

Short communication

Severe IgE-mediated anaphylaxis following consumption of fried frog legs: definition of α -parvalbumin as the allergen in cause

Background: IgE-mediated allergic reactions to bullfrog and edible frog have been reported. The implicated allergens have not been defined so far. The frog material and the patient's serum from a case of severe food-induced anaphylaxis were used to define the implicated allergen at the protein and DNA level.

Methods: Immunoblotting techniques and *N*-terminal protein microsequencing were used to define the allergen recognized by the patient's serum. Back translation from the identified protein sequence was used to design degenerated primers to amplify the allergen's cDNA by polymerase chain reaction (PCR). We defined the nucleotide sequence of the allergen from the frog of Indonesian origin that was consumed by the patient, and the homologous cDNA from *Rana esculenta*.

Results: Protein microsequencing revealed that the implicated frog allergen belonged to the parvalbumin family. cDNAs coding for α - and β -parvalbumin of *R. esculenta* and *Rana species* were cloned. Recombinant proteins were expressed in *Escherichia coli*. The patient's serum IgE antibodies recognized parvalbumin prepared from frog muscle and recombinant α -parvalbumin from *R. species* but not from *R. esculenta*. Recombinant β -parvalbumin was not recognized by the IgE antibodies.

Conclusion: This work defines at the protein and DNA levels α -parvalbumin as the allergen implicated in a case of IgE-mediated anaphylaxis to frog muscle. It also shows that a protein belonging to the parvalbumin family is implicated in type I allergies outside the fish species.

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Key words: allergens; angiotensin-converting enzyme inhibitor; beta-blocker; cDNA; food allergy; frog; parvalbumin; *Rana esculenta*.

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Accepted for publication 13 May 2002

IgE-mediated allergic reactions (1) to frogs or frog material have been the topic of a number of case reports. The symptoms and situations in which these allergic reactions occur fall into two categories: airborne and contact-mediated reactions provoked by catching or handling frogs, and food-induced anaphylactic reactions following consumption of frog meat. Asthma, rhinitis, conjunctivitis, and skin reactions have been described in a laboratory technician (2, 3), a professional frog-catcher (4), and a female frog-skinner (5). A cook presented eczematous reactions after preparing frog legs; when he tasted some he developed constriction and severe itching of the throat, and difficulty in swallowing (6). Recently a severe anaphylactic reaction was reported in a 6-year-old child after eating boiled frogs (7).

The exact nature of the antigen remains elusive. Nevertheless in the cases of occupational allergic reactions, the allergen was shown to be present in the skin (2, 5), in venom expelled from skin glands (4), or in brain extract (3). In one case of occupational asthma the

patient's serum recognized skin extract proteins of 19 and 55 kDa (5). However the subclass of the reacting antibodies was not defined. In the reports on IgE-mediated food allergic reactions the allergen was present in frog muscle (6, 7). We report the case of a near fatal IgE-mediated anaphylactic reaction in an adult that developed within minutes of starting a meal of fried frog legs. IgE immunoblotting, *N*-terminal protein sequencing, cDNA isolation and expression of the corresponding recombinant proteins allowed us to pin down the responsible allergen.

Material and methods

A couple of minutes after eating several fried frog legs, a 47-year-old man felt itching and discomfort in his throat. He rapidly developed breathing problems, with edema of the face and tongue, and a few minutes later he fainted. In the emergency ambulance he was intubated, ventilated, and given epinephrine (adrenaline). On admission to the intensive care unit the patient was "semicomatous", with a blood pressure of 60/40 mmHg, and heart frequency 130 beats/min. He had angioedema, with edema of the tongue, and generalized urticaria. During the next 24 h, despite treatment, it was

Parvalbumin cDNA sequences have been deposited in EMBL under accession numbers AJ 315960, AJ 315959, AJ 414731, AJ 414730.

difficult to maintain normal blood pressure. The patient had been treated regularly with sotalol 160 mg and perindopril 4 mg once daily for hypertension. The electrocardiogram showed ischemia in lateral areas associated with a significant rise in heart muscle enzymes. He developed signs of intravascular coagulopathy.

