

Letter to the Editor

A general strategy for the generation of hypoallergenic molecules for the immunotherapy of fish allergy

To the Editor:

Surveys in the United States and Europe show that up to 0.5% of the population suffers from fish allergy,^{1,2} which is a frequent cause of severe and life-threatening anaphylactic reactions. Parvalbumin, a small calcium-binding protein, which is extremely resistant to heat and digestion, represents the major fish allergen. Because of the high degree of sequence homologies and cross-reactivities between parvalbumins from different fish species, consumption of various fish species leads to clinical symptoms in fish-allergic patients.³ Several immunotherapy trials investigating the applicability of recombinant hypoallergenic vaccines are currently ongoing, and the clinical immunotherapy studies for birch pollen in particular indicate that recombinant allergen-based specific immunotherapy is a promising concept for the effective and safe treatment of respiratory allergy.⁴

Specific immunotherapy is less frequently used for the treatment of food allergies because fewer standardized allergen extracts are available and the risk of inducing severe anaphylactic reactions is high. To develop a hypoallergenic vaccine for the subcutaneous treatment of fish allergy, we generated a hypoallergenic derivative of carp parvalbumin, a highly cross-reactive

fish allergen,⁵ by introducing 4 point mutations into the 2 calcium-binding regions of Cyp c 1.⁶

However, there are reports that some patients show a selective reactivity to parvalbumins from only certain fish species.^{7,8} We therefore investigated whether the mutation of exactly the same amino acids as in the Cyp c 1 mutant may be a generally applicable strategy for converting parvalbumins from other fish species to hypoallergenic molecules. For this purpose, parvalbumins from commonly consumed, but distantly related fish species (Sal s 1 beta 1, accession number Q91482.1 from Atlantic salmon; Thu a 1, accession number FM178217.1 from tuna; Sco j 1, accession number P59747 from chub mackerel; Clu h 1, accession number FM178220 from herring; and Gad c 1, accession number AY035584 from codfish) were selected. In the sequences of these allergens, the calcium-binding aspartic acids (D) at the first and third positions of the 2 calcium-binding domains were replaced by alanines (A) (Fig 1). Synthetic genes that had been codon-optimized for expression in *Escherichia coli* coding for the wild-type and mutated fish allergens were synthesized by GenScript (Piscataway, NJ). The synthetic genes were cloned into the *Nde*I and *Eco*RI restriction sites of plasmid pET-27b (Novagen, Madison, Wis), which were then transformed into the *E. coli* strain BL21 (DE3) and expressed by addition of isopropyl- β -D-thiogalactopyranoside in liquid culture. Coomassie-staining of protein gels containing extracts of bacteria expressing

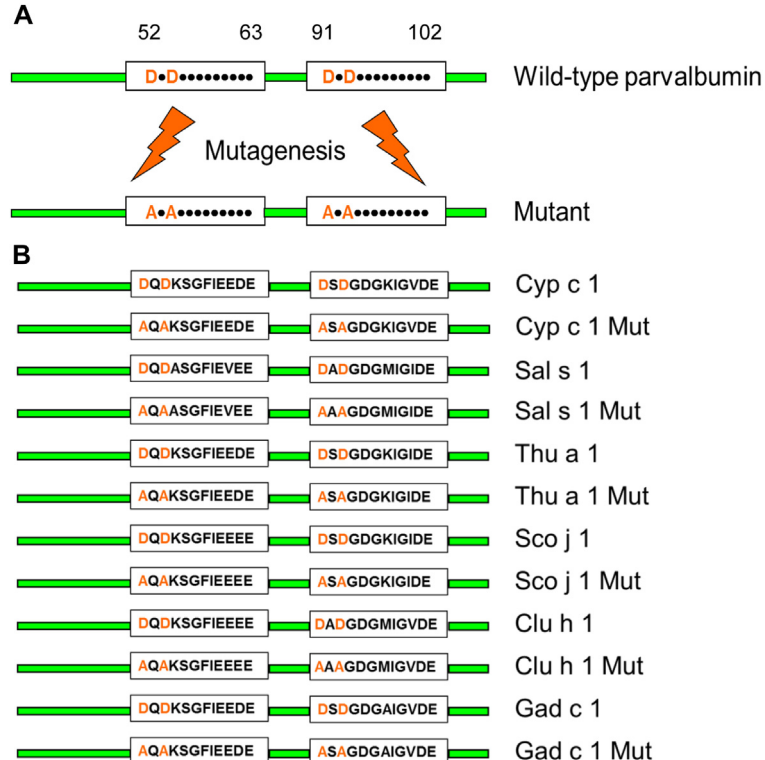


FIG 1. A, Position of the conserved aspartic acids in the 2 calcium-binding regions in fish parvalbumins and their exchange to alanines in the mutant proteins. B, Sequences of the 2 calcium-binding domains of wild-type and mutated parvalbumins Cyp c 1 and Cyp c 1 Mut (carp), Sal s 1 and Sal s 1 Mut (Atlantic salmon), Thu a 1 and Thu a 1 Mut (tuna), Sco j 1 and Sco j 1 Mut (chub mackerel), Clu h 1 and Clu h 1 Mut (herring), and Gad c 1 and Gad c 1 Mut (codfish).

