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Molecular and immunological characterization of β′-component (Onc k 5), a major IgE-binding protein in chum salmon roe

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Abstract

Salmon roe has a high allergic potency and often causes anaphylaxis in Japan. The major allergic protein of salmon roe is β′-component, which is a 35 kDa vitellogenin fragment consisting of two subunits. To elucidate structural information and immunological characteristics, β′-component and the subunit components were purified from chum salmon (*Onchorhincus keta*) roe and vitellogenin-encoding mRNA was used to prepare β′-component subunit-encoding cDNA. This was PCR-amplified, cloned and sequenced and the deduced amino acid sequence compared with partial sequences of β′-component obtained by peptide mapping. The recombinant β′-component subunit was produced by bacterial expression in *Escherichia coli* and its IgE-binding ability was measured by ELISA using the sera of a patient allergic to salmon roe. This was then compared with that of the native β′-component with and without carboxymethylation. Following successful cloning of the cDNA encoding the β′-component subunit, 170 amino acid residues were deduced and matched with the amino acid sequences of 121 and 88 residues in the 16 kDa and 18 kDa subunits, respectively. The sequences of both β′-component subunits were almost identical, and the predicted secondary structure of the β′-component showed a high content of β-pleated sheets and no α-helices. There was no difference in IgE-binding ability between the native and recombinant β′-component subunits at the same protein concentration, regardless of carboxymethylation. In conclusion, β′-component is a homodimer protein composed of two isoform subunits having the same level of IgE-binding ability and, therefore, allergenic identity.

Keywords: β′-component, IgE-binding ability, recombinant allergen, salmon roe allergy, vitellogenin

Introduction

Several kinds of fish roes, including sturgeon, paddlefish, salmon, cod, lumpfish, capelin and herring, are becoming popular seafood worldwide (1). However, as individuals have been widely reported to experience immediate allergic reactions following the consumption of king salmon caviar (2), Russian beluga caviar (3) and the roe of white fish and rainbow trout (4), the trend could cause a new risk of fish roe allergies at a global level. In Japan, where more than 500 kinds of marine bioresources are consumed, the cases of allergy to salmon roe, particularly among children, have increased over the last decade and anaphylaxis by ingestion of salmon roe also has been reported (5). On the basis of the number of cases of actual illnesses and the degree of seriousness, salmon roe is listed as a potential allergen (one of subspecific allergenic ingredients) under Japanese food sanitation laws (6).

Lipovitellin (Lv) and β′-component (β′-c) are major constituents of yolk proteins in teleost fish roe (7, 8), and have high IgE-binding ability (9). β′-c in particular is a major allergic protein of chum salmon (*Oncorhynchus keta*) roe as specific IgE reactions to β′-c occur in the sera of almost all patients with salmon roe allergies (9). Additionally, IgE

cross-reactivities among fish roes, such as those from salmon (*O. keta*), herring (*Clupea pallasii*) and walleye pollock (*Theragra chalcogramma*), have been reported in case studies (10). The authors also confirmed that yolk proteins from Atka mackerel (*Pleurogrammus azonus*), dusky sole (*Pleuronectes mochigarei*), slime flounder (*Microstomus achne*), shishamo smelt (*Spirinchus lanceolatus*) and Atlantic capelin (*Mallotus villosus*) were bound to specific IgE of salmon-roe-allergic patient sera (11). These findings clearly indicate the importance of research on β′-c as a food allergen, and β′-c was registered as a new allergen 'Onc k 5' in the official allergen list of the World Health Organization and International Union of Immunological Societies Allergen Nomenclature Subcommittee in 2012 (http://www.allergen.org/).

Lv and β′-c are degradation fragments of vitellogenin (Vg), a protein synthesized in fish liver that is carried to the oocytes through the bloodstream. Accumulated Vg in fish oocytes is proteolytically degraded to the three major yolk proteins: Lv, β′-c and phosvitin (Pv) (7, 8, 12, 13) at proteolytic cleavage sites. Lv and β′-c amino acid sequences present in Vg have previously been confirmed in barfin flounder (*Verasper moseri*) (14), haddock (*Elanogrammus aeglefinus*) (15), Japanese conger (*Conger myriaster*) (16), red sea bream (*Pagrus major*) (17) and yellowfin goby (*Acanthogobius flavimanus*) (18). Lv and Pv are sources of embryonic nutrients in oviparous vertebrates, while β′-c appears to be stable to proteolysis during development of the teleost fish embryo since it remains during oocyte growth and the early cleavage stage of the embryo (8, 12). The proteolytic tolerance of β′-c is probably related to its high allergenicity. Indeed, pepsin–trypsin digestion had little effect on the IgE-binding ability of β′-c prepared from chum salmon roe (19).

Thus far, there is little information regarding the biochemical characteristics of allergic proteins in fish roe compared with other food allergens. Most cases of fish roe allergy are classified as Type I allergies that are triggered by the binding of an allergen to a specific IgE and the recognition of specific amino acid sequences in allergic proteins. Therefore, clarifying the structure of β′-c is the first step toward understanding fish roe allergy.

