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Original Article

Heat stability of *Anisakis simplex* larvae antigens

The aim of the present work was to investigate the heat stability of the various allergens of the parasite *Anisakis simplex* so as to eventually detect patients that might tolerate without risk sufficiently cooked fish. *Patients:* Six patients with a diagnosis of urticaria or anaphylaxis due to sensitisation to the parasite *Anisakis simplex* were selected for this study. *Methods and Results:* The study was carried out with a commercial extract and with a further one prepared in our own laboratory. Aliquots of both extracts were heated to 40°C and 60°C for ten minutes, and a further three aliquots were boiled for 5, 10 and 30 minutes, respectively. Skin tests were performed with all the aliquots on all the patients, with positive results in all cases. The skin reactivity of the extracts did not change with the heat treatment. The affinity of the extracts for the serum IgE (assessed by ELISA) was statistically lower only for those aliquots processed at 100°C. The quantitation of specific IgG under the same conditions disclosed increased affinity for the samples heated to 40°C and 60°C, with no loss of binding for the boiled extracts. The SDS-PAGE and immunoblot studies revealed a great variability in the recognition of the antigenic bands by the sera of the various patients. Generally, the antigens of medium or high molecular weight were shown to be more sensitive to heat treatment, while those with molecular weight below 20 kDa were more resistant. The behaviour of these small molecules varied among the patients. It is possible that the various antigenic determinants of each of these molecules may have varying responses to heat. *Conclusions:* The *in vitro* tests reveal the ability of a number of *Anisakis simplex* allergens to resist heat, although there is some variability between the patients. The advice to be given to patients will probably have to be individualised, but this aspect is not yet definitively settled.

Key words: *Anisakis simplex*. Antigens. Heat stability.

Termoestabilidad de los antígenos de la larva de *Anisakis simplex*

El objetivo de este trabajo fue explorar la sensibilidad al calor de los diferentes alérgenos del parásito *Anisakis simplex* para eventualmente detectar pacientes que toleren sin riesgo los pescados suficientemente cocinados. *Pacientes:* Seleccionamos seis pacientes diagnosticados de urticaria o anafilaxia por sensibilización al parásito *Anisakis simplex*. *Métodos y Resultados:* Realizamos el estudio con un extracto comercial y otro extracto elaborado en nuestro laboratorio. Se procesaron alícuotas a 40°C y 60°C durante diez minutos, y otras tres alícuotas se hirvieron durante 5, 10 y 30 minutos, respectivamente. Se realizaron pruebas cutáneas a todos los pacientes, que fueron positivas con todos los extractos procesados. La re-

actividad cutánea de los extractos no varió con el tratamiento por calor. La afinidad del extracto por la IgE sérica (ELISA) sólo fue estadísticamente inferior a temperaturas de 100°C. La determinación de IgG específica en las mismas condiciones reveló un aumento de la afinidad al calentar el extracto a 40 y 60°C, sin pérdida de unión al hervir el extracto. Los estudios de SDS-PAGE e inmunodetección detectaron una gran variabilidad en el reconocimiento de las bandas antigénicas por cada enfermo. En general, los antígenos de peso molecular medio o elevado se mostraron más sensibles al calor, siendo más resistentes aquellos de PM inferior a 20 kDa. El comportamiento de estas moléculas pequeñas varió entre los enfermos. Es posible que los diferentes determinantes alergénicos de cada una de estas moléculas tengan diferente respuesta al calor. **Conclusiones:** Los ensayos *in vitro* demuestran la capacidad de varios alérgenos de *Anisakis simplex* para resistir al calor, aunque existe una variabilidad entre los enfermos. El consejo a los pacientes probablemente tendrá que ser en el futuro individual, pero este aspecto todavía no está definido.

Palabras clave: *Anisakis simplex*. Antígenos. Termoestabilidad.

INTRODUCTION

Awareness of the existence of allergic sensitisation to the parasite *Anisakis simplex* (AS) has brought with it a number of problems that require solution. One of these problems concerns the diagnosis of the condition. There are still no sufficiently precise tests for selecting that group of patients who are at risk of developing an allergic reaction when they come into contact with the parasite. A considerable number of patients are being detected who have positive skin tests and/or specific IgE antibodies to the parasite and who have never had any form of allergic complaint related to the ingestion of fish or of any other possible vector or source of contamination with AS. The possible risk these patients have of developing clinically manifest sensitisation in the future is unknown.