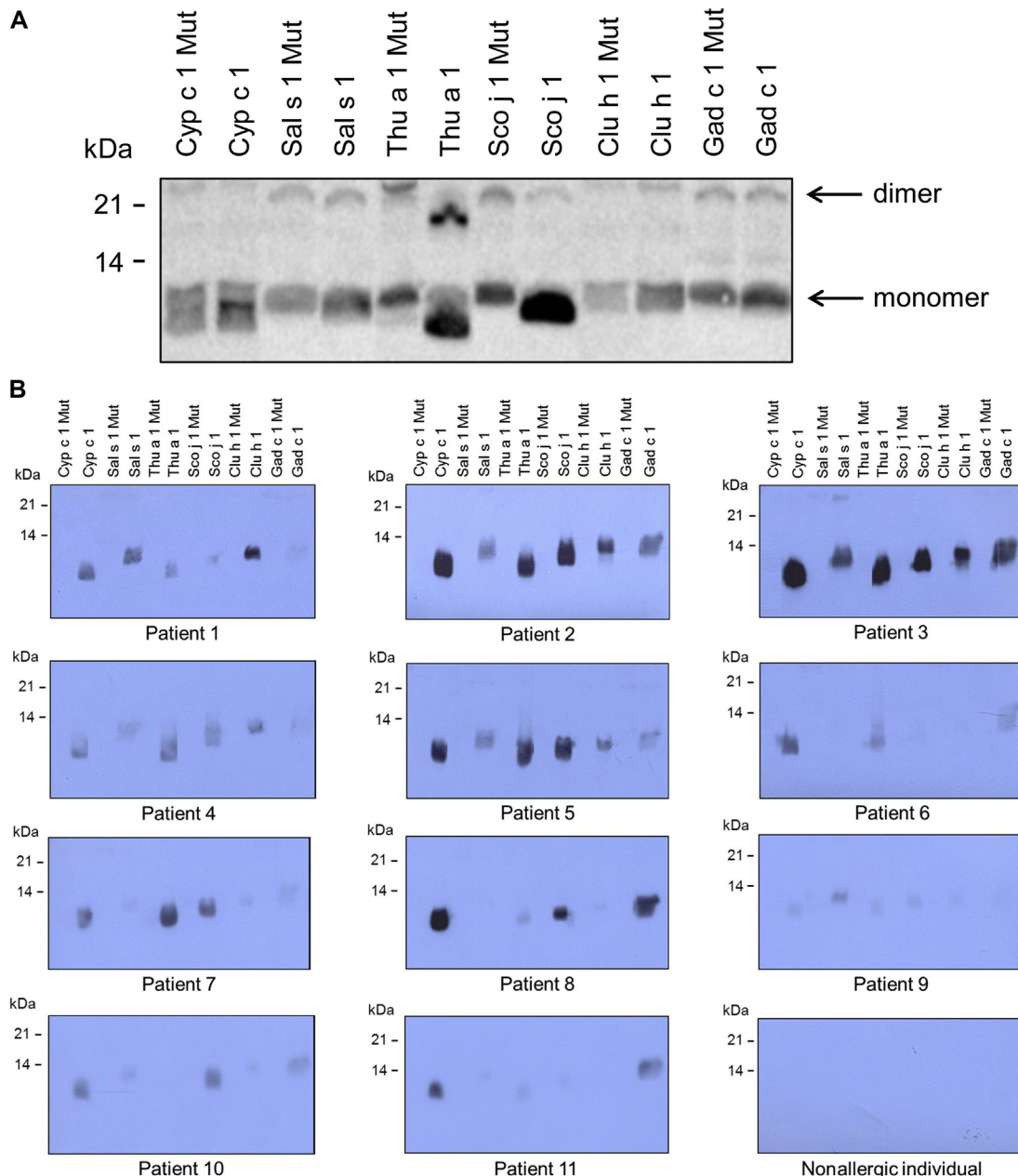


FIG 2. IgE reactivity and reactivity to antiparvalbumin mutant antibodies of the wild-type and mutant proteins assessed by immunoblot analyses. Wild-type and mutated parvalbumins from Atlantic salmon (Sal s 1, Sal s 1 Mut), tuna (Thu a 1, Thu a 1 Mut), chub mackerel (Sco j 1, Sco j 1 Mut), herring (Clu h 1, Clu h 1 Mut), codfish (Gad c 1, Gad c 1 Mut), and carp (Cyp c 1, Cyp c 1 Mut) were exposed to anticarp parvalbumin mutant mouse antibodies (**A**) and to serum IgE from fish-allergic patients and 1 nonallergic individual (**B**). Arrows mark parvalbumin monomers and dimers.

