

O-glycans as a source of cross-reactivity in determinations of human serum antibodies to *Anisakis simplex* antigens

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Summary

Background *Anisakis simplex* is a seafood-borne parasite that may both infect humans and cause allergy. Serodiagnosis of anisakiasis and allergy caused by this nematode is difficult since most *Anisakis* antigens show cross-reactivity problems.

Objective To analyse the possible role of sugar epitopes contained in *Anisakis simplex* antigens as causes of false-positive results in serodiagnostic assays.

Methods The antigens UA2R and UA3R recognized by two anti-*Anisakis* monoclonal antibodies were used in this study. Capture ELISA techniques were used to compare the reactivities with native or O-deglycosylated antigens of sera from *Anisakis*-free children (most of them infected by several other parasites) and from *Anisakis* allergy patients. O-deglycosylation was done by mild alkali treatment with NaOH. SDS-PAGE and immunoblotting were used to characterize the effects of NaOH or N-glycanase F treatment on UA3R.

Results Native UA2R was recognized by IgG1 and IgM antibodies in the sera of both *Anisakis*-free subjects and allergy patients. Native UA3R was recognized by most sera from allergy patients (92% considering immunoglobulin (Ig) G1, 100% considering IgE), but also by a significant proportion of sera from *Anisakis*-free subjects (36% considering IgG1, 14% considering IgE). O-deglycosylation of UA3R greatly improved specificity: none of the sera from *Anisakis*-free patients showed either IgG1 or IgE reactivity with O-deglycosylated UA3R, while the proportion of sera from allergy patients showing IgE reactivity with this antigen was practically unaffected. O-deglycosylation of UA2R did not improve the specificity of assays using this antigen. Our results also show that the protein core of glycoproteins may be altered by even very mild alkali treatment, depending on the nature of the protein.

Conclusion Native glycoproteins of *A. simplex* should not be used for diagnostic purposes. O-deglycosylated UA3R seems to be an excellent candidate for use as target antigen in the serodiagnosis of anisakiasis and *A. simplex* allergy.

Keywords: *Anisakis simplex*, immunoglobulin E, immunoglobulin G, monoclonal antibodies, nematode, N-glycans, O-glycans, serodiagnosis

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Introduction

The availability of suitable target antigens is of crucial importance to the development of specific and sensitive

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Table 1 Characteristics of the 28 subjects in the *Anisakis*-free Argentinean child population, showing parasite infections, age and percentage eosinophils. The rightmost column shows the prevalence of each infection in this population, mean age and mean percentage eosinophils

Parasite	Patient number																				Percentage or Mean						
	5			10			15			20			25														
<i>Giardia</i>	●	●	●	●	○	●	○	○	○	●	○	○	○	●	●	●	○	○	○	○	●	○	○	●	○	●	12/26 (46.1)
<i>B. hominis</i>	○	○	●	○	●	○	●	●	●	○	●	○	●	●	●	●	○	○	○	●	○	●	●	●	●	●	16/26 (61.5)
<i>H. nana</i>	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1/26 (3.8)
<i>S. stercoralis</i>	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	3/26 (11.5)
<i>Uncinaria sp.</i>	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	5/26 (19.2)
<i>A. lumbricoides</i>	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	4/26 (15.4)
<i>E. vermicularis</i>	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1/26 (3.8)
<i>T. trichiura</i>	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1/26 (3.8)
<i>Toxocara sp.</i>	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	10/21 (47.6)
Age	5	8	4	7	9	3	7	13	7	6	13	11	7	6	8	5	9	1	8								7.26
Eosinophils (%)	5	3	3	8	10	5	1	10	12	6	2	8	34	9	15	5	3	10	39								11.2

immunoassays for serodiagnosis of parasite infections. The identification of suitable antigens is particularly difficult in the case of nematode infections due to the marked cross-reactivity within this group. Notably, most nematode proteins contain a variety of highly reactive sugar epitopes which cross-react with identical or analogous epitopes present in other related or unrelated organisms. This seems to be the case with *Anisakis simplex*, a nematode present in sea fish that may infect humans if swallowed alive, and that has more recently been recognized as a significant cause of food-related allergy [1–3].

