

# Characteristics of hypersensitivity reactions and identification of a unique 49 kd IgE-binding protein (Hal-m-1) in abalone (*Haliotis midae*)

Andreas L. Lopata, MSc, Caryn Zinn, BSc(Hons), and Paul C. Potter, MD, FCP(SA)

Cape Town, South Africa

**Background:** There is a paucity of published data on the clinical presentation and the nature of the allergens involved in hypersensitivity to mollusks. This study reports the clinical and immunologic findings in 38 patients with reported immediate and delayed adverse reactions to abalone (*Haliotis midae*, Class Gastropoda).

**Methods:** Patients were recruited as part of a South African seafood allergy survey. Allergic symptoms were assessed by a self-administered questionnaire. A total of 38 patients with abalone sensitivity were recruited. Specific IgE responses to abalone and other mollusks were studied by using RAST and inhibition ELISAs. Skin prick tests and lymphocyte proliferation assays were also performed on several of the subjects. Allergenic components of *Haliotis midae* were identified with Western blotting.

**Results:** Twenty five of the 38 patients in the study were first seen with immediate symptoms, and 13 had delayed reactions. Seventeen of the sera tested were RAST positive. Skin prick tests responses with abalone extract were positive in all subjects with positive RAST responses ( $n = 8$ ) and in 6 of 13 subjects with negative RAST responses. Five of the subjects with positive RAST responses had positive results on Western blotting and demonstrated binding to two major allergens with molecular weights of 38 and 49 kd. The 49 kd IgE-binding protein has been designated as Hal-m-1.

**Conclusions:** Abalone allergens are heat-stable proteins with molecular weights of 38 and 49 kd, later designated as Hal-m-1 according to International Union of Immunological Societies allergen nomenclature regulation. Our studies indicate a clear clinical and immunologic heterogeneity in patients reactive to abalone. (J Allergy Clin Immunol 1997;100:642-8.)

**Key words:** Food allergy, IgE, mollusks, abalone, shellfish hypersensitivity, lymphocytes

Shellfish elicit severe allergic reactions in sensitized individuals.<sup>1,2</sup> Invertebrate species that are known to elicit adverse food reactions belong mainly to two phyla, the Arthropods and Mollusks.<sup>3-5</sup> This grouping is generally referred to as "shellfish." There are over 80,000 living

## Abbreviations used

PBMCs:	Peripheral blood mononuclear cells
PBs:	Phosphate-buffered saline
SPTs:	Skin prick tests

species of mollusks, which are second only to arthropods in the number and diversity of living species. Of the mollusks, oysters, clams, scallops, and mussels have gained widespread culinary acceptance. In Africa and Asia, other types of mollusks in addition to these are frequently eaten including snails, limpets, squid, and abalone.<sup>6-9</sup> In a recent South African survey, we identified 38 patients who reported hypersensitivity to abalone.<sup>10</sup>

Because little is known about the clinical features of abalone hypersensitivity and the allergens involved, we have investigated these patients and their immune responses using a modified RAST, skin prick tests (SPTs), Western blotting, and lymphocyte proliferation assays. Allergenic components of abalone extract were defined by immunoblotting.

## METHODS

### Patients

This study forms part of a large survey of 108 individuals with perceived hypersensitivity to seafood in the Western Cape, South Africa.<sup>10</sup> The questionnaire sought details of exposure to or contact with different mollusks, crustaceans, and fish species; their clinical presentation (12 different symptoms to be selected); other atopic disorders (e.g., hay fever, asthma, eczema); and family history of allergies. The subjects had to describe, in detail, a typical attack (i.e., time between food ingestion and symptom onset, how and where the food was prepared). Thirty-eight patients who specifically reported symptoms after ingestion of abalone were recruited on the basis of this survey for our in-depth study.

A comparative group consisted of 10 subjects from the survey who reported sensitivity to fish (*Teleostei*) but not to mollusks or crustaceans. The members of the comparative group were recruited as control subjects for the in vitro and in vivo tests.