Lack of knowledge of the characteristics of the sensitising AS antigens as to their source (structural antigens of the parasite or antigens secreted by it), potency, lability or stability in contact with enzymes, heat, acid environments, etc., generates another considerable problem leading to a lack of medical consensus at the time of providing advice to the patients regarding their future alimen-

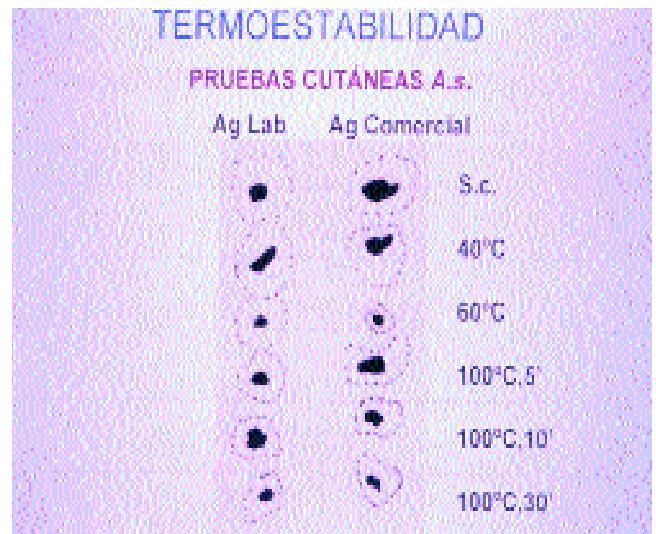


Fig. 1. Skin tests performed with the commercially acquired *Anisakis simplex* antigen (I.P.I. Laboratories) and with the antigen prepared in our own laboratory, after heat pre-treatment at various temperatures.

tary and cooking habits once they have been diagnosed. Many patients who have experienced a clinical picture highly suggestive of AS sensitisation have later tolerated the ingestion of deep-frozen or even fresh fish over months or years. This has led to thinking that the presence of the live parasite in the stomach is required for triggering the reaction. However, some authors have reported about 10% recurrences over a period of several years in patients who had been advised to freeze the fish (thus killing the parasite) prior to consumption. On the other hand, there have been reports of asthma caused by inhala-



Fig. 2. Mean wheal area (mm²) of the skin tests in the six patients with *Anisakis simplex* extract processed at different temperatures.

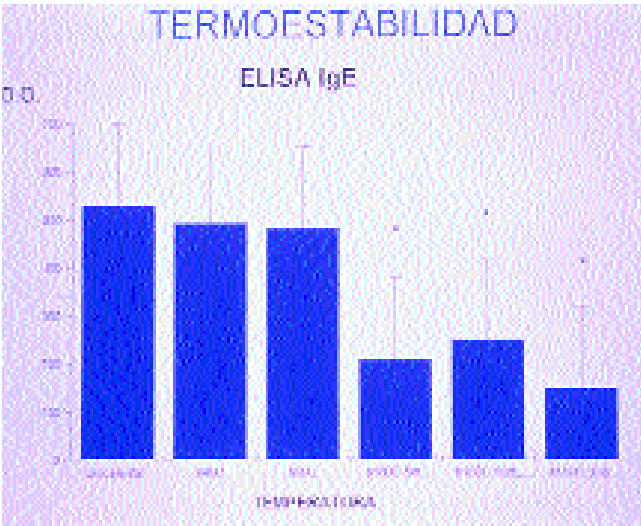


Fig. 3. Mean optical density (at 492 μ m) detected after IgE ELISA with AS extracts treated at different temperatures and the sera of the six patients.

tion of AS antigens, with demonstration by inhalational challenge. It would seem that a perhaps not overly large number of patients may develop allergic sensitisation to antigens present in the dead parasite or that had been secreted into the environment by the parasite larvae.