This study therefore aimed to elucidate structural information and immunological characteristics of chum salmon β′-c. Vg mRNA was sampled from fresh livers of female chum salmon, and 170 amino acid sequences corresponding to the β′-c subunit were analyzed by cDNA cloning and sequencing. The primary structure and IgE-binding ability of recombinant β′-c were then examined by comparison with native β′-c.

Methods

Salmon roe and total RNA from liver

Chum salmon roe from fresh mature individuals (*O. keta*) was washed with cold 0.16 M NaCl and frozen at −60°C until required. Liver for total RNA sampling was collected from a mature female fish caught within 8 h and was immediately treated by RNAlater (Qiagen, Hilden, Germany).

Sera from patients allergic to salmon roe

Sera from patients diagnosed with salmon roe allergies were used for this study. Patient clinical data are listed in Table 1. Total and specific IgE levels were determined by the capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST system; Phadia AB, Uppsala, Sweden). After being stored at < −60°C for 2–12 months, the patients' sera were mixed with the same volume of phosphate-buffered saline (PBS, pH 7.5) containing 0.2% NaN_3 and then stored at 4°C until required. We confirmed that the specific IgE was contained in all sample sera by ELISA using purified β′-c. The study was approved by the local ethical committee, and all subjects provided written informed consent before enrollment in the study.

Preparation of β′-c and its subunit components

β′-c was prepared from chum salmon roe according to the method of Hiramatsu and Hara (20). Briefly, roes were homogenized in 0.5 M NaCl and 20 mM Tris–HCl (pH 8.0), and the yolk protein extract was dropped into 10 × volumes of cold distilled water. The precipitate generated in this step was collected by centrifugation at 20 000 × *g* for 30 min, dissolved in 0.5 M NaCl (pH 8.0), and the 67%-saturated ammonium sulfate precipitate was collected by centrifugation at 20 000 × *g* for 30 min. The precipitate was redissolved in 0.5 M NaCl (pH 8.0) and loaded onto a Sephacryl S-200HR column (GE Healthcare, Piscataway, NJ, USA) to purify β′-c. The protein fractions were detected at 280 nm, and the concentration was determined by the Biuret method (21). All steps were performed at temperatures <5°C, and the purified proteins were frozen at −30°C until required. The subunit components of purified β′-c were separated with a preparative SDS–PAGE system (AE-6750S, Atto Corp., Tokyo, Japan) in the presence of 2-mercaptoethernol. The purified subunits were dialyzed

Table 1. Characteristics of allergic patients with hypersensitivity to chum salmon roe

Serum	Age (years)	Gender	Total IgE (IU ml^{-1})	Specific IgE IU ml^{-1} (CAP-RAST score)			Symptoms
				Salmon roe	Egg yolk	Egg white	
P1	1	Male	66	20.0 (4)	4.6 (2)	0.5 (1)	AD
P2	6	Male	1017	18.9 (4)	2.5 (2)	1.1 (1)	BA, OAS
P3	1	Male	221	35.9 (4)	4.0 (2)	0.7 (1)	AD
P4	3	Male	337	84.2 (5)	2.6 (2)	6.0 (3)	Ur
P5	4	Male	—	>100 (6)	—	—	AD, BA

AD, atopic dermatitis; BA, bronchial asthma; OAS, oral allergy syndrome; Ur, urticaria.

against 1 mM sodium bicarbonate, lyophilized, and stored at −60°C until required.

SDS–PAGE analysis and immunoblotting

SDS–PAGE was performed according to the method of Laemmli (22), using 4.5% and 12.5% polyacrylamide slab gels for stacking and resolving gels, respectively. The protein bands were stained with 0.25% Coomassie Brilliant Blue R (Sigma Aldrich, St Louis, MO, USA) dissolved in 9% acetic acid and 45% methanol. Proteins detected by SDS–PAGE were transferred onto a polyvinylidene difluoride membrane and reacted with the patient's serum (P1 in Table 1). The blotting picture was gray scaled and reversed using a computer.

Circular dichroism spectroscopy

The circular dichroism spectrum of β′-c (0.8 mg ml^{-1}) was measured at 25°C using a spectropolarimeter (J-725, Jasco Inc., Tokyo, Japan).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Mass spectrometry (MS) was performed using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) system (AB4700, Applied Biosystems Inc., CA, USA) equipped with a 335-nm YAG laser in the reflection mode. α-Cyano-4 hydroxycinnamic acid (α-CHCA) was used as the matrix. Samples, desalted with a C18-micro column (ZipTip, Millipore Corp., MA, USA) and dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid, were spotted onto a sample–target plate using the dry droplet method. The sample-coated plate was subsequently subjected to the MALDI-TOF system under the positive ion mode, after applying a drop of the matrix-only solution (10 mg ml^{-1} of α-CHCA dissolved in acetonitrile-trifluoroacetic acid) to the sample droplet.