We have developed a panel of anti-*Anisakis* monoclonal antibodies (mAbs) which appear to be specific for this parasite [4]. Indeed, studies with sera from healthy individuals, *Anisakis* allergy patients and *Anisakis*-infected patients [5] indicate that the antigens recognized by one of these mAbs (UA3, recognizing two major glycoproteins of 139 and 154 kDa) are immunodominant (i.e. antigenic for more than 90% of patients tested) for both circulating immunoglobulin (Ig) G1 and IgE antibodies, without known cross-reactions. However, since sea fish is eaten at least occasionally by the great majority of people in developed countries, it is very difficult to assess whether the presence of anti-UA3R antibodies in the serum of a given individual is by itself indicative of *Anisakis* infection and/or oral immunization with *Anisakis* antigens. Ideally, the specificity of the 139/154-kDa antigen (or any other candidate antigen) should be confirmed by analysing sera from a population of definitely *Anisakis*-free individuals (that is,

individuals who have never eaten sea fish) before using for human immunodiagnostic tests. We have recently had the opportunity of testing sera of children from an Argentinian population (Corrientes, Chaco) in which contact with *Anisakis* antigen is highly improbable due to geographical location (about 1000 km from the Atlantic coast) and poverty. Furthermore, and ideally for our purposes, most of these children are infected with one or more species of intestinal parasite, some related to *A. simplex* and some unrelated. In the present work we used these sera to test the specificity of the antigens UA2R and UA3R recognized by our anti-*Anisakis* mAbs UA2 and UA3, and to analyse the possible role of the sugars present on these antigens (especially O-glycans) in the induction of cross-reactivity. A Spanish population of patients showing allergy to *A. simplex* was also tested for comparative purposes.

Materials and methods

Monoclonal antibodies

The mAbs used were UA2 (IgM/ κ) and UA3 (IgG1/ κ), both specific for *A. simplex*, and US4 (IgG1/ κ), specific for *T. spiralis*. UA2 recognizes two glycoproteins of MWs 48 and 67 kDa in *A. simplex* somatic antigens, while UA3 recognizes two major glycoproteins of 139 and 154 kDa [4]. US4 recognizes an N-glycan epitope present on TSL-1 excretory-secretory glycoprotein 53 (gp53).

A. simplex allergy sera

Twenty-six sera from patients showing allergy to *A. simplex* were obtained from the Allergology Department of the Santiago Apóstol Hospital (Vitoria-Gasteiz, Spain). All patients included in the study showed clinical symptoms of *Anisakis simplex* allergy, including (a) urticaria/angioedema or anaphylaxis within 6 h after ingestion of fish, (b) positive reaction in skin prick test with *A. simplex* extract, (c) presence of IgE to whole *Anisakis* antigens as measured by the CAP system FEIA (Pharmacia & Upjohn, Uppsala, Sweden), and (d) absence of known allergy to fish or other suspected agents. CAP IgE values ranged from 3.6 (class 3) to > 100 kU/L (class 6).

A. simplex-free population sera

Twenty-eight sera from children aged 1–13 years (see Table 1) from the suburbs of Resistencia (NE Argentina) were provided by Dr José Mario Alonso of the Instituto de Medicina Regional, Universidad Nacional del Nordeste, Resistencia, Chaco (Argentina). Most of these children had multiple parasite infections (see Table 1), in accordance with the fact that they lived in poor hygienic conditions without adequate sanitation. These poor conditions were particularly extreme for cases 22–28, who also had malnutrition problems. Intestinal parasites were determined in these subjects by coprological examination. Anti-*Toxocara* antibodies in serum were determined with the LMD *Toxocara* serology kit (Alexon-Trend Inc., Minneapolis, MN, USA).

To facilitate comparisons, these subjects can be grouped as follows (see Table 1): cases 1–10 showed intestinal parasites but were *Toxocara*-negative; cases 11–16 showed intestinal parasites and were *Toxocara*-positive; cases 18–21 showed no intestinal parasites (though note that coprological examination was not done for cases 18 and 19) and were *Toxocara*-positive; cases 22–28 showed multiple intestinal parasites but anti-*Toxocara* antibodies were not evaluated; and case 17 showed neither intestinal parasites nor anti-*Toxocara* antibodies.

Parasites

Third-stage larvae (L3) of *Anisakis simplex* were extracted manually from the viscera and body cavity of blue whiting (*Micromesistius poutassou*) purchased in a local market.

Parasite antigens

The somatic antigen (SA) preparation of *A. simplex* was obtained as follows. Parasites were suspended in phosphate-buffered saline (PBS, pH 7.2) containing 1 mmol/L phenylmethylsulphonyl fluoride (Sigma-Aldrich, Madrid, Spain),

5 mmol/L *O*-phenanthroline (Sigma), 1 µmol/L pepstatin A (Sigma), 10 µmol/L E64 (Sigma), 3 µmol/L aprotinin (Sigma) and 1 mmol/L EDTA (Sigma) as protease inhibitors, then ruptured by homogenization in a Braun S Potter homogenizer with a Teflon pestle followed by sonication on ice (100 W, 5 × 30 s) (Branson Ultrasonic Corporation, Danbury, CT, USA). The sonicated homogenate was centrifuged (10 000 *g* for 30 min at 4 °C) and the resulting pellet discarded. The supernatant was ultracentrifuged (105 000 *g* for 60 min at 4 °C) and the resulting soluble fraction stored at –30 °C until use.