With treatment, the patient recovered within 48 h from all his complications. Coronography showed no sign of stenosis. He was transferred to the Immunology and Allergy unit for further work-up. Clinical history revealed that he had previously experienced mild seasonal conjunctivitis. Several times during the last year he had eaten frog legs of Indonesian origin, purchased in a supermarket. On the previous occasion of eating fried frog legs he had experienced slight oral itching. Skin prick tests (SPT) performed with an extensive series of food and respiratory allergens (Allerbio, Neuilly sur-Seine, France) showed a weak positive reaction to grass pollen, birch pollen and hazelnuts. SPT with the fluid left in the bag that wrapped the skinned frog legs was strongly positive, causing a wheal diameter of 10 mm. Total IgE titer was 80 kU/l; specific-IgE determination by ImmunoCAP method (Pharmacia-Upjohn Uppsala, Sweden) was 1.1 kU/l for meadow grass, 0.8 kU/l for birch, and was below 0.35 kU/l for hazelnut, peanut, pepper, garlic, cod, tuna, and several other food allergens.

Patient serum and frog material

Serum samples were obtained at days 1, 2 and 4 after anaphylaxis and frozen in aliquots at -80°C until testing. The serum of a grass-pollen allergic patient was used as a negative control. Frog legs originating from the same batch as the ones consumed by the patient were kept frozen. Frog legs from *R. esculenta* were obtained from an animal breeding center, immediately frozen on dry ice, and stored at -80°C .

Preparation of protein extracts

Frog skeletal muscle was disrupted in single detergent lysis buffer (50 mM TrisHCl pH 8, 150 mM NaCl, 1% Triton X-100, 100 $\mu\text{g}/\text{ml}$ Phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) by a rotor-stator homogenizer (Kinematica; Lucerne, Switzerland). The extract was centrifuged for 10 min at 5300 g to remove insoluble particles and the protein concentration of the supernatant was determined by Bio-Rad protein assay (Biorad, Nazareth, Belgium).

Protein sequencing

Frog muscle extract (370 μg) was separated by a 18% preparative SDS-PAGE gel and blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). A piece of membrane containing the band of interest was isolated. The protein was subjected to automated N-terminal sequencing. Microsequencing was performed by Edman degradation using an Applied Biosystems 473 A sequencer (Paris, France).

Purification of parvalbumin by SDS-PAGE

Frog muscle extract was centrifuged for 1 h at 50 000 g at 4°C and concentrated with an Ultrafree concentrator (Millipore, Bedford, MA), and 40 mg of extract were separated by a 18% PAGE in a MiniPrep Cell (Bio-Rad). Eluted proteins were analyzed by SDS-PAGE followed by silver staining (Amersham Pharmacia, Uppsala, Sweden). Fractions were pooled and concentrated by centrifugation as above.

SDS-PAGE and immunoblot

Proteins were separated by 18% SDS-PAGE gels under denaturing conditions and visualized by Coomassie Brilliant Blue colloidal staining. For immunoblotting, proteins were separated by 18% SDS-PAGE and transferred onto PVDF membranes. To reduce nonspecific binding, blotted membranes were incubated in blocking buffer (PBS/Tween 20 0.05%, 2% cold water fish gelatine (Sigma, St. Louis, MO). The PVDF membrane was cut into strips which were incubated over night with patients' sera diluted 1/10 in blocking buffer. Control strips were incubated with buffer alone or with serum from a grass-pollen allergic patient. Bound IgE were detected with antihuman IgE antibody labeled with alkaline phosphatase (Sigma) and the blots were developed by addition of nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Promega, Madison, WI). A mouse mAb directed against frog skeletal muscle parvalbumin (clone Parv-19, Sigma) was used for detection of parvalbumin.

