CROSS-REACTIVITY BETWEEN ANTIGENS OF *ANISAKIS SIMPLEX* S.L. AND OTHER ASCARID NEMATODES

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Summary:

A study of the cross-reactivity among somatic and excretory-secretory antigens of the third stage larvae of *Anisakis simplex* s.l. and somatic antigens of other ascarid nematodes (*Ascaris lumbricoides*, *A. suum*, *Toxocara canis*, *Anisakis physeteris*, *Hysterothylacium aduncum* and *H. fabri*) was carried out by immunoblotting. It was revealed a high degree of cross-reactivity among ascarids in the 30 and > 212 kDa range by using sera against somatic and excretory-secretory antigens of *A. simplex* s.l. It has been revealed also specific components of the *Anisakis* genus (< 7.2, 9, 19 and 25 kDa) that will be interesting in diagnosis.

KEY WORDS : ascarids, antigen, immunoblotting, cross-reaction.

Résumé : RÉACTION CROISÉE DES ANTIGÈNES D'*ANISAKIS SIMPLEX* S.L. ET D'AUTRES NÉMATODES ASCARIDÉS

Dans ce travail nous étudions les réactions croisées des antigènes du troisième stade larvaire d'*Anisakis simplex* s.l. et des antigènes somatiques d'autres nématodes ascarides par la technique d'immunoblot (*Ascaris lumbricoides*, *A. suum*, *Toxocara canis*, *Anisakis physeteris*, *Hysterothylacium aduncum* et *H. fabri*). On a ainsi révélé une très importante réactivité croisée parmi des ascarides dans les bandes de 30 et dans celles supérieures à 212 kDa en employant des immunosérums anti-*A. simplex* s.l. (antigènes somatiques et antigènes excrétés-sécrétés ou métaboliques). De même, nous avons révélé des fractions spécifiques (< 7.2, 9, 19 et 25 kDa) dans le genre *Anisakis* qui seront intéressantes dans le diagnostic.

MOTS CLÉS : ascaridés, antigène, immunoblotting, réaction croisée.

One of the main drawbacks of immunological diagnosis of parasite infection is caused by the complexity of the parasite antigens, especially the existence of antigens shared between species with varying degrees of phylogenetic relationship and between the parasite and host (Capron *et al.*, 1968; Pascual *et al.*, 1997; Favre *et al.*, 1998). Immunological cross-reactions among ascarid nematodes are widespread and infection by a species could potentially affect on the nature of the immune response by another species and produce false positives in the diagnosis (Kennedy *et al.*, 1988; Kennedy *et al.*, 1989; Iglesias *et al.*, 1996; Nunes *et al.*, 1997; Fernandez *et al.*, 1998; Lorenzo *et al.*, 1999; Kennedy, 2000). Anisakiasis of the human alimentary tract is frequently caused by the ingestion of raw or undercooked fish infected by *Anisakis simplex* larvae (L3) (Van Thiel *et al.*, 1960; Ishikura *et al.*, 1992). On the other hand, recently several human infections due to *A. simplex* larvae have been reported in Spain (Domínguez Ortega *et al.*, 2000; Daschner *et al.*, 2000; Castan *et al.*, 2002).

For this reason, in the present work, we have studied the cross-reactivity between somatic (SA) or excretory-secretory (ES) antigens of the third stage larvae of *A. simplex* s.l., and other ascarid nematodes (*Ascaris lumbricoides*, *A. suum*, *Toxocara canis*, *Anisakis physeteris*, *Hysterothylacium aduncum* and *H. fabri*) using immunoblotting to detect specific antigens that could be applied to serologic diagnosis in human anisakiasis.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

3 larvae of the following nematodes were isolated: *A. simplex sensu lato* (s.l.), (blue whiting, *Micromesistius poutassou*), *H. aduncum* (horse mackerel, *Trachurus trachurus*), *A. physeteris* (hake, *Merluccius merluccius*), *H. fabri* (striped mullet, *Mullus barbatus* and striped red mullet, *M. surmuletus*). The hosts are given in parentheses.

Adults of *T. canis*, *A. suum* and *A. lumbricoides* were obtained from dogs, pigs and humans, respectively.

ANTIGENS

Somatic antigens (SA) of the larvae and adults helminths: all the helminths were washed several times

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with phosphate saline buffer (PBS) pH 7.2, and homogenized in PBS supplemented with protease inhibitors: 5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulphonyl fluoride and 0.1 trypsin inhibitor unit/ml aprotinin (Sigma). The homogenates were applied to several cycles of ultrasound sonicator and centrifuged at 20,000 r/min for 30 min at 4°C. The supernatant was harvested and conserved at -70°C until use.

Excretory-secretory antigens of *A. simplex* s.l. (ES): viable L3 larvae of *A. simplex* s.l. were placed in an antibiotic-antifungal solution and axenized (Iglesias *et al.*, 1997). Then, 200-250 larvae/2.5 ml were placed in culture flask containing sterile saline solution at 0.9 %, and maintained at 37°C and 5 % CO₂ (Valero *et al.*, 2003). The worms were observed daily for mobility, moulting and survival; after 7-9 days of culture, the larvae were separated and the supernatant was filtered and centrifuged at 1,900 g for 30 min at 4°C and stored at -70°C until use.