A *Trichinella spiralis*-purified antigenic fraction (P4) was obtained from the nematode cytosolic extract by affinity chromatography (see below) using the mAb US4 produced in our laboratory. UA3R was obtained from the *A. simplex* SA by affinity chromatography using the mAb UA3.

The protein concentration in the antigen preparations was estimated by the method of Lowry *et al.* [6] using bovine serum albumin (BSA; Sigma) as the internal standard.

Mild alkali deglycosylation

Mild alkali treatment to cleave *O*-glycosidic linkages was performed as described by MacDonald *et al.* [7]: briefly, 4 mg of *A. simplex* SA was dissolved in 500 µL of 0.01 mol/L NaOH and incubated for 24 h at 50 °C.

N-glycosidase F (PNGase F) treatment

N-glycosidase F (EC 3.5.1.52) was obtained from Boehringer Mannheim (Mannheim, Germany). Purified *A. simplex* and *T. spiralis* antigen fractions (UA3R and P4) were dissolved in PBS containing 0.1% SDS (10 µg glycoprotein/100 µL buffer) following the supplier's instructions, and denatured by heating at 95–100 °C for 5 min. Then 0.5% Triton X-100 buffer was added and the samples were divided into two aliquots, one of which was treated with 0.5 U of *N*-glycosidase F to cleave *N*-glycosidic linkages. Both aliquots were then incubated at 37 °C for 15–20 h and analysed by SDS-PAGE. The control for effective treatment was the *N*-glycan-containing *T. spiralis* P4 antigen.

Affinity chromatography

The affinity columns used were prepared by coupling mAbs US4 and UA3 to CNBr-activated Sepharose 4B (Pharmacia) following the supplier's instructions. Protein was eluted from the US4 column with 0.1 mol/L glycine/HCl (pH 2.5) and from the UA3 column with 0.1 mol/L glycine/8 mol/L urea (pH 1.8). After elution, samples were brought immediately to pH 7 with Tris or NaOH, respectively. Eluted fractions were dialysed in distilled water and then lyophilized and stored at –30 °C.

Determination of serum antibody levels

To evaluate levels of IgG1, IgM and IgE antibodies to native and *O*-deglycosylated UA2R and UA3R antigens of *A. simplex* in serum from *Anisakis*-free and allergy patients, we used the sandwich ELISA described by Lorenzo *et al.* [5]. First, 100 μ L of PBS containing appropriate amounts of mAb (0.8 μ g of UA2 or 1.4 μ g of UA3) was added to each well of an ELISA plate and incubated overnight at 4 °C. After washing, either (a) 100 μ L of undiluted *A. simplex* SA (500 μ g total protein for both native and *O*-deglycosylated antigens) for IgE determination, or (b) 100 μ L of TBS-T (TRIS-buffered saline containing 0.2% Tween 20) containing 3% dry skimmed milk (TBS-T₁) and 100 μ g of *A. simplex* native SA or 250 μ g of *O*-deglycosylated SA, for determination of IgG1 and IgM, were added to each well and incubated for 1 h at 37 °C. After a washing step and the addition of human serum (100 μ L of pure serum for IgE determination; 1/100 dilution in TBS-T₁ for the other isotypes), the plates were incubated for 90 min at 37 °C.

For determination of IgE and IgG1 isotypes, the plates were then washed and 100 μ L of a monoclonal mouse antihuman-IgE serum (Ingenasa, Madrid, Spain; 1/2500 in TBS-T₁) or antihuman-IgG1 serum (Sigma-Aldrich; 1/500 in TBS-T₁), in both cases labelled with fluorescein isothiocyanate (FITC), was added. After incubation for 1 h at 37 °C and washing, bound Ig-FITC was detected by incubation first with peroxidase-conjugated rabbit anti-FITC Ig (Dakopatts A/S, Glostrup, Denmark; dilution 1/1000 in TBS-T; 1 h incubation at 37 °C) and then with *O*-phenylenediamine (OPD; Sigma).

For determination of IgM isotype, bound serum antibodies were detected by incubation for 1 h at 37 °C with a polyclonal peroxidase-conjugated mouse antihuman-IgM serum (Dakopatts) diluted 1/3000 in TBS-T, and then with OPD as for the other isotypes.

Optical densities (OD) at 492 nm were calculated by subtracting the OD values given by the same serum in the absence of antigen. All isotype determinations were done in duplicate. For data analysis, sera were considered positive if the OD was more than $\bar{x} + 4\text{SD}$, where $\bar{x}$ and SD are the corresponding mean OD and standard deviation for 25 control sera from normal blood donors aged 18–24 years. All of the normal blood donors were from Galicia (north-west Spain), where sea fish is an important component of the diet (mean consumption ≥ 35 kg per person per year). None of these sera showed IgE to whole *Anisakis* antigens in CAP FEIA (Pharmacia & Upjohn).