### Ethical approval

Ethical approval for the study was obtained from the Ethics and Research Committee of the University of Cape Town.

### Preparation of extracts

Abalone (*Haliotis midae*), snail (*Helix aspersa*), black mussel (*Mytilus galloprovincialis*), white mussel (*Donax serra*), oyster (*Crassostrea gigas*), crawfish (*Jasus lalandii*), sea urchin (*Pare-*

From the University of Cape Town and Groote Schuur Hospital, Cape Town.

Supported by the Abalone Packers and Marketers Association, the Medical Research Council, and the Allergy Society of South Africa-UCB.

Received for publication Nov. 5, 1996; revised June 5, 1997; accepted for publication June 6, 1997.

Reprint requests: Andreas Lopata, MSc, Department of Clinical Science and Immunology, Allergology Unit, University of Cape Town, Medical School (Groote Schuur), Observatory 7925, South Africa.

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0091-6749/97 \$5.00 + 0 1/1/84003

*chinus angulosus*), and squid (*Loligo vulgaris*) used in this study were caught on the South African Coast and fresh frozen before preparation of the extracts. Edible portions were removed, cut into small pieces, and tumbled overnight at 4° C in phosphate-buffered saline (PBS). After centrifugation at 1000g for 15 minutes, the extracts were sterilized by sterile filtration (0.45 µm; Millipore), and the protein content was determined by using a dye-binding method (BCA-protein assay, Pierce) and stored in aliquots (100 µl) at -20° C until further use. For SPTs the extracts were diluted in 50% glycerol (vol/vol) and stored at -20° C. The extracts were retested after 6 months by sodium dodecylsulfate-polyacrylamide gel electrophoresis and demonstrated no degradation whatsoever.

### SPTs

SPTs were performed with informed consent on the forearms of 24 of 38 subjects by using five in-house extracts (abalone, oyster, black mussel, white mussel, and squid) diluted in 50% glycerol (vol/vol) and one commercial blue mussel (*Mytilus edulis*) extract (Soluprick, ALK Laboratories, Horsholm, Denmark). SPTs were not performed on the 10 subjects whose sera displayed abalone RAST results exceeding 5% binding in view of a possible risk of inducing a generalized reaction. Four subjects did not come to the clinic for the skin test because of the long distance to travel. Histamine dihydrochloride (10 mg/ml) was used as a positive control, and a diluent of glycerol and saline solution was used as a negative control. Subjects were instructed not to take any antihistamines for 48 hours before the SPTs. The SPTs were read after 15 minutes and were considered to be positive if the diameter of the wheal was at least 3 mm greater than that produced by the negative control. Blood pressure, pulse, and peak expiratory flow rate were monitored before and after the SPTs.

### Specific IgE RAST

RASTs were performed with the Pharmacia CAP System radioimmunoassay (Pharmacia, Uppsala, Sweden) for blue mussel, oyster, squid, and snail. These RAST results were regarded as positive when binding values exceed 0.35 ku/L, representing an increase in the concentration of allergen-specific antibodies. By definition, 1 U of IgE equals 2.4 ng.

Abalone-specific IgE levels were determined with duplicate serum samples and in-house RASTs. CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) were coupled with raw and cooked abalone extracts containing 4 mg/ml and 2 mg/ml of protein, respectively. Sepharose 4B was activated according to the manufacturer's instructions, apart from the inactivation step, which was performed with glycine rather than with ethanolamine. A volume of 100 µl of serum was incubated with 100 µl of the allergen-coupled Sepharose suspension in duplicate, while shaking, at room temperature overnight. The Sepharose was then washed three times with saline solution containing 0.05% Tween-20. Twenty microliters of iodine 125-labeled anti-IgE (Pharmacia) containing approximately 20,000 cpm were added and incubated overnight. Excess <sup>125</sup>I-labeled anti-IgE was eliminated by washing and centrifuging, as described above, and bound radioactivity was counted with a Beckman gamma counter. Results were expressed as the percent bound of total counts per minute added. A positive reaction was regarded as percentage of binding exceeding by three times the binding obtained with the negative control.