Primer design for RACE

Back translations from the α - and β -parvalbumin protein sequences of *R. esculenta* (PRVA ranes and PRVB ranes, edible frog) were used to design degenerated 5' and 3' primers for polymerase chain reaction (PCR). Parv-24 α (TTC AAC CAC AAG AAR TTY TTY GAR) and Parv-96 α (AAT YTT NCC RTC NCC RTC YTT RTC) are located at amino acid positions 24 and 96. Parv-25 β (TTT AAC TAY AAR ATH TTY TTY CAR AAR) and Parv-96 β (GCC ATC TCC RTC NSW RTC NCC NGC YTT) are located at amino acid positions 25 and 96.

RNA extraction, cDNA synthesis and RACE

Frog skeletal muscle was disrupted in lysis buffer by a rotor-stator homogenizer and RNA was extracted according to the RNeasy kit procedure (Qiagen, Hilden, Germany). cDNA synthesis and PCR were performed using the SMART RACE cDNA amplification kit (Rapid Amplification of cDNA Ends by SMART technology; Clontech, Palo Alto, CA) according to the manufacturer's instructions. PowerScript reverse transcriptase (Clontech) was used for reverse transcription of total RNA.

α -Parvalbumin

PCR was performed with sense (Parv-24 α) and antisense (UPM, Universal Primer Mix) primers for 3'RACE and sense (UPM) and antisense (Parv-96 α) primers for 5' RACE. In a first step these RACE reactions failed and only an internal cDNA fragment delimited by the primers Parv-24 α and -96 α could be amplified. Sequencing of the cloned fragment provided enough sequence data to design new nondegenerated primers for the RACE protocol.

β -Parvalbumin

PCR was performed with sense (Parv-25 β) and antisense (UPM) primers for 3'RACE and sense (UPM) and antisense (Parv-96 β) primers for 5'RACE. The 3'RACE failed, but in the 5'RACE a 200-base pair fragment was amplified. The fragment was cloned and sequenced and the sequences obtained were used to design new nondegenerated primers for the RACE protocol.

Expression of recombinant α - and β -parvalbumin in *Escherichia coli*

After 5' and 3' RACE products had been characterized by sequencing, new primers were designed to amplify the whole protein coding region of the genes coding for α - and β -parvalbumin. The cDNAs were inserted into the pQE40 expression vector (Qiagen, Hilden, Germany) digested with *Bam*HI/*Sal*I. Recombinant parvalbumin was expressed in *E. coli* M15 with an *N*-terminal 6-histidine tail. The recombinant protein was purified by Nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Hilden, Germany) under denaturing conditions according to the manufacturer's protocol.

Sequence analysis and database searches

Database homology searches, multiple sequence alignments, and sequence analyses were done with HUSAR (Heidelberg Unix Sequence Analysis Resources, DKFZ, Heidelberg, Germany).

Results

Allergen characterization by immunoblot and protein sequencing

Frozen frog legs provided by the patient were used for protein extraction. Proteins were separated by SDS-PAGE under denaturing conditions. The patient's serum IgE antibodies recognized protein bands of about 10 kDa in the frog muscle extract (data not shown).

The predominant band in this region was prepared for protein sequencing. The *N*-terminal sequence was PMTDVLAAGDISKAXXAFXAXEXFNH. The peptide sequence was used for searching the protein database SwissProt with the program BLAST. Two sequences with high sequence identity were retrieved: α -parvalbumin of *R. esculenta* (PRVA ranes) with an homology of 76%, and parvalbumin α of *R. catesbiana* (PRVA ranca), homology 72%.

Mouse monoclonal antiparvalbumin antibody and patient IgE antibodies recognized a protein purified from frog muscle extract.