SDS-PAGE and immunoblotting

The *A. simplex* antigen preparations (SA and UA3R), P4 and the proteins fetuin (Sigma) and BSA were separated by

sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 5–20% linear gradient gels (70 $\times$ 80 $\times$ 0.75 mm) in a MiniProtein II cell (Bio-Rad, Richmond, CA, USA) as described previously [8]. Following electrophoresis, proteins were transferred at 15 V for 55 min to nitrocellulose membranes (0.45 μ m pore size) in a Trans-Blot SD transfer cell (Bio-Rad). The membranes were incubated with appropriate dilutions of the corresponding mAbs, then with peroxidase-conjugated rabbit antimouse Ig (Dakopatts; dilution 1/800), and finally with 3,3'-diaminobenzidine tetrahydrochloride (Sigma).

When immunoblotting was not done, proteins separated electrophoretically were fixed with 12% trichloroacetic acid (Merck; Darmstadt, Germany) for 1 h at room temperature and then stained with Coomassie blue G-250 (Sigma).

Statistical analysis

Proportions were compared between groups by the Fisher exact test using Woolf's approximation. Statistical significance is taken to be indicated by $P \leq 0.05$.

Results

Anti-UA2R and anti-UA3R antibodies in *Anisakis*-free children and in allergy patients

To confirm the specificity of the antigens captured by our UA2 and UA3 mAbs, we first compared levels of antibodies to these antigens in the serum of *Anisakis*-free children and in *Anisakis* allergy patients. As mentioned above, the former population was chosen because its members do not eat sea fish, but typically suffer from a range of other intestinal helminthoses (Table 1).

The results presented in Fig. 1 and Table 2 show that most allergy patients and most *Anisakis*-free patients had IgM and IgG1 antibodies to the UA2R antigen. This assay was limited to IgM and IgG1 antibodies, since less than 60% of *Anisakis* allergy patients show IgE antibodies to this antigen (unpublished results).

When we tested levels of antibody to the UA3R antigen (Fig. 2, Table 2), 100% of the allergy patients showed IgE antibodies, but in addition 92% showed IgG1 antibodies and 58% showed IgM antibodies. The UA3R antigen was more specific than the UA2R antigen, but by no means entirely so: about one third of the 28 *Anisakis*-free patients showed anti-UA3R IgM and/or IgG1, and four patients (14%) showed detectable levels of IgE antibodies.

Statistical analysis of the data shown in Table 1 indicate that the proportion of false-positives was significantly higher among *Uncinaria*-positive patients than *Uncinaria*-negative patients (IgG1 assays, $P \leq 0.01$; IgE assays, $P \leq 0.05$), and among *Toxocara*-positive patients than

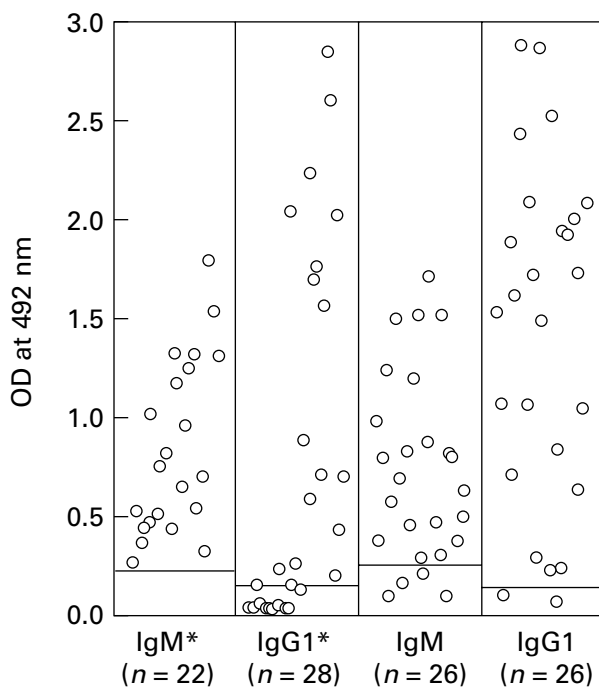


Fig. 1. Serum IgM and IgG1 responses to the native UA2R antigen in the *Anisakis*-free group (*) and the *A. simplex* allergy group. Each point represents the OD value for a given patient. Horizontal bars represent the cut-off values (mean OD + 4SD) for each isotype (IgG1 = 0.150 and IgM = 0.250), as obtained on the basis of results for 25 control sera (see Material and methods)

among *Toxocara*-negative patients (IgG1 assays, $P \leq 0.05$; IgM assays, $P \leq 0.05$).