### IgE inhibition ELISAs

Immunochemical analyses of various shellfish extracts were performed with an IgE inhibition ELISA by using raw abalone protein extract as the solid-phase antigen (Polysorp plates, Nunc). Dilutions of patient sera (1:5) were preincubated with tenfold serial dilutions of the various extracts from 0.5 mg/ml to 5 ng/ml. IgE binding was detected by using an in-house monoclonal mouse anti-human IgE antibody and biotinylated rabbit anti-mouse antibody (DAKO).

### Western blotting

Immunoblots were prepared by electrophoresis of the allergen extracts on 11% sodium dodecylsulfate-polyacrylamide gel electrophoresis.<sup>11</sup> After electrophoresis, proteins were electroblotted onto polyvinylidene difluoride membrane (Hybond-PVDF, Amersham). The allergen-bound membranes were then cut into 5 mm strips, blocked for 30 minutes in 1% blocking reagent (BM Chemiluminescence assay, Boehringer Mannheim), washed twice in PBS-Tween, and incubated overnight with 200 µl of serum sample in 3 ml of PBS. The strips were then washed twice with PBS-Tween for 10 minutes each and incubated for 2 hours with a mouse anti-human IgE antibody (1:3000 in PBS). After washing twice, the strips were incubated for 30 minutes in biotinylated rabbit anti-mouse anti-serum (1:5000), washed, and finally incubated again in streptavidin-peroxidase (1:10,000) for another 30 minutes. After an overnight wash, the strips were developed by using a chemiluminescence detection system (Boehringer Mannheim) and exposed to Kodak x-ray film. Fresh raw, cooked, and commercially dried abalone extracts were studied in the Western blots.

### In vitro proliferation assay of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood and cultured at 10<sup>5</sup> cells/well for 6 days with 100 µg/ml protein extract of raw and cooked abalone. The cells were pulsed with 0.5 µCi of tritiated thymidine per well 18.5 hours before harvesting on filter paper and counting for 1 minute in a Beta-counter (Beckman, LS 3800). The results are expressed as stimulation index.

$$SI = \frac{\text{Stimulation induced, disintegrations per minute}}{\text{Control, dpm}}$$

## RESULTS

### Study group and symptoms

The ages of the 38 subjects participating in this study ranged from 17 years to 74 years with a mean age of 44 years. Twenty-four subjects (63%) were female and 24 (63%) subjects gave positive histories of atopic diseases. Fourteen (38%) had a family history of allergy to other foods.

The comparative group consisted of 10 subjects who reported sensitivity to fish only. These subjects had a mean age of 36 years, six were female, and eight had atopic diseases. As part of the questionnaire, the subjects were asked to describe, in detail, the symptoms they experienced during an adverse reaction. Symptoms were divided into four categories: cutaneous, gastrointestinal, respiratory, and others. Respiratory reactions were more frequent in subjects who also had other atopic diseases (58% vs 21%). Although most of the subjects (66%) reported symptoms within 2 hours of food ingestion, a

**TABLE I.** RAST reactivity to different mollusk species

	In-house RAST (%-bound)*	Commercial RAST (ku/L)	<i>n</i> = 38
Abalone			
<10%	4.8 ± 1.1	n/a	10
>10%	24.3 ± 9.9	n/a	7
Snail	n/a	2.4 ± 2.1	10
Oyster	n/a	7.7 ± 4.5	4
Blue mussel	n/a	2.7 ± 1.6	5
Squid	n/a	2.1 ± 2.0	5

\*Percent of total counts added. Values given ± standard deviation.  
n/a, Not available.

significant number of subjects (13; 34%) only reacted between 2 and 7 hours. In both groups the majority of the subjects (76% and 69%, respectively) experienced symptoms associated with type I hypersensitivity (i.e., cutaneous and respiratory symptoms), whereas the remaining subjects were first seen with gastrointestinal symptoms.