The predominant low molecular weight protein in frog muscle extract was purified by preparative SDS-PAGE. Figure 1 shows the silver stained SDS-gel with the frog muscle extract and two eluted protein fractions (A), and the corresponding immunoblots incubated with the mouse antiparvalbumin antibody and the patient's serum (B). The mouse monoclonal antibody (mAb) identifies the purified protein as parvalbumin. As the IgE antibodies from the patient recognize the same protein fraction, it seems highly probable that the allergen is parvalbumin.

Cloning of the cDNA coding for α -parvalbumin

Total RNA from frozen frog muscle provided by the patient was used to synthesise and amplify the parvalbumin cDNA using a RACE protocol. Degenerated sense and antisense primers were designed based on the back translation of the parvalbumin protein sequence of *R. esculenta*. For primer localization, two regions were chosen with high sequence homology between the two species *R. esculenta* and *R. cates.* and with less degenerated codons. The amplification of the cDNA ends failed, but the gene-specific primers could amplify an internal cDNA fragment of 220 bp. This DNA fragment was cloned and the DNA sequence determined. The sequence information was used to design new nondegenerated gene specific primers. cDNA synthesis, and amplification with these new primers was done using the RACE method. A parallel cDNA cloning was done with RNA extracted from *R. esculenta*.

The cDNA was cloned and the nucleotide sequences determined for several clones. The two sequences have an identity of 91.8% on the nucleotide sequence level and

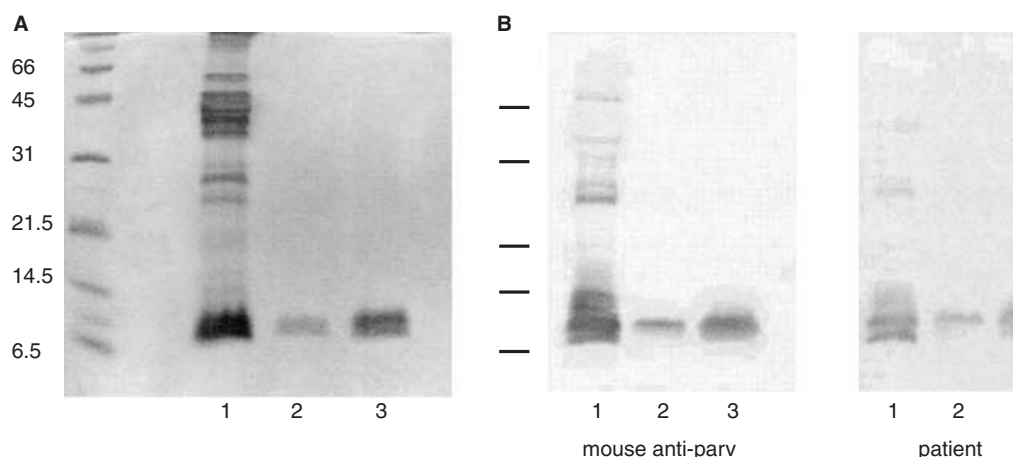


Figure 1. Purification of frog parvalbumin (A) by preparative electrophoresis monitored by SDS-PAGE and silver-staining. (B) Immunoblot analysis of frog muscle extract and fractions of purified parvalbumin. Proteins blotted onto polyvinylidene difluoride (PVDF) membranes were exposed to a mouse antiparvalbumin mAb (mouse antiparv) and to the patient's serum for detection of IgE binding. 1, frog muscle extract (*Rana species*); 2 and 3, protein fractions eluted from preparative electrophoresis.

82.7% on the amino-acid sequence level. The calculated molecular weights of the two proteins are 12.002 and 11.93 kDa; the calculated isoelectric point is 4.69 for both. At amino-acid positions 10 (C) and 16 (M) the deduced cDNA sequence differs from the amino-acid sequence found by protein sequencing. This probably reflects the fact that the quantity of the isolated protein was too small to allow an unequivocal interpretation of the sequencing data.