The protein concentration of the antigens was determined by the method of Lowry (Lowry *et al.*, 1951).

ANTI-SA AND ANTI-ES IMMUNE SERA

To obtain anti-SA and anti-ES sera, New Zealand rabbits weighing about 2 kg were immunized intramuscularly with doses of 1 ml/rabbit of a 1:1 (v/v) mixture of complete Freund's adjuvant and a solution containing 6 mg of antigen. Both at 21 and 31 days the same antigen dose except adjuvant was injected. After one month from the last immunization, they were administered a booster dose and the animals were bled at seven days. For each antiserum, a pool with immune sera (from three rabbits) obtained from successive bleedings was done. Before the first inoculation, the rabbits were bled via incision into the marginal vein of the ear, using this serum as a negative control.

IMMUNOBLOTTING

The protein electrophoresis was carried out in gels of polyacrylamide with reduced condition and in the presence of SDS (sodium dodecylsulphate) (Laemli, 1970). The gels were discontinuous with a first stacking gel (4 % of acrylamide) and a second with 10 % of acrylamide.

The samples loaded was mixed with equal amounts of the antigenic solution and sample buffer, and then heated in a bath of boiling water for 4 min. The electrophoresis was carried out at 4°C and 200 V for 45 min and the separated proteins were transferred to nitrocellulose membranes (Bio-Rad) of 0.22 µm (Towbin *et al.*, 1979). The transfer lasted 12 h at 4°C and 30 V, in a buffer of 20 % methanol, 25 mM of Tris, and 192 mM of glycine, pH 8.3.

The nitrocellulose membrane was blocked for one hour at room temperature, with skimmed powdered milk at 5 % in PBS, and then washed for five minutes in PBS-Tween at 0.3 % (PBS-T). The membranes placed on a Mini Protean II Multiscan (Bio Rad., Richmond, CA, USA) were incubated with a 1:200 dilution of anti-SA or anti-ES of *A. simplex* s.l. sera (primary antibodies). After washed for three times with PBS-T, the membranes were incubated with a 1:30,000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) (secondary antibody) for one hour at room temperature with light stirring. With washings as the above, antigen bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BICP-NBT) (Bio-Rad).

RESULTS

Rabbit anti-SA and anti-ES sera of *A. simplex* s.l. had similar antigen recognition patterns and numerous bands against the somatic antigens of *A. lumbricoides*, *A. suum*, *T. canis*, *A. simplex* s.l., *A. physeteris*, *H. aduncum* and *H. fabri* and against the excretory-secretory antigens of *A. simplex* s.l. were detected. These results are shown in the Table I.

ASSAYS WITH ANTI-SA SERUM OF *A. SIMPLEX* S.L.

Most of the shared bands were found between 30 and > 212 kDa (Fig. 1A). Bands of approximately 60, 75, 110 kDa and two bands greater than 212 kDa were observed in the majority of the antigens. However, the anti-SA serum recognised a 25 kDa band only in SA of *A. simplex* s.l. and low Mw bands (19 and 22 kDa) in both antigens of *A. simplex* s.l. and in the somatic antigens of *A. physeteris*.

ASSAYS WITH ANTI-ES SERUM OF *A. SIMPLEX* S.L.

The results obtained with the different antigens are presented in Fig. 1B. This immune serum recognises common bands of 7.2, 36, 75, 110, 212 and > 212 kDa,

	Anti AS <i>A. simplex</i> s.l. serum	Anti ES <i>A. simplex</i> s.l. serum
<i>A. simplex</i> s.l. (AS)	20	23
<i>A. simplex</i> s.l. (ES)	19	26
<i>A. lumbricoides</i>	12	19
<i>A. suum</i>	13	20
<i>T. canis</i>	10	15
<i>A. physeteris</i>	18	18
<i>H. aduncum</i>	11	14
<i>H. fabri</i>	12	14

Table I. - Common bands, between nematode species, obtained by immunoblot.

Fig. 1. – Cross-reactivity revealed by immunoblotting between *Anisakis simplex* s.l. and other nematodes. A) Immunoblot of helminth extracts with anti-SA serum of *A. simplex* s.l. B) Immunoblot of helminth extracts with anti-ES serum of *A. simplex* s.l.: (lane a) *Ascaris suum*; (lane b) *A. lumbricoides*; (lane c) *Toxocara canis*; (lane d) *A. simplex* s.l. -SA-; (lane e) *A. simplex* s.l. -ES-; (lane f) *A. physeteris*; (lane g) *Hysterothylacium aduncum*; (lane h) *H. fabri*. Arrows show specie-specific and genus-specific bands. The molecular weight markers are indicated on the left.

in the different species of nematodes studied. Also it revealed a band of low Mw (14 kDa) in *A. simplex* s.l., *A. physeteris* and *Hysterothylacium* antigens, two bands of < 7.2 and 19 kDa in both antigens of *A. simplex* s.l. and a band of 9 kDa only in the ES antigen of *A. simplex* s.l.