### Specific IgE assessment

The RAST reactivity to five different mollusks in abalone-sensitive subjects is demonstrated in Table I. Elevated RAST percent-binding to abalone was detected in 45% of all sera tested (17 of 38). None of the 10 comparative subjects who were hypersensitive to fish displayed specific IgE binding to any of the five mollusks in Table I. For the comparative subjects the mean RAST value was 0.6% (SD ± 0.3%, *n* = 10). Eight of 17 patients with positive RAST responses to abalone also had elevated IgE to snail. The RAST ratios, compared and analyzed by linear regression, showed a positive correlation ( $r = 0.82$ ,  $p < 0.01$ ).

### SPTs

The reactivity of SPTs to in-house mollusk extracts is presented in Table II. Seventeen subjects had positive RAST responses, of which eight had positive SPT responses. All of these subjects had high IgE binding in abalone RAST tests. The mean abalone RAST value for subjects with positive SPT responses was 3.7% (SD ± 3.7%) and the mean abalone RAST value for subjects with negative SPT responses was 0.5% (SD ± 0.5%). These results show that individuals with negative SPT responses had little or no reactivity to the abalone RASTs in general. However, SPTs were more sensitive. Six patients who had negative responses to RAST tests, had positive responses to SPTs. Therefore a negative RAST response does not rule out an immunologic sensitivity to abalone.

In the comparative group, 1 of 10 subjects had a positive response to oyster, squid, black mussel, blue mussel, and white mussel (Table II). No positive reaction was obtained with the abalone extract.

**TABLE II.** Positive SPT reactivity to different mollusk extracts

Extract	Abalone-sensitive ( <i>n</i> = 24)	Comparative subjects ( <i>n</i> = 10)
Abalone	14	0
Oyster	9	1
Black mussel	10	1
Blue mussel	6	1
White mussel	5	1
Squid	7	1

### Inhibition ELISA

To assess specificity of the IgE binding, an inhibition ELISA was performed on two subjects by using seven different extracts. These two subjects were representatives of the group of eight subjects who had positive RAST responses to abalone and to snail, whereas the remaining subjects with positive RAST responses to abalone had negative responses to snail. Inhibition data for one of these subjects is presented in Fig. 1. Comparison of the dose of an antigen extract required to inhibit 50% of the abalone ELISA IgE binding, provides an index of the antigenic relationship. The most potent inhibitors are, as expected, raw and cooked abalone at a concentration of less than 1 µg/ml, followed by snail at a concentration of about 9 µg/ml. The required concentration for crawfish and black mussel is much higher (about 25 µg/ml). However, sea urchin and house dust mite inhibit by only 10% with a concentration 500 times higher (500 µg/ml).

Some of the sera with a high percent binding in the abalone RAST did not recognize antigens bound to the ELISA plate or the PVDF membrane (immunoblot), possibly as a result of changes in the conformational epitopes of these proteins on the solid phase.

### In vitro proliferation assay of PBMCs

To compare the immunologic response of lymphocytes of subjects with positive responses to RAST with those of subjects with negative responses to RAST and a convincing history of hypersensitivity to abalone, we exposed PBMCs to extracts of abalone. The PBMCs of subject "Si" show a significant proliferative response to raw and cooked abalone extract, as do those of subject "Vi" who had a negative RAST response to abalone (Fig. 2). The two extracts induced no significant response in the control subjects.

### Western blots

Protein extracts of abalone and other mollusks were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis according to their molecular weights, and the IgE-binding components were analyzed by immunoprinting with different sera. The blot in Fig. 3 demonstrates the IgE-binding pattern among six different mollusk species in one patient's serum. Two proteins with molecular weights of 49 and 38 kd were consistently