Role of O-glycans in the cross-reactivity shown by UA2R and UA3R A. simplex antigens

Sugar epitopes coupled to proteins are a very frequent cause of false-positive reactions in serodiagnosis. As is well known, glycans from glycoproteins belong to two groups. Those linked to the hydroxyl group of either serine or threonine are called *O*-linked glycans, while those linked to the amide group of an asparagine residue are called *N*-linked glycans [9]. Most of the *N*-linked glycans can be easily removed from glycoproteins by the action of peptide-*N*-(*N*-acetyl- β -glucosaminyl) asparagine amidase (*N*-glycosidase F) [10], but it is a very expensive procedure to cleave large amounts of antigen for subsequent serodiagnostic use. Alternatively, glycans can be cleaved by chemical deglycosylation [9], but the integrity of the core protein cannot be guaranteed after such treatment [11]. Recently, however, it has been reported that *O*-glycans can be cleaved from glycoproteins under very mild alkaline conditions without alteration of the protein core [7]. We therefore used this deglycosylation technique in the present study.

In Figs 3 and 4, and in Table 2, it can be seen that after removal of *O*-glycans from UA2R the observed cross-reactivities dropped in both *Anisakis*-free and allergy populations though in no case was the drop statistically significant. After removal of *O*-glycans from UA3R, however, the proportions of sera testing positive for IgG1 and IgE dropped to zero in the *Anisakis*-free population ($P \leq 0.01$ for IgG1; not significant for IgE, probably due to the small amount of sera testing positive in this population). By contrast, the proportion testing positive for IgE

Table 2 Numbers of subjects showing IgG1, IgM and IgE antibodies to native and *O*-deglycosylated UA2R and UA3R antigens, in the *Anisakis*-free group and the *A. simplex* allergy group. Percentages of patients testing positive are shown in brackets. ND = not determined. *Significantly different ($P \leq 0.01$; Fisher exact test) from the number obtained using the same assay but with the native antigen

Antigen	Isotype	<i>Anisakis</i> -free children		<i>A. simplex</i> allergy patients	
		UA2	UA3	UA2	UA3
Native	IgM	22/22 (100)	5/22 (22.7)	22/26 (84.6)	15/26 (57.7)
	IgG1	18/28 (64.3)	10/28 (35.7)	24/26 (92.3)	24/26 (92.3)
	IgE	ND	4/28 (14.3)	ND	26/26 (100)
<i>O</i> -deglycosylated	IgM	19/22 (86.4)	1/22 (4.5)	18/26 (69.2)	2/26* (7.7)
	IgG1	14/28 (50.0)	0/28* (0)	19/26 (73.0)	18/26 (69.2)
	IgE	ND	0/28 (0)	ND	26/26 (100)

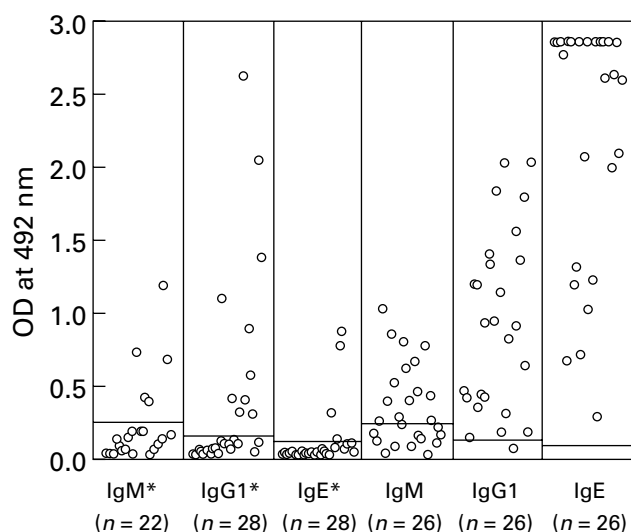


Fig. 2. Serum IgM, IgG1 and IgE responses to the native UA3R antigen in the *Anisakis*-free group (*) and the *A. simplex* allergy group. Each point represents the OD value for a given patient. Horizontal bars represent the cut-off values (mean OD + 4sd) for each isotype (IgG1 = 0.135, IgM = 0.256 and IgE = 0.105), as obtained on the basis of results for 25 control sera (see Material and methods)

remained at 100% in the allergy population and even the proportion testing positive for IgG1 was not significantly reduced (to 76.9% vs 92.3% for non-deglycosylated UA3R). In addition, the proportion of sera testing positive for IgM was reduced from 22.7 to 4.5% in the *Anisakis*-free population and from 57.7 to 7.7% in the allergy population; however, only this latter decrease was statistically significant.

Effect of alkali treatment on glycoprotein integrity

Although the proportions of allergy patients showing IgE and, to a lesser extent, IgG1 antibodies were maintained after treatment of UA3R with mild alkali, the OD values obtained in ELISA were significantly lower than those obtained with untreated antigen. Thus a reasonable doubt arises as to whether this phenomenon is due to release of the sugar epitopes from the protein core, and/or to partial damage to the protein epitopes themselves. To assess these possibilities, we investigated whether alkali treatment alters *Anisakis* antigens; as a control, we also evaluated the effect of this treatment on bovine serum albumin and fetuin.