The safest –and most restrictive– solution in order to prevent the risk of anaphylaxis, particularly in those patients who have already experienced one severe episode and in whom exaggeratedly high specific serum IgE levels are detected, would be to forbid contact with any possible source of contact with the parasite altogether. However, in adopting this attitude a number of further very significant problems

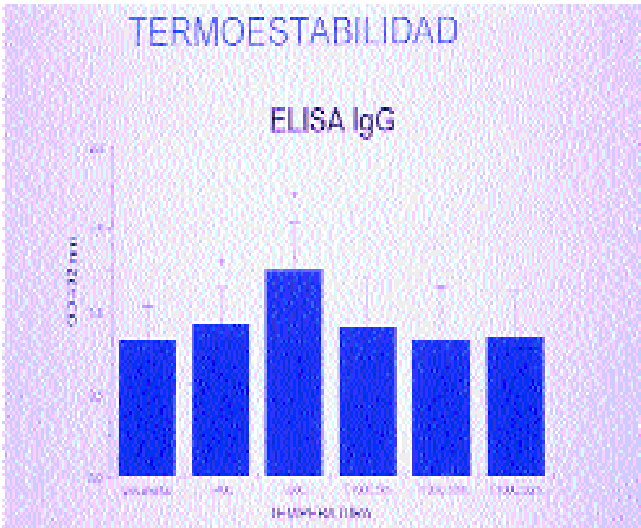


Fig. 4. Mean optical density (at 492 μ m) detected after IgG ELISA with AS extracts treated at different temperatures and the sera of the six patients.

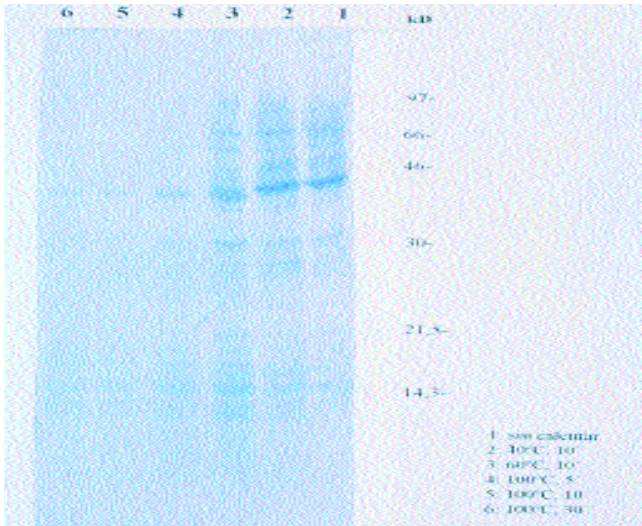


Fig. 5. . SDS-PAGE (14%) of the various extracts treated at different temperatures. Amido black development stain.

are created, either through the inconveniencies caused to the family by definitively and drastically modifying the diet of the patient or through the lipid intake and metabolism levels that might be induced particularly in at-risk patients.

The parasite antigens detected in the transference and immunodetection studies with the sera of various patients are multiple, and the response patterns elicited in the patients themselves are quite varied. Not all patients generate IgE antibodies directed at the same molecules, and the characteristics of each of the allergenic molecules eliciting antibody responses may be different. The identification and characterisation of these allergens will beyond doubt represent a help in solving the present diagnostic and therapeutic problems.

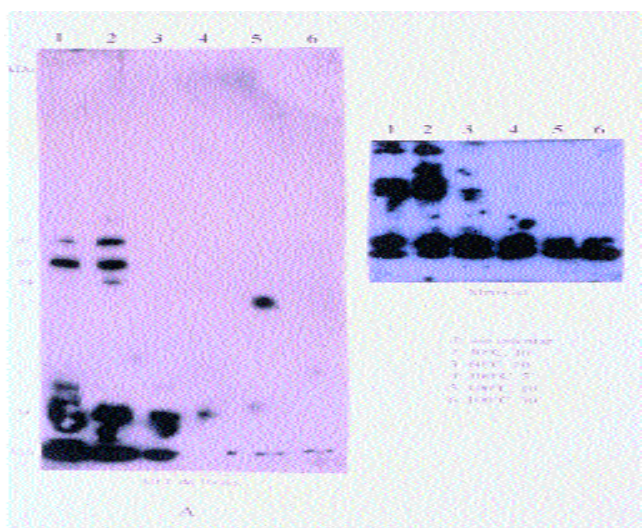


Fig. 6. IgE immunoblot with heat-processed AS antigen on a 16-cm SDS-PAGE (left) and a minigel (right), with 5 and 15 min exposure time, respectively, with the serum of Patient 1.