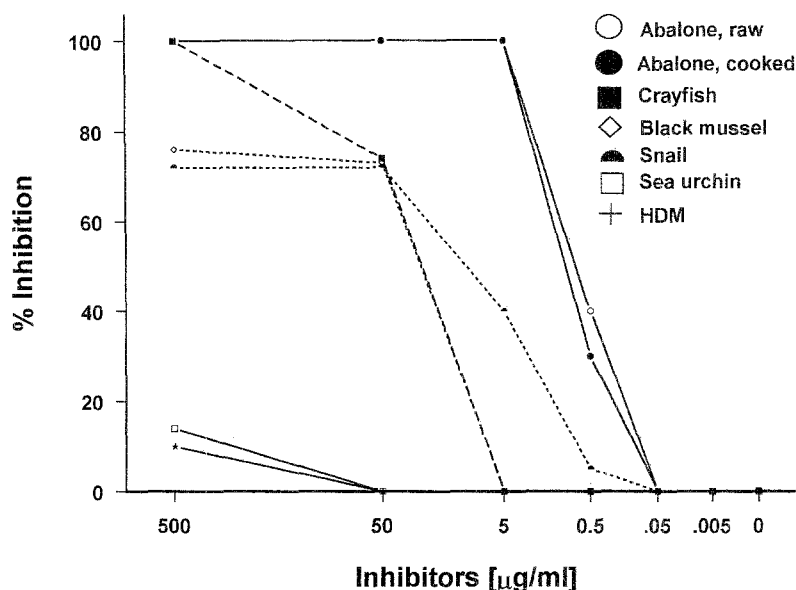


FIG. 1. Allergenic activity of different extracts as compared by inhibition ELISA. Increasing concentrations of abalone (raw and cooked), snail, crayfish, black mussel, sea urchin, and house dust mite extracts were tested for their ability to inhibit the abalone RAST. HDM, House dust mite.

identified and show the main allergens that were identified by five sera that demonstrated specific IgE binding on the Western blots (Fig. 4). In addition, other allergens were found in the immunoblotting experiments with molecular weights above 50 kd.

The identified proteins are heat-resistant and exceptionally stable as immunoblots with raw, cooked, and dried abalone confirm (Fig. 5). We have designated the 49 kd protein Hal-m-1. Specific IgE binding to the 49 kd protein was identified on Western blots of the South African abalone (*Haliotis midae*) and the Australian abalone (*Haliotis rubra*).

## DISCUSSION

Although adverse reactions after ingestion of abalone have been reported,<sup>7</sup> specific reactivity of IgE antibodies from sensitized patients with specific abalone allergens and related mollusks has not been investigated. Our results indicate that hypersensitivity to abalone can be confirmed by an in-house RAST in about half of our patients (17 of 38; 45%). Sensitivity of testing was improved by using an additional SPT in subjects with negative responses to RASTs, which identified an additional six patients with abalone hypersensitivity (23 of 38; 61%). When the subjects were divided into a clinically atopic group and a clinically nonatopic group according to their detailed questionnaire and their symptoms were analyzed, the atopic subjects reported significantly more respiratory symptoms (58% vs 21%).

The association of respiratory symptoms with positive SPT or RAST responses to abalone is high compared with subjects with negative results (92% vs 60%). A previous study on subjects who had allergic symptoms

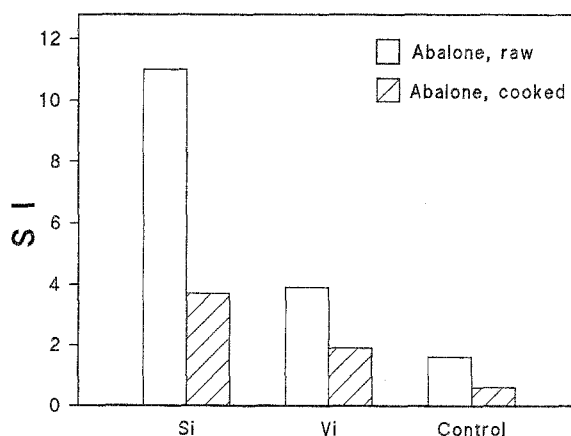


FIG. 2. Proliferative response of PBMCs of subjects Si and Vi to fresh and cooked abalone extract. Subject Si had a strong positive reaction to the abalone RAST, whereas subject Vi had a negative response. The control subject demonstrated no significant response. Results are given as ratios of stimulation index (SI): allergen-induced proliferation, dpm per spontaneous proliferation of control, dpm.

after ingestion of snails (*Helix terrestris*) reported that 8 of 10 patients had pulmonary symptoms.<sup>9</sup> Another study of snail-sensitive subjects reported that only 15% experienced respiratory symptoms and none had gastrointestinal problems.<sup>12</sup> Interestingly, in the latter study, a different snail species (*Euparipha pisana*) provoked the food hypersensitivity.