Cloning of the cDNA coding for β -parvalbumin

Most muscles contain parvalbumin of either α - and β -lineage, although in frog skeletal muscle both α and β parvalbumins have been found (8). To determine if the patient's IgE were directed against α - and β -parvalbumin or both, we cloned the corresponding, β -parvalbumin gene both from *R. species* and *R. esculenta* and expressed the corresponding protein in *E. coli*. The sequence identity is 94% on the nucleotide sequence level and 88.1% on the amino-acid sequence level. The calculated molecular weight and isoelectric point is 11.88 kDa and 4.51 for *R. species* and 11.71 kDa and 4.53 for *R. esculenta*.

Expression of recombinant parvalbumin in *E. coli*

The cDNAs coding for α - and β -parvalbumin from both frog species were inserted into an expression vector and

recombinant proteins were produced in *E. coli*. Purified proteins were separated by SDS-PAGE and subjected to immunoblotting with the patient's serum and the mouse antiparvalbumin mAb. The mouse mAb recognized parvalbumin of α - and β -lineage from *R. esculenta* but also from *R. species*. However, IgE antibodies of the patient only recognized α -parvalbumin from *R. species* (Fig. 2), the frog material that had initially provoked the anaphylactic reaction.

Sequence comparison of the deduced α - and β -parvalbumin amino acid sequences

R. sp. Parv- α and *R. sp. Parv- β* isolated from *R. species* represent new parvalbumin sequences. *R. esc. Parv- α* and *R. esc. Parv- β* , the deduced protein sequences of the cDNA clones isolated from *R. esculenta*, are identical to the α - and β -parvalbumin sequences of *R. esculenta* retrieved from the SwissProt database. Protein sequences of the α - and β -parvalbumins of *R. species* and *R. esculenta* were aligned (Fig. 3). The two EF-hand domains that bind Ca^{2+} ions, are conserved regions. α - and β -parvalbumins of both species present many more sequence identities (82.7% and 88.1%) than α - and β -parvalbumins of one species (53.3% for *R. species* and 52.3% for *R. esculenta*). The frog-allergic patient's serum