The results presented in Figs 5 and 6 show that the effect of NaOH treatment was different depending on the nature of the antigen: the treatment severely damaged most *A. simplex* proteins and fetuin, but seemed not to affect BSA. Some of the *Anisakis* protein bands were maintained after NaOH treatment (Fig. 5), but these bands were faint and smudgy, indicating partial degradation.

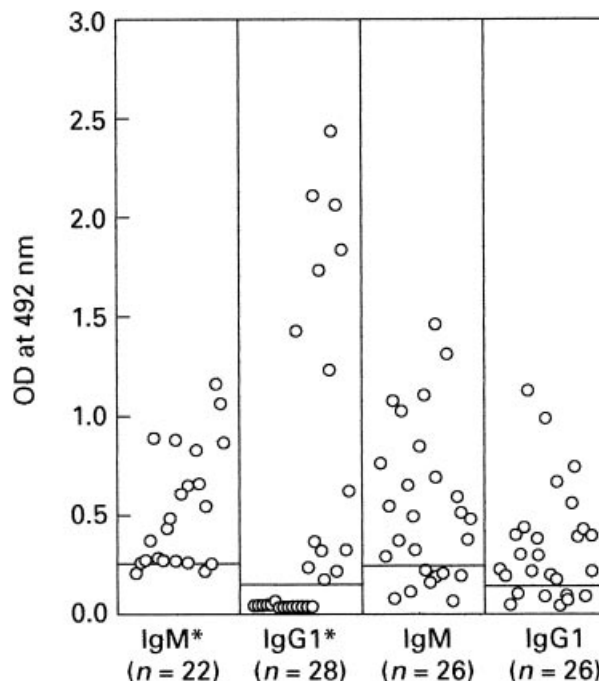


Fig. 3. Serum IgM and IgG1 responses to the *O*-deglycosylated UA2R antigen in the *Anisakis*-free group (*) and the *A. simplex* allergy group. Each point represents the OD value for a given patient. Horizontal bars represent the cut-off values (mean OD + 4sd) for each isotype (IgG1 = 0.150 and IgM = 0.250), as obtained on the basis of results for 25 control sera (see Material and methods)

Effects of N-glycanase F treatment on UA3R

Having found that most (if not all) cross-reactivity shown by the UA3R antigens was abolished after elimination of *O*-glycans, we investigated whether this antigen also bears *N*-glycans. To this end, putative *N*-glycans were removed from affinity-purified UA3R by enzymatic cleavage with *N*-glycanase F. The immunoblotting results presented in Fig. 7 show that treatment with this enzyme did not alter the molecular weight of the antigen; this indicates that *N*-glycans are absent or present in only negligible amounts on this antigen. That the enzyme was effective was confirmed by the fact that it abolished binding by our mAb US4, which recognizes an *N*-glycan on the P4 excretory/secretory antigen of *Trichinella* (see Fig. 7b, lane 2).

Discussion

Anisakis simplex is currently recognized as an important human pathogen that may cause not only gastrointestinal infection but also allergic disease. Despite the fact that it induces a strong antibody response when it infects mice [8] or humans [5,12,13], there is still no definitive test available

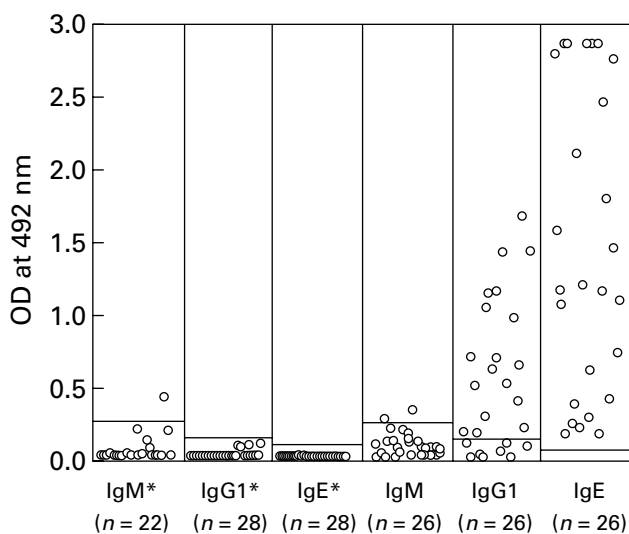


Fig. 4. Serum IgM, IgG1 and IgE responses to the *O*-deglycosylated UA3R antigen in the *Anisakis*-free group (*) and the *A. simplex* allergy group. Each point represents the OD value for a given patient. Horizontal bars represent the cut-off values (mean OD + 4SD) for each isotype (IgG1 = 0.135, IgM = 0.256 and IgE = 0.105), as obtained on the basis of results for 25 control sera (see Material and methods)