In a study of crustacean allergy, Morgan et al.<sup>13</sup> reported a similar prevalence of skin test positivity with more respiratory reactions to shrimp in atopic subjects (85.7% vs 54.5%). These different symptom profiles

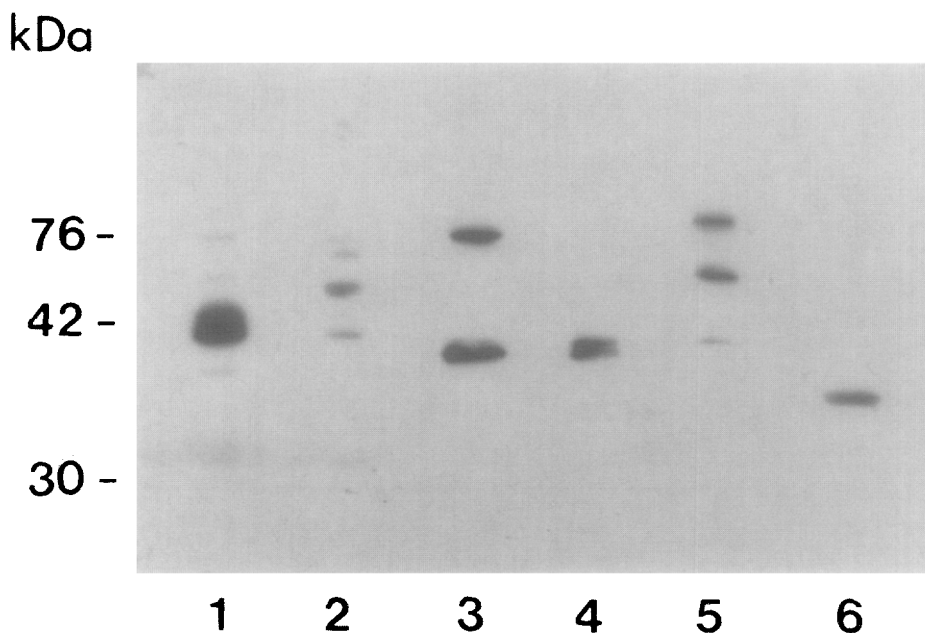


FIG. 3. Western blots showing IgE antibody binding by an abalone-sensitive serum to different mollusk extracts: abalone (1), snail (2), white mussel (3), black mussel (4), oyster (5), and squid (6). Molecular weight markers are indicated in kilodaltons on the left.

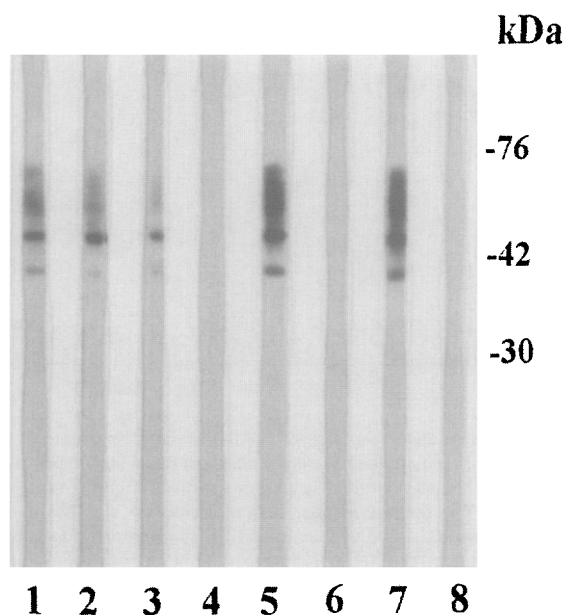


FIG. 4. Immunoblotting of sera from abalone-sensitive subjects against extract from abalone. Lanes 1 through 7 represent seven abalone-sensitive sera. Lane 8 represents a negative control of normal sera. Molecular weights are indicated in kilodaltons.

demonstrate that even within the shellfish groups and among the same mollusk classes, in this case gastropods, symptoms can be highly variable.