DISCUSSION

The studies of cross-reactivity by different methods confirm the large antigenic homology among the nematodes. In this work, somatic and excretory-secretory antigens of the third stage larvae of *A. simplex* s.l. and the somatic antigens of the nematodes: *A. physeteris*, *H. aduncum*, *H. fabri*, *A. lumbricoides*, *A. suum* and *T. canis*, have been studied by immunoblotting, using rabbit anti-SA and anti-ES sera of *A. simplex* s.l. A study of this kind had not been done with *A. physeteris* and *H. fabri*. Although this study has been carried out with immunized rabbits sera only, we believe that the results obtained can apply to a natural infection. Thus, sera from mice infected with live larvae and immunized with dead larvae recognized a similar pattern of bands in immunoblots of ES and SA antigen preparations (Iglesias *et al.*, 1993); also, Pascual *et al.*, 1999, demonstrated high, medium and low molecular weigh bands in the sera of 12 patients with gastric anisakiasis. Otherwise, the larvae L3 were maintained of 7-9 days *in vitro* cultivation to obtain a greater concentration of excretion-secretion antigens

and we should admit that they were obtained so much antigens of L3 as of L4, since L3 moulting at 3-5 days (Iglesias *et al.*, 1997; Iglesias, 1999; Iglesias *et al.*, 2001).

Cross-reactivity was detected with all the heterologous antigens tested, although the greatest antigenic similarity was obtained with the somatic antigen of *A. physeteris* that revealed 18 common bands (Table I). In the case of the anti-ES serum, a strong reactivity with the antigens of *A. suum* and *A. lumbricoides* (20 and 19 bands, respectively) was also demonstrated.

The results obtained with anti-SA and anti-ES sera demonstrate the existence of common bands in the ascarids of 60, 75, 110 and > 212 kDa. Some of these bands had been also revealed by other authors (Akao *et al.*, 1990; De Baere *et al.*, 1992; García *et al.*, 1997; Carretero *et al.*, 1998; Iglesias *et al.*, 1996; Iglesias, 1998). However, the high Mw bands (212 and > 212 kDa) observed by us in the most of the ascarids studied both by electrophoresis (unpublished data) and by blotting, have not been detected by other authors either in cross-reactivity studies or in sera of patients with invasive anisakiosis, but they have been revealed with the serum of patients allergic to *Anisakis* (Montoro *et al.*, 1997; Valero *et al.*, 2003).

Specie-specific components have also been revealed: one < 7.2 kDa in the two *A. simplex* s.l. antigens, a band of 9 kDa only in the ES antigen and another one of 25 kDa in the AS antigen (Figs 1A, 1B).

Anti SA and anti ES sera, detect a 19 kDa protein in *A. simplex* s.l. antigens and the anti SA serum also

revealed this component in the somatic antigen of *A. physeteris*. Therefore, this protein could be considered to be specific of the *Anisakis* genus; we think that it will be interesting to look deeply into the study of these low Mw antigens in view of possible use in immunodiagnostic. Other authors also demonstrated that the specificity is found in the low molecular mass proteins, so Hwang *et al.*, 2003, reported a specific antigen in L3ESP, located at less than 13 kDa and recognized by sera from persons diagnosed with anisakiasis. Also, specific bands with low Mw were detected in somatic antigens of *Anisakis* (Iglesias *et al.*, 1993; Iglesias *et al.*, 1996); *A. suum* and *T. canis* (Maizels *et al.*, 1984; Magnaval *et al.*, 1991) and in the sera of patients allergic to *Anisakis* (Del Pozo *et al.*, 1996; Montoro *et al.*, 1997; Carretero *et al.*, 1998).

Anti-ES serum revealed a protein of 14 kDa in the somatic antigens of *A. simplex* s.l., *A. physeteris* and *H. aduncum* and in the excretory-secretory antigens of *A. simplex* s.l (Fig. 1B); otherwise, the anti SA serum did not detect this protein in any case. The fact that this component was only detected in the antigens of *Anisakis* and *Hysterothylacium* and with the ES serum, seems to indicate that this is released by the larvae, into the maintenance medium in sufficient amounts, to produce an antibody response in immunized rabbits and that it is present, in a lower concentration, in the somatic antigens. This protein, characteristic of the Ascaridoidea, was not detected in the other parasitic nematodes supporting the findings of Kennedy *et al.*, 1988 and Kennedy *et al.*, 1989, about the 14 kDa antigen which is produced in different quantities by the members of this superfamily and can even presents structural differences in the molecule, that would condition its immunogenicity.

ACKNOWLEDGEMENTS

This work was partly funded by the Research Groups Grant from Junta de Andalucía. The translation into English was by C. Coope.

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Reçu le 23 juin 2003

Accepté le 20 janvier 2004