the recombinant wild-type and mutated fish allergens as well as rCyp c 1⁵ and the Cyp c 1 mutant⁶ (data not shown) and corresponding immunoblots probed with antiparvalbumin mutant mouse antisera (Fig 2, A) showed that wild-type and mutated proteins were expressed at comparable levels and had similar migration behavior. They migrated as monomers and as dimers (indicated by arrows in Fig 2, A). Binding of parvalbumin-specific antibodies was visualized with an alkaline phosphatase-conjugated goat antimouse antiserum (Jackson ImmunoResearch, West Grove, Pa) and a Lumi-phos WB chemiluminescent substrate detection system (Pierce, Rockford, Ill).

Nitrocellulose-blotted protein extracts were also exposed to sera from 11 allergic patients suffering from IgE-mediated allergy clearly attributable to fish ingestion and from 1 nonallergic individual without any allergic symptoms, total IgE levels below 100 kU/L, and without positive reactions in an IgE-based multiallergen test.^{5,6} Bound IgE antibodies were detected with ¹²⁵I-labeled antihuman IgE antibodies and visualized by autoradiography.^{5,6}

Fig 2, B, indicates that none of the mutated variants was recognized by IgE antibodies from the fish-allergic patients. Sera from patients 1 to 5 showed IgE cross-reactivity of varying intensity

with each of the recombinant parvalbumins, whereas patients 6 to 11 showed a more selective IgE recognition of parvalbumins from certain fish species, indicating that certain patients are indeed sensitized preferentially to specific fish species (Fig 2, B). In fact, for patients 2 and 3, who showed extensive IgE cross-reactivity, it was reported that they had clinical symptoms upon consumption of a variety of fish species, whereas for patients 8, 9, 10, and 11, who showed weak or no IgE reactivity to tuna, it was determined by oral challenge that they tolerated this fish. Serum from the non-allergic person did not react to any of the blotted proteins, confirming the specificity of the IgE detection system (Fig 2, B).

Interestingly, IgE recognition of parvalbumins was not associated with the levels of homology between the different wild-type allergens. The analysis of percentages of identity between the wild-type parvalbumins (see Table E1 in this article's Online Repository at www.jacionline.org) showed that identities varied between 64% (between Sco j 1 and Sal s 1) and 89% (between Cyp c 1 and Thu a 1). However, high levels of sequence identity (eg, between Cyp c 1 and Thu a 1) were not necessarily associated with cross-reactivity. For example, patients 8 and 11 showed a rather selective IgE reactivity to Cyp c 1 but not to Thu a 1, whereas parvalbumins with lower sequence identity (eg, Sco j 1 and Sal s 1 64%) were recognized by several patients.

These results indicate that the exchange of 4 calcium-coordinating aspartic acids, which are highly conserved in fish parvalbumins, to alanines represents a generally applicable strategy for the generation of hypoallergenic allergen derivatives from different fish species to be used as allergy vaccines.

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Ines Swoboda, PhD^{a,b}
Nadja Balic^c
Christoph Klug, MSc^b
Margit Focke, PhD^a
Milena Weber, MSc^a
Susanne Spitzauer, MD^c
Angela Neubauer, PhD^d
Santiago Quirce, MD^e
Nikolaos Douladiris, MD^f
Nikolaos G. Papadopoulos, MD^f
Rudolf Valenta, MD^a

From ^athe Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria; ^bthe Molecular Biotechnology Section, University of Applied Sciences, Campus Vienna Biocenter, Vienna, Austria; ^cthe Clinical Institute for Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Austria; ^dBiomay AG, Vienna, Austria; ^ethe Department of Allergy, Hospital La Paz Health Research Institute (IdiPAZ), Madrid, Spain; and ^fthe Second Department of Pediatrics, University of Athens, Greece. E-mail: ines.swoboda@meduniwien.ac.at.

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TABLE E1. Percentage amino acid sequence identities between wild-type parvalbumins from carp (Cyp c 1), tuna (Thu a 1), codfish (Gad c 1), chub mackerel (Sco j 1), herring (Clu h 1), and Atlantic salmon (Sal s 1)*

	Cyp c 1 (%)	Thu a 1 (%)	Gad c 1 (%)	Sco j 1 (%)	Clu h 1 (%)	Sal s 1 (%)
Cyp c 1 (AJ292211)	100	89	81	76	72	66
Thu a 1 (FM178217.1)		100	77	71	73	67
Gad c 1 (AY035584)			100	71	74	66
Sco j 1 (P59747)				100	73	64
Clu h 1 (FM178220)					100	71
Sal s 1 (Q91482.1)						100

*The accession numbers of the parvalbumins are indicated in parentheses.