In this study the majority of subjects with positive RAST and SPT responses were atopic. Similar findings were reported in a study on crustacean-sensitive pa-

tients.<sup>5</sup> None of our subjects with negative SPT responses had elevated RAST values. These results demonstrate that the SPTs and RASTs correlate well with a history of abalone sensitivity and that a higher prevalence of positive reactions is found in clinically atopic subjects who are first seen with respiratory symptoms.

An uninvestigated aspect of abalone sensitivity is the relatively frequent occurrence of delayed onset of symptoms (up to 7 hours) reported in 13 (34%) of our subjects. Type I sensitivity could only be confirmed in 38% of these subjects by positive SPT responses, positive RAST responses, or both compared with 71% of the subjects with immediate reactions. Interestingly, more than 69% (9 of 13) of the subjects who experienced delayed reactions demonstrated predominantly respiratory and cutaneous symptoms. Delayed reactions have been reported with other mollusks but not with crustaceans.<sup>7, 14</sup> Delayed type I symptoms are a frequent and characteristic feature of mollusk hypersensitivity and may account for the lack of apparent sensitivity of SPTs and RASTs in these patients.

Testing the abalone-sensitive subjects for sensitivity to four different mollusk species by using commercial RASTs reflected both the specificity and coreactivity of our abalone-sensitive subjects. The closest related mollusk species available commercially is the snail (*Helix aspersa*), which is also a gastropod. Abalone sensitivity could have been detected in only half of the subjects with positive abalone RAST responses (10 of 17) by using the snail RAST only. Two of the subjects with negative snail RAST responses had weakly positive responses to squid only, whereas the other six subjects had negative responses to all mollusks tested except abalone. A signif-

ificant number of abalone-sensitive subjects (15 of 38; 39%) had neither SPT nor RAST reactivity in spite of a convincing type I reaction on history. Mechanisms of their food hypersensitivity are thus not clear. The lack of RAST reactivity in some subjects with histories of abalone sensitivity may be partially related to the amount of time that had elapsed since they had last eaten abalone. Most of the subjects with positive RAST responses (62%) had avoided contact with abalone for between 2 and 10 years but still demonstrated significant skin and RAST reactivity. Another important consideration for lack of skin reactivity was the dose of extract necessary to elicit a response. We found that for seven subjects, a protein concentration of abalone extract as low as 100  $\mu\text{g/ml}$  generated a wheal of about 50% of the size induced by the extract used in our study (3000  $\mu\text{g/ml}$ ; data not shown). Other reasons for the lack of immunologic responses in some of the patients included the fact that some individuals may have falsely indicated abalone as offending seafood or may have reacted to other allergens or irritants ingested with abalone such as food additives<sup>1</sup> or algae toxins that may elicit adverse reactions by nonimmunologic mechanisms.<sup>15</sup>

We have demonstrated that for certain subjects with negative RAST and SPT responses and a convincing history of allergy to abalone the lymphocytes display strong proliferative responses toward abalone antigens (Fig. 2). Further studies need to be done to assess whether proliferative assays are a reliable alternative to confirm food hypersensitivity in subjects with negative RAST responses who report delayed onset of symptoms after exposure to mollusks.