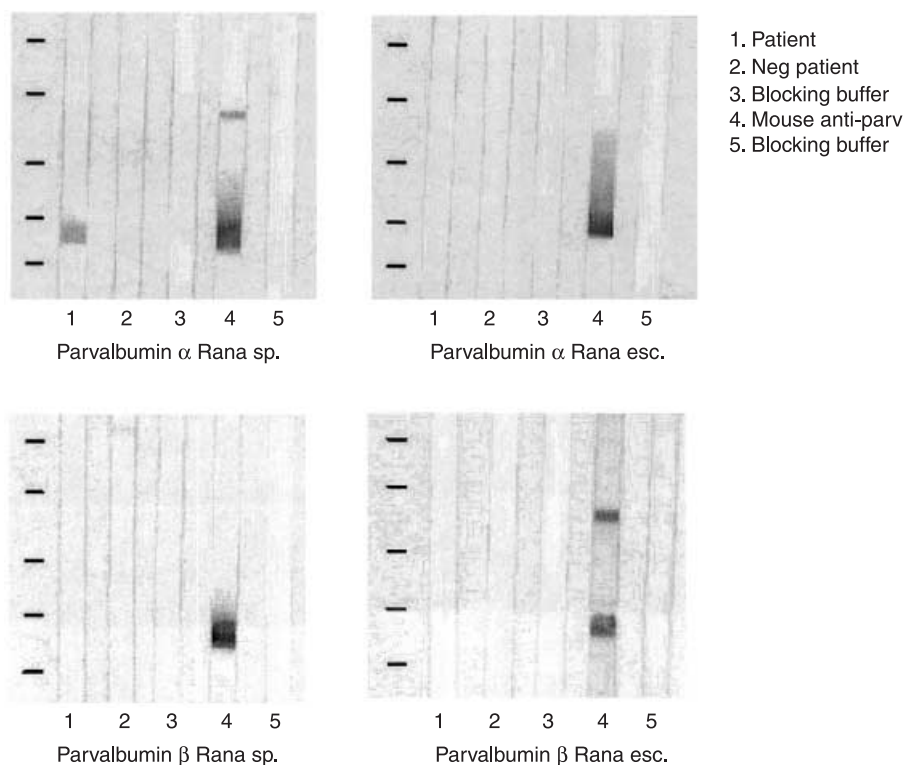


Figure 2. Immunoblot analysis of recombinant α - and β -parvalbumin. Polyvinylidene difluoride (PVDF) membrane strips with recombinant α -parvalbumin from *Rana species* and *Rana esculenta* and β -parvalbumin from both were incubated with different sera. 1, frog-allergic patient; 2, grass-pollen allergic patient; 3, negative control; 4, mouse antiparvalbumin mAb, and 5, corresponding negative control.

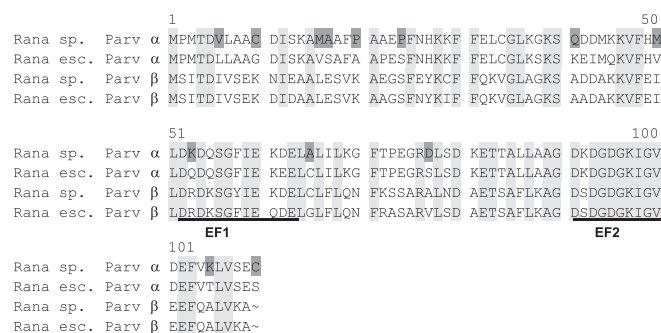


Figure 3. Alignment of the amino acid sequences deduced from the cloned cDNAs coding for α - and β -parvalbumin of *Rana species* and *Rana esculenta* EF-hand motifs (two perpendicularly placed α -helices E and F which form together with an interhelical loop a single Ca^{2+} binding site) are underlined. Amino acid identities are shown in light grey, amino acid positions unique to *R. species* Parv- α are in dark grey. R. sp. Parv- α and R. sp. Parv- β are parvalbumins from *R. species*; R. esc. Parv- α and R. esc. Parv- β are parvalbumins from *R. esculenta*.

reacted specifically to recombinant α -parvalbumin of *R. species* (R. sp. Parv- α). A total of 13 amino acid positions are unique to this protein in comparison to the other 3 recombinant α - and β -parvalbumins, resulting in a limited number of possible continuous as well as discontinuous IgE epitopes.

Discussion

The reported case of anaphylaxis induced by frog meat was especially severe in its intensity and its resistance to treatment. The patient was being treated for hypertension and was taking a α , β -blocker drug as well as an inhibitor of the angiotensin-converting enzyme. Particularly severe anaphylactic reactions and resistance to treatment in patients treated with, β -blockers are well known (9–11). The aggravating effect of angiotensin-converting enzyme inhibitor (ACE inhibitor) treatment on anaphylactic reactions and especially on its edematous manifestations has also been reported (12, 13).

Microsequencing of the purified protein band recognized by the patient's serum IgE antibodies on the immunoblot performed with frog muscle extract, revealed high sequence identity with α -parvalbumin of *R. esculenta* and α -parvalbumin of *R. catesbiana*. This suggested that α -parvalbumin, a well-known frog muscle component, whose protein sequence but not DNA sequence was known, was the culprit allergen. Parvalbumins are Ca^{2+} binding proteins of low molecular weight, initially detected in relatively high amounts in the white muscle of lower vertebrates (14). Parvalbumins were subsequently found, although in lesser amounts, in the fast-twitch skeletal muscles of higher vertebrates (15). Parvalbumins were suggested to be important in fiber relaxation (16).