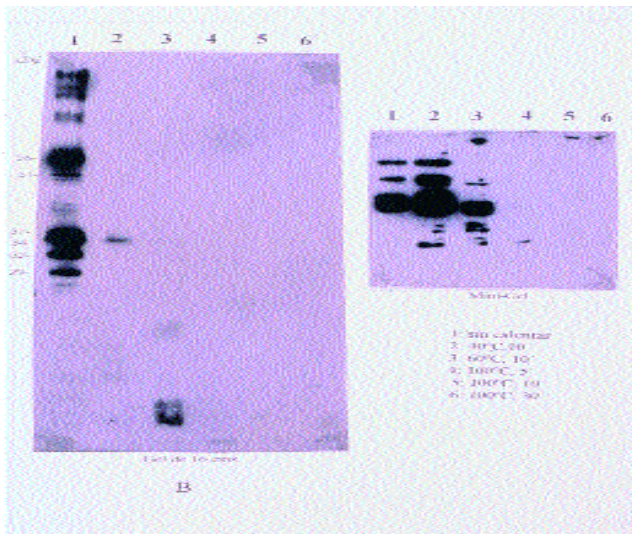


Fig. 7. IgE immunoblot under the same conditions as in Fig. 6, with the serum of Patient 2.

Heat treatment of the fish to be consumed may alter the antigenic determinants of the parasite proteins, which would cease to be allergenic, and this might enable certain patients to consume fish that has been cooked at certain prescribed temperatures.

The aim of the present study was to try to identify those AS antigens that are heat-sensitive, and thus to be eventually able to detect patients who might tolerate without risk fish that have been sufficiently cooked.

The studies on heat-stability of AS antigens performed by Dr. M. García and co-workers have revealed persistent positiveness of the skin tests with AS antigens despite previous heating, and a differential loss of IgE affinity

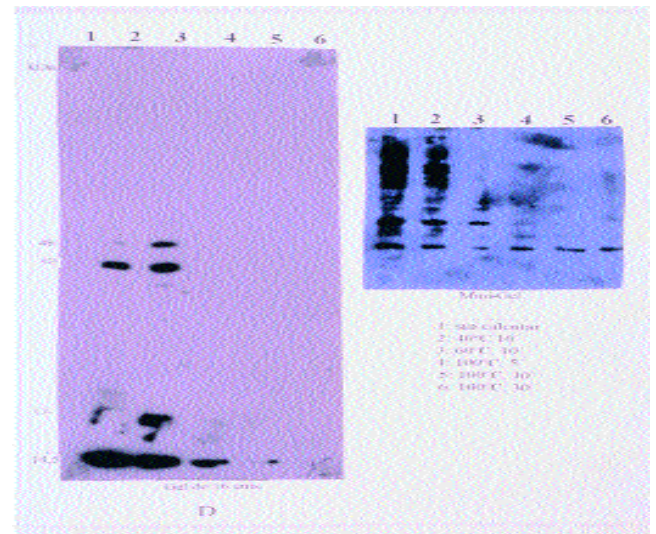


Fig. 9. IgE immunoblot under the same conditions as in Fig. 6, with the serum of Patient 4.

in the antigens of higher as compared to lower molecular weight. The latter would cause the overall group of parasite antigens to be heat-stable.

We designed the present study in order to investigate the varying behaviour of a number of patients in relation to each allergen.