that guarantees detection of anti-*Anisakis* antibodies in serum. The results of the present study show that when the 139/154-kDa antigen recognized by our mAb UA3 is *O*-deglycosylated it becomes a specific target for anti-*Anisakis* immunoglobulin determinations, even when testing sera from patients having several *Anisakis*-related infections. By contrast, the UA2R antigen (which appeared specific for *Anisakis* in previous studies using a healthy Spanish population as control) was invalidated for diagnosis, in view of the results obtained with *Anisakis*-free children. These findings are of particular interest since three of the currently available methods for identifying serum IgE antibodies induced by *A. simplex* (CAP-FEIA, Pharmacia; IgE Western blotting; and the prick test [2,14–16]) use a whole antigenic fraction derived from this parasite. Whether treatment of whole antigenic preparations with alkali might improve the specificity of these techniques merits further investigation. However, the results obtained with UA2R (which was not affected by alkali treatment) suggest that the presence in whole *Anisakis* extracts of this and other antigens bearing immunoreactive *N*-glycan epitopes and/or cross-reactive protein epitopes could make these assays invalid for specific IgE determinations, particularly in view of the fact that studies of plant allergens have shown that *N*-glycan epitopes can induce IgE production [17–19]. Cross-reactive antigens present in parasite extracts include the biotinyl-enzymes. We have recently demonstrated that these molecules induce cross-reactive antibodies

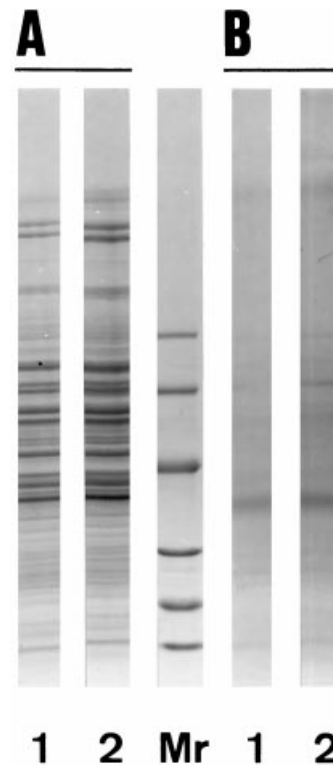


Fig. 5. SDS-PAGE analysis of native (A) and *O*-deglycosylated (B) *Anisakis simplex* somatic antigen. Proteins were separated on 5–20% polyacrylamide gels under reducing conditions. Protein amounts were 13 µg (lane 1) or 39 µg (lane 2). Relative molecular masses (M_r) of standard markers (97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa from top to bottom) are shown in the middle

of all classes in humans [20]. However, it has yet to be demonstrated that the biotinyl-enzymes of parasites (e.g. *Toxocara* and *Uncinaria*) bear *O*-glycans; and even if this is indeed the case, it does not necessarily explain the observed cross-reactivity. Nevertheless, the present results allow us to conclude that the IgE response to *O*-deglycosylated UA3R may permit allergologists to differentiate true *Anisakis* allergy from other cases in which positive test results are due to cross-reactivity with sugar or protein epitopes that are present in other organisms and immunize the host by the oral route. This is of special interest in view of the immunodominance of the 139/154-kDa antigen: specifically, we have demonstrated in previous studies that 100% of patients with a clear history of allergy to *Anisakis* have detectable serum IgE antibodies to this antigen [5].

On the other hand, anti-UA3R IgG1 levels were positive in about one third of *Anisakis*-free children and in more than 90% of allergy patients. These percentages dropped to zero and 69%, respectively, after *O*-deglycosylation of the antigen, indicating that IgG1, like IgE, also becomes specific after this antigen treatment. Nevertheless, since OD values shown by allergy sera are often slightly decreased after

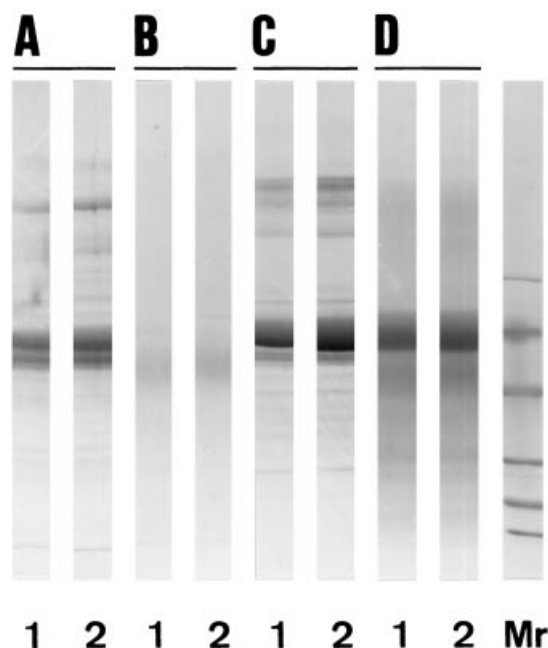


Fig. 6. SDS-PAGE analysis of native (A) and *O*-deglycosylated (B) fetuin and native (C) and *O*-deglycosylated (D) bovine serum albumin. Proteins were separated on 5–20% polyacrylamide gels under reducing conditions. Protein amounts were 20 µg (lane 1) or 30 µg (lane 2). Relative molecular masses (M_r , in kDa) of standard markers (97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa from top to bottom) are shown to the right