Quantification of shellfish-specific IgE antibodies illustrates a different approach to the evaluation of shared allergenic determinants among different species in general.<sup>16,17</sup> The abalone inhibition ELISAs illustrated a high degree of specificity of the patients' IgE antibodies towards abalone antigens. The snail extract demonstrates, as the correlation of abalone RAST with snail RAST suggests, a high degree of cross-reactivity compared with crawfish and black mussel. Homologous and heterologous crossreactivity has been described between shrimp and oyster allergens<sup>16</sup> but also between limpet, crustacean, and mussels.<sup>6</sup> We found no significant cross-reactivity between abalone and the house dust mite (*Dermatophagoides pteronyssinus*), as has been previously suggested for limpet and house dust mite.

The serum IgE reactivities of abalone-sensitive subjects to different mollusk species on Western blots showed similar but not identical binding patterns. Some of the dominant allergens of the abalone extract may also be present in snails, and this will need to be studied further with absorption experiments. It is expected that closely related seafood species may share similar allergenic epitopes, and this could account for a degree of coallergenicity. This is particularly likely for the 38 kd protein because other mollusks also demonstrated binding to this protein (Fig. 3). The remarkable thermal stability of abalone allergens was demonstrated by the

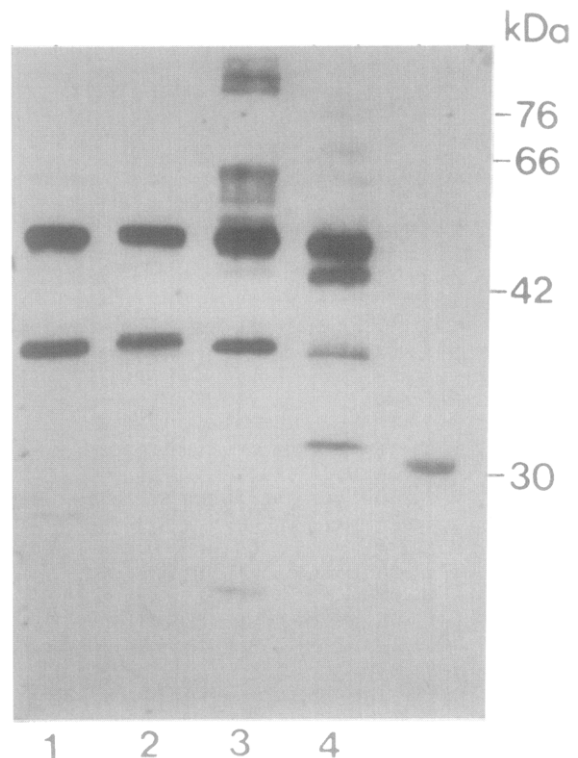


FIG. 5. Western blot of IgE antibody reactivity to four different abalone extracts: abalone *Haliotis midae* (1), abalone *Haliotis rubra* (2), cooked *H. midae* (3), and dried *H. midae* (4). Molecular weight markers are displayed on the right.

significant binding of IgE to the Western blots (Fig. 5) after boiling or drying. This stability is comparable with a similar property in the shrimp<sup>18</sup> and snail<sup>12</sup> allergens. The latter study on snails identified a major allergen with a molecular weight of about 24 kd, which is different from the allergens identified in this study.

Major allergens identified in studies of different shrimp species have a molecular weight of 36 to 38 kd. The shrimp allergen has been identified as tropomyosin, a muscle protein that functions as an actin-binding protein in muscle and nonmuscle tissues and is highly conserved during the process of evolution.<sup>19,20</sup> It is possible that the 38 kd protein in abalone could also be a tropomyosin from muscle tissue. Further studies with purified muscle proteins and monoclonal antibodies currently in progress will address this question. We have designated the unique 49 kd IgE-binding protein as Hal-m-1 because it was recognized by sera from five patients on Western blots. Further studies of the biochemical and immunologic characterization of the identified abalone allergens with monoclonal antibodies are in progress and will provide further information about the degrees of coallergenicity between the different mollusk species. Furthermore, the isolated abalone allergens will help to develop better diagnostic tests and treatment for abalone-sensitive patients.

We thank Mr. Alistair Oaksholt of ALK (Denmark) for skin prick testing solutions and Mrs Jacqui Higgins for typing the final manuscript.

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