PATIENTS

Six non-atopic patients aged 35 to 70 years were selected for the study. Two of them had evidenced anaphy-

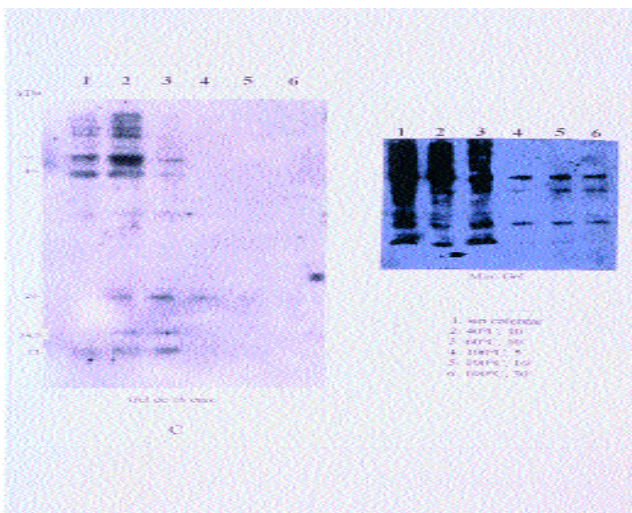


Fig. 8. IgE immunoblot under the same conditions as in Fig. 6, with the serum of Patient 3.

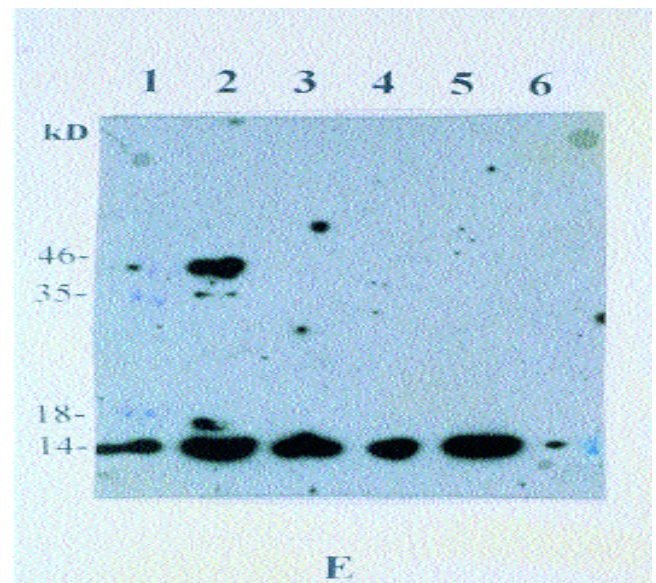


Fig. 10. IgE immunoblot (minigel) with the serum of Patient 5.

laxis and three acute urticaria due to *Anisakis simplex* sensitisation, and the sixth, a female, had had an episode of gastric anisakiasis associated to facial erythema and pruritus. All of them had positive skin tests to the parasite, as well as specific serum IgE levels in excess of 45 kU/l. The skin tests and specific IgE quantitation to the presumably involved fish had in all six cases been negative.

METHODS AND RESULTS

Anisakis simplex antigens

The study was carried out with two different extracts, one of them commercially acquired from I.P.I. Laboratories (1 mg/ml) and the other one prepared in our own laboratory according to the procedure described by Desowitz. The larvae for the latter one were isolated from fresh hake (*Merluccius merluccius*), and the final protein concentration was 0.8 mg/ml.

Aliquots of both extracts were processed for ten minutes at 40°C and 60°C, and a further three aliquots of each were boiled for 5, 10 and 30 minutes, respectively.

The extracts thus processed were used for performing skin tests, for specific IgE and IgG quantitation (ELISA) and to study the varying response to each of the antigens in relation to the applied heat treatment in each of the patients by SDS PAGE transfer and immunodetection with a chemoluminescent substrate.

Skin tests

Skin tests were performed with the two types of extract after pre-treatment at different temperatures (Fig. 1). The skin tests were positive with both extracts and, although there were slight variations in each of the patients, the mean areas of the wheals elicited with the various extracts pre-treated at different temperatures were similar, without any significant difference (Fig. 2).

Specific IgE and IgG quantitation (ELISA)

The affinity of the extracts for the serum IgE of the patients did not change upon pre-treatment at 40 or 60°C, but it was significantly reduced by boiling already after five minutes (Fig. 3).

The determination of specific IgG under the same conditions disclosed, in three independent experiments,



Fig. 11. Close-up detail of the low molecular weight bands in the IgE immunoblot study (14% on 16 cm-gel) with the serum of Patient 5.

an increased affinity for the extract after heating to 40°C and 60°C, without loss of this affinity upon boiling (Fig. 4).