To obtain confirmatory evidence that α -parvalbumin was the allergen responsible for the patient's anaphylactic reaction, two complementary approaches were taken: one at the protein level, the other at the DNA level. Parvalbumin was isolated from the frozen frog muscle provided by the patient. The nature of the isolated protein was confirmed by a commercial mAb recognizing frog parvalbumin. This antiparvalbumin mAb as well as the patient's serum recognized the same protein in a highly purified protein preparation, confirming that the patient's IgE were indeed directed against parvalbumin.

As parvalbumins comprise α - and β -parvalbumin which have both been shown to coexist in frog muscle (8), further characterization of the allergen was performed at the DNA and protein level. In a multistep procedure cDNAs from α - and β -parvalbumins were cloned from frog muscle of Indonesian origin and from the common edible frog *R. esculenta*. Recombinant molecules of the α - and β -parvalbumins were produced in *E. coli*. All four recombinant parvalbumins were recognized by a commercial mAb directed against frog parvalbumin. The patient's serum IgE antibodies recognized only recombinant α -parvalbumin of the *R. species* from Indonesia that he had been consuming, showing that the patient was specifically sensitized to this protein. There was no reactivity to a muscle extract from *R. esculenta*, confirming the specific sensitization to the consumed frog species (data not shown). α -Parvalbumin from *R. species* and *R. esculenta* have an amino-acid identity of 83%. Extensive homologies between α - and β -parvalbumin are mainly found in the conserved regions of the EF-hand motifs (amino acids 52–63 and 91–102).

Parvalbumins have been shown to represent the major allergen in fish (17). Parvalbumin from fish is a very stable allergen: extremes in pH, temperature, or random folding of the molecule following exposure to dissociating agents do not significantly alter its allergenicity (18, 19). Parvalbumins from different fish species, for instance cod, tuna, salmon have been shown to contain cross-reactive as well as species specific epitopes when tested with the sera of fish allergic patients (20). The patient analyzed in our study presented only antibodies specifically directed at the *R. species* from Indonesia without any cross-reactivity for *R. esculenta*.

Even more, the reactivity was limited to α -parvalbumin, with no cross-reactivity to β -parvalbumin. In the case of a laboratory technician described by Charpin (2), skin tests performed with the skin of *R. esculenta* were positive while tests with *R. temporaria* were negative. Although this narrow specificity might not be the rule, one must be cautious about including the correct frog species in a diagnostic procedure to avoid false-negative results.

The clinical cases reported so far in the literature were caused by *R. esculenta*, but also by *R. catesbiana*. Incriminated antigen sources are skin (2, 5), venom (4),

brain (3), and legs (6, 7). The distribution of parvalbumin in different frog tissues should be evaluated in further studies. In rat, the presence of parvalbumin has been described in a variety of tissues, including endocrine glands, skin and neurons (21–24). It has also to be seen whether other molecules besides parvalbumin are responsible for allergic reactions to frogs.

Allergic reactions to fish are frequent, especially in countries with a developed fish industry, affecting up to 3% of children in Finland (25). Frog meat is a delicacy, consumed by a limited number of people; this might explain the limited reports of allergic reactions to frog meat. Parvalbumins are present in greater amount in fish and frog muscle than in other species (15). It remains to be seen whether parvalbumins of other species are also capable of eliciting allergic reactions.

This study shows that α -parvalbumin from frog tissue, a protein previously not known as an allergen, was

involved in a case of severe IgE-mediated food anaphylaxis. This is the first report that implicates parvalbumins in a case of immediate-type allergy outside the fish species. The α - and β -parvalbumin cDNA sequences of *R. esculenta* and *R. species* from Indonesia have been defined. We found that the corresponding recombinant parvalbumin molecules produced in *E. coli* are recognized by IgE, as well as murine mAbs, showing that the recombinant molecules may be useful diagnostically.

Acknowledgments

We gratefully acknowledge the collaboration of Dr André Steinmetz in protein microsequencing and the assistance of Patrick Schmit with protein purification. This work was supported by grants from the CRP-Santé (97-12) and the Fondation Recherche Cancer et Sang (98-03), in Luxembourg.

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