target-antigen deglycosylation, without loss of positivity, it is not at first clear whether this reduction is due to loss of *O*-glycans or to partial degradation of the protein core of the antigen, making it suboptimal for ELISA. The available data seem to indicate that both effects may occur simultaneously. First, it is clear that removal of *O*-glycans from UA3R eliminates false-positive results, but a 2.5-fold increase in antigen concentration was necessary to achieve saturation in ELISA. However, this increase in antigen concentration did not increase cross-reactivity with sera from the *Anisakis*-free population (results not shown), thus confirming the validity of our assay. Second, partial degradation of UA3R is suggested by the fact that most of the OD values obtained with allergy sera using the *O*-deglycosylated antigen as target are lower than those obtained with the native antigen as target; however, as already mentioned, this slight decrease in OD did not alter the proportion of sera (100%) that tested positive.

All sera from the *Anisakis*-free population showed IgM antibodies to native UA2R, while only 23% of these sera showed IgM antibodies to native UA3R. After *O*-deglycosylation of the antigen, the percentages dropped to 86 and 5% (one serum), respectively. The slight positive reactivity of this latter serum is probably attributable to the presence of one or several *N*-glycans present in the minor glycoprotein

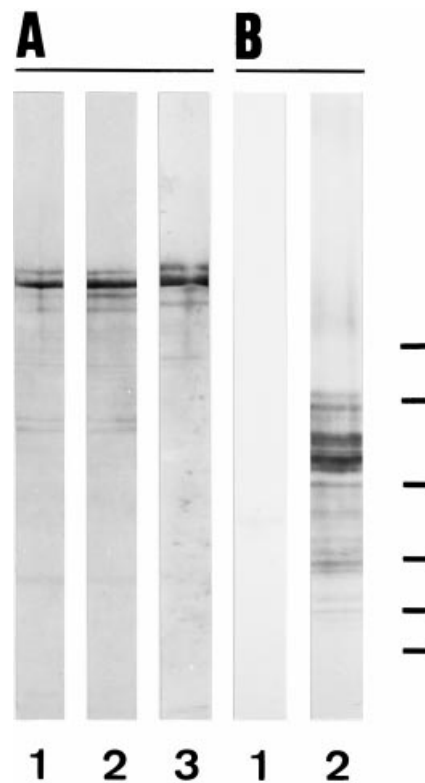


Fig. 7. Immunoblots of *A. simplex* UA3R with mAb UA3 (lanes A) or of *T. spiralis* P4 with mAb US4 (lanes B), after treatment of the antigen with *N*-glycanase F (lanes 1), treatment of the antigen as for lanes 1 but without enzyme (lanes 2), or without any treatment of the antigen (lane 3; migration control). In all cases protein separation was on 5–20% polyacrylamide gels under reducing conditions. Relative molecular masses of standard markers (97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa from top to bottom) are shown to the right

bands that copurify with the major UA3R glycoproteins (see Fig. 7). It is expected that such reactivity would be completely eliminated if the UA3R antigen were produced without glycosylation using recombinant technology.

Determination of specific anti-*Anisakis* IgM antibodies may be of interest in primary infections in which the patient has clinical symptoms and the larvae cannot be visualized by fibroscopy or other means. However, confirmation of the diagnostic value of UA3R in this context is problematic, since it cannot be confirmed that a given patient has a primary or secondary infection with *Anisakis*. Such problems are aggravated if, as seems likely, primary infections may occur without appreciable clinical manifestations [21].

In a previous study of Japanese patients with confirmed gastric anisakiasis [5], we observed that only one patient had exclusively IgM anti-*Anisakis* antibodies and no IgG antibodies, and even in this case the patient showed anti-*Anisakis*

IgE (as detected by CAP). The diagnostic value of IgM determinations thus needs to be further investigated.

Another interesting aspect of our results is related to the sensitivity of the different proteins to beta-elimination with a mild alkali. It has been reported recently that mild alkali treatment removes O-glycans from glycoproteins without significantly altering the protein core [7]. The results presented here clearly show that the effect of this treatment is broadly dependent on protein structure. The protein core of the UA3R antigen, unlike that of other *Anisakis* antigens, is relatively resistant to NaOH treatment. This means that NaOH treatment of this antigen, to assess its utility for diagnosis of anisakiasis and allergy, can be performed without significant cost.

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