PROTEIN AND ANTIGEN PATTERN (SDS-PAGE, TRANSFER AND IMMUNODETECTION)

Staining of the separated proteins after SDS-PAGE of the heat-processed extracts disclosed a progressive loss of all protein bands, which was perhaps more evident in those of intermediate or high molecular weights (Fig. 5). The assessment of the individual affinity of these bands to the serum IgE from each patient showed remarkably variable results.

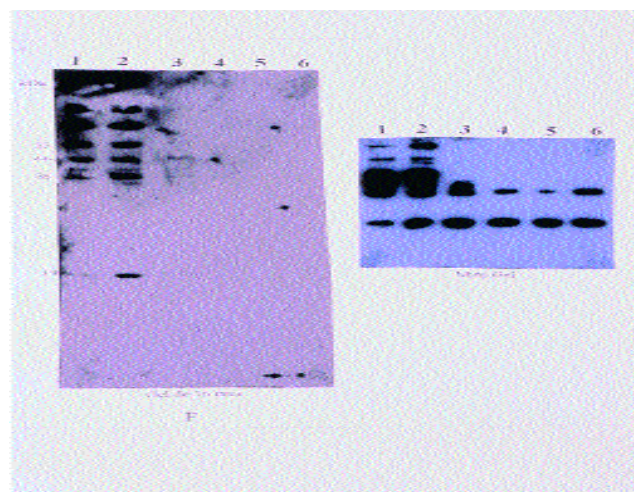


Fig. 12. IgE immunoblot under the same conditions as in Fig. 6, with the serum of Patient 6.

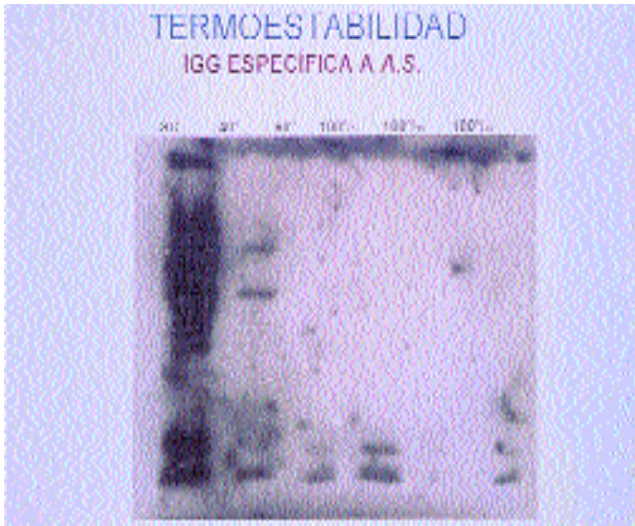


Fig. 13. IgG immunoblot (minigel) with heat-processed AS antigen and the serum of one of the patients.

Patient 1. This was one of the cases of acute urticaria. In this study, the patient's serum recognised antigens of intermediate molecular weight (37, 32 and 29 kDa) and two further bands of 14 and 10.5 kDa MW. The intermediate molecular weight bands begin to fade at 60°C and disappear upon boiling. The low MW bands also seem to be heat-sensitive, and perhaps more so the 14 kDa one than the 10.5 kDa one. However, if the exposure time is forced (Fig. 6) these bands are seen not to disappear even after boiling the extract for 30 minutes. At this exposure level other high-MW bands become visible that are also heat-sensitive.

Patient 2. This was a further case of urticaria, after the ingestion of vinegar-marinated anchovies (Fig. 7). In this particular case, the patient's serum recognises intermediate MW antigens (37, 34, 32 and 29 kDa), further antigens with MW close to 50 kDa, and even those antigens of higher MW that evidence heat-sensitivity already upon heating to 40°C. Forcing of the exposure time caused these bands to persist even after heating to 60°C, while further bands in the 20 kDa MW range became visible.

Patient 3. This was the one case of gastric anisakiasis, in which the infestation occurred after the ingestion of vinegar-marinated anchovies (Fig. 8). The antigens recognised by this patient's serum are grouped in the 40 kDa, 50 kDa and higher MW range, with weaker recognition of 20 kDa and 14 and 12 kDa antigens. All these bands appeared to be sensitive to heat treatment at any of the temperatures, but two intermediate-weight bands and the 20 kDa one resisted even 30 minutes' boiling.

