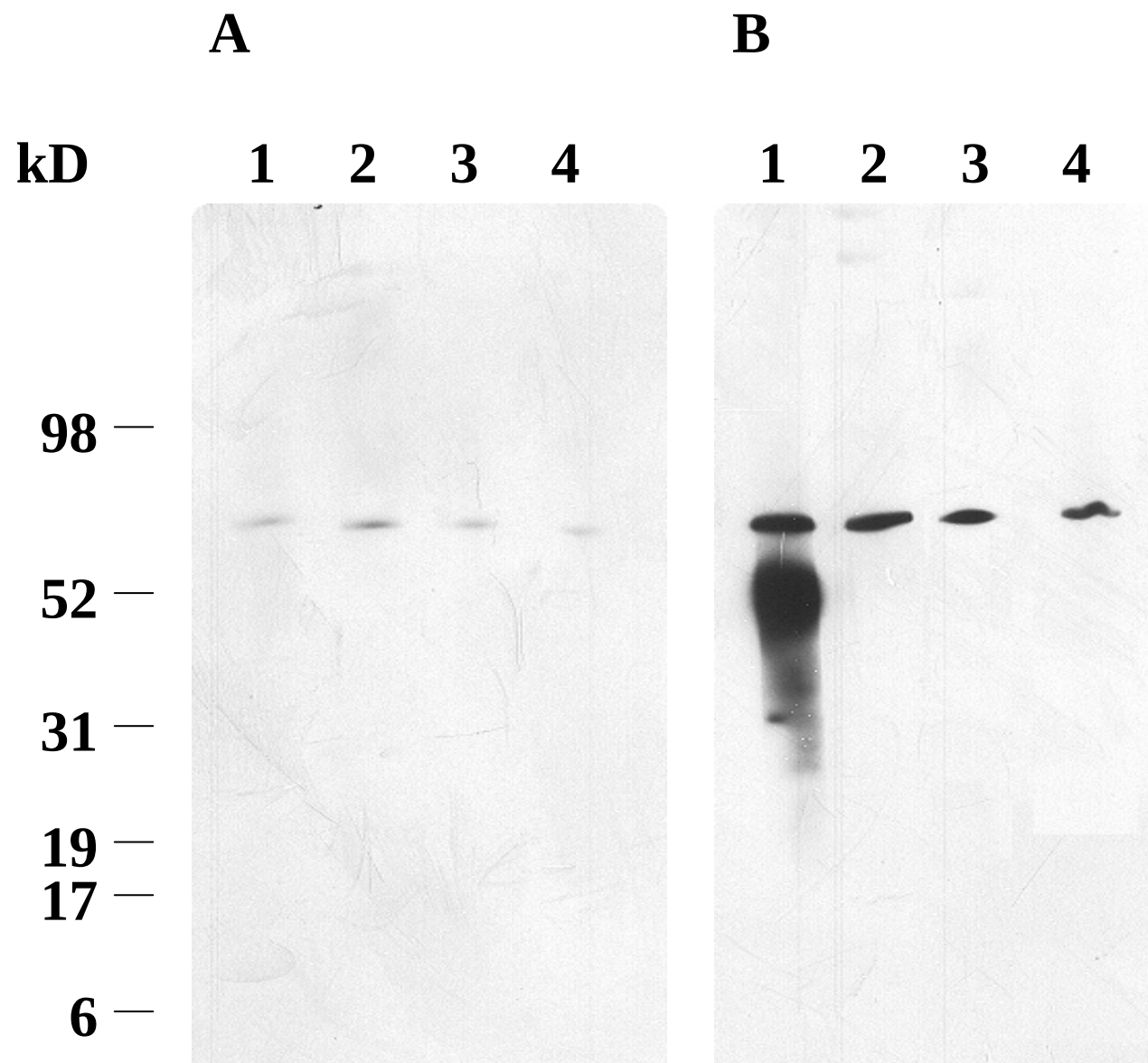
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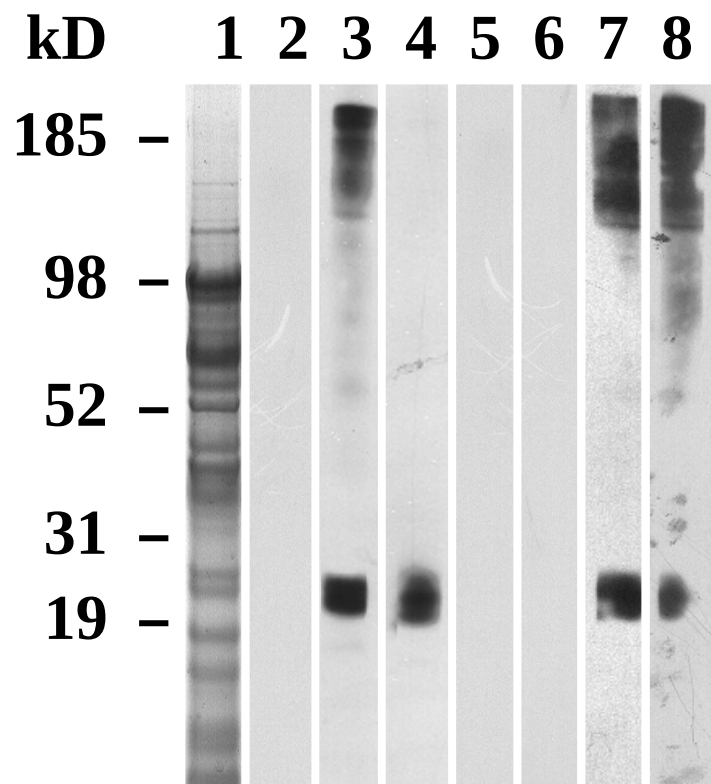
Figure 1.

SDS-PAGE profile of larval *As* extract (A) (lane 1) and metabolic *As* extract (B) (lane 1). Immunoblot inhibition experiments on 15 µg/lane of larval *As* extract (A) (lanes 2-8) or metabolic *As* extract (B) (lanes 2-8). Sera from healthy donors (lane 2) and *As/Dp* IgE-positive patient (lanes 3-8) were preincubated with saturating buffer (2, 3) or 400 µg of *Dp* (4), larval *As* (5), metabolic *As* (6) extracts, hem (lane 7), tr (lane 8). Molecular weight markers are indicated on the left of each panel.

Figure 2.

Immunoblot on *Dp* extract (15 µg/lane) incubated with control serum (A) or *As/Dp* IgE-positive patient's serum (B) preincubated with saturating buffer (lane 1), or 400 µg of larval *As* (lane 2), metabolic *As* (lane 3) or *Dp* extracts (lane 4). Molecular weight markers are indicated on the left.



A**B**