Patient 4. This patient had evidenced urticaria/angioedema after the ingestion of john dory (Fig. 9). The intermediate MW antigenic bands (42 and 48 kDa) disappear upon heating to 60°C. The lower MW band (17 kDa) disappeared upon heating to 100°C, while the 14 kDa one withstood boiling.

Patient 5. The diagnosis in this case was anaphylactic shock after eating fried whiting (Fig. 10). This patient's serum recognised intermediate MW bands (42 and 38 kDa), which were sensitive to heating to 60°C, and low MW bands (10 and 14 kDa), which were heat-resistant (Fig. 11).

Patient 6. In this last patient, the diagnosis had been anaphylaxis after the ingestion of anchovies (Fig. 12). His specific IgE recognised bands in the high and intermediate MW ranges and also an 18 kDa one. The latter two groups appeared to be resistant to boiling.

Only one example of the antigen bands recognising specific IgG is shown (Fig. 13). This patient's serum recognises molecules of varying molecular weights that appear to be sensitive to the temperatures achieved upon boiling. As in the previous cases (IgE affinity), the affinity of the lower MW molecules for the specific IgG appears to be more heat-resistant than that of the higher MW ones. A further possibility would be that, as the temperature increases, some large molecules become fractionated without losing their immunogenicity.

Summarising the results of the immunoblot studies, the protein bands with intermediate molecular weights (30–60 kDa) appear to be rather heat-sensitive, even though the determinants recognised by some patients' sera (Patient 3 and Patient 6) seem to withstand boiling.

The smaller molecular weight allergens appear to be rather more variable in their recognition by the various patients' sera. It is possible that several different allergens with similar molecular weights exist or, alternatively, that some molecules bear more than one antigenic determinant with different characteristics, causing the protein to evidence varying behaviour upon heat treatment. This might explain the varying behaviour of the sensitivity of the patients' sera to proteins of the same molecular weight upon heat treatment, such as the 10.5 kDa band, which appears to be heat-sensitive with the serum from Patient 1 and heat-resistant with that of Patient 5. In the case of the 14 kDa band, the determinant is heat-sensitive with the sera from Patients 1 and 3, and heat-resistant with those from Patients 4 and 5.

TOLERANCE TO FISH

At the time of writing, Patients 1, 2 and 6 tolerate the ingestion of deep-frozen fish; Patients 3 (gastric anisakiasis) and 5 tolerate fresh fish, and Patient 4 does not eat fish at all. All these patients are currently asymptomatic. They all are patients who had had severe episodes in relation to fish ingestion, and it is quite possible that they may be implementing measures that preclude their again encountering the parasite: eating less fish, cooking the fish to a greater degree, consuming preferently the tail, examining the fish more carefully prior to cooking, etc. For these reasons, it cannot be stated with certainty whether these patients at present tolerate the antigen, or whether they are having the good luck not to have contact with it. In all of them, the specific serum IgE levels have evidenced a reduction over time (months). The challenge tests with the lyophilised parasite do not at present seem to represent the gold standard for the diagnosis of these patients, and thus it cannot be stated with certainty whether *in vitro* data such as ours may or not be safely extrapolated to the clinical situation.

CONCLUSION

1.- The *Anisakis simplex* antigens do not lose their ability to bind to mast-cell IgE upon boiling; for this reason, the skin tests continue to be positive.

2.- The parasite's antigens show reduced ability to bind to the specific serum IgE after boiling, but the affinity is not fully abolished.

3.- The antigenic determinants recognised by specific IgG are heat-resistant, although the molecules may not be complete and intact.

4.- The intermediate- or high-MW protein bands appear to be more sensitive to heat treatment than those of low MW, but the antigenic heat stability varies among the patients possibly due to the recognition of different antigenic determinants within the same molecule.

5.- It is at present unknown how these *in vitro* data may be reflected in the clinical situation. Dietary advice to the patients may in the future have to be individualised, but this aspect is not yet fully defined.