Molecular cloning of β′-c subunit cDNA

Vitellogenin is a precursor of β′-c as described in the introduction, and the DNA sequences encoding major yolk proteins in salmonids are located in the Vg gene in the following order: NH_2-(Lv heavy chain)-(Pv)-(Lv light chain)-(β′-c)-(C-terminal peptide)-COOH (8). Although we have no structural information about chum salmon Vg, the N-terminal 20 amino acid sequence of chum salmon β′-c was almost identical to that of rainbow trout (*Oncorhynchus mykiss*) β′-c (9), suggesting high similarity of the primary structures. Thus, the partial base sequence of rainbow trout Vg (European Molecular Biology Laboratory: X92804) was used as a cloning reference (23). The cDNA cloning strategy is shown in Fig. 1. Briefly, the forward primer (5′-CCCTGTTCTCTGCCATTTGA-3′) was designed upstream of the coding to the N-terminal amino acid sequence of β′-c, and the reverse primer (5′-CTGGGTGCTTCCTTCTGATA-3′) was designed downstream from the sequence encoding the 170th amino acid residue in order to cover the whole amino acid sequence of the 18 kDa subunit. cDNA cloning was carried out as described previously (24), with the exception of primer differences. The nucleotide sequence was determined using the DNA Sequencer 3130 (Life Technologies, Carlsbad, CA, USA) after labeling the DNA with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies).

Carboxymethylation of β′-c

Native and recombinant β′-cs were carboxymethylated according to the modified method of Crestfield *et al.* (25). β′-c was dissolved in 20 mM Tris–HCl (pH 8.5) containing 8 M urea, 10 mM 2-mercaptoethanol and 5 mM EDTA, then incubated for 3 h at room temperature. The β′-c solution was then treated by 10 mM monoiodoacetic acid for 30 min with shaking. After dialysis against 1 mM $NaHCO_3$, the β′-c solution was lyophilized and stored at −60°C until required.

Peptide mapping of native β′-c subunits

The 16 kDa and 18 kDa subunits of β′-c were digested with lysyl endopeptidase (Wako, Osaka, Japan) dissolved in 20 mM Tris–HCl (pH 8.0), endoproteinase Glu-C (Sigma Aldrich) dissolved in 50 mM PBS (pH 7.8) or trypsin (Sigma Aldrich) dissolved in 20 mM Tris–HCl (pH 8.0), at 1% of the enzyme-to-substrate weight ratio and at 37°C for 2 h. The digested peptides were lyophilized, dissolved in 1% acetonitrile containing 0.1% trifluoroacetic acid and then applied to reverse-phase high-performance liquid chromatography (HPLC) on Mightysil RP-18 GP columns (4.6 × 250 mm: Kanto Chemical Co., Inc., Tokyo, Japan). Columns were eluted at a flow rate of 1.0 ml min^{-1} by a linear gradient of acetonitrile (1%–60% in 120 min) containing 0.1% trifluoroacetic acid. Peptides were monitored at 228 nm with a UV detector. Digested peptides fractionated with HPLC thus obtained

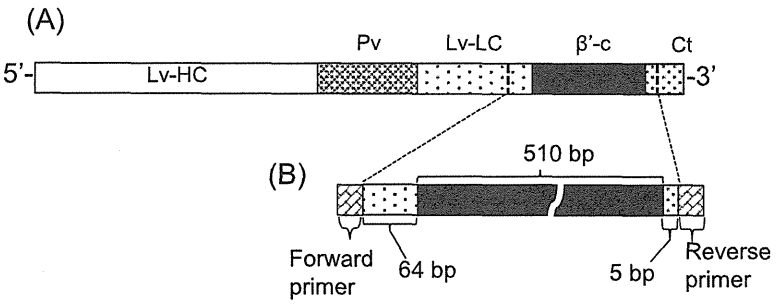

Fig. 1. DNA sequences encoding major yolk proteins located in the vitellogenin gene (**A**) and cDNA cloning strategy for the expression of β′-component subunit gene (**B**).

were subjected to the automatic Edman sequence analyzer (Procise 492, Perkin Elmer, Waltham, MA, USA) to identify the amino acid sequences. Up to 20 residues from the N-terminus were detected in this experiment.

Preparation of recombinant β′-c subunit (Rβsub)

The coding region of β′-c was amplified by RT–PCR using the forward primer (5′–TTAGGATCCGAAGTCAACGCAGT–3′), reverse primer (5′–TTAGGCAAAGCTGACTGAGCTCT–3′) and PrimeSTAR™ HS DNA polymerase (Takara Bio Inc., Shiga, Japan). The ampli-fied fragment was then inserted into the *BamH* I and *Sma* I sites of the pGEX-6P-1 vector (GE Healthcare). The vector was transformed into competent *Escherichia coli* [BL21 (DE3), Nippon Gene Co., Ltd, Tokyo, Japan], which were cultured in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 50 μg ml^{-1} ampicillin. The expression of the glutathione-S-transferase (GST)-fused protein was induced by 1.0 mM isopropyl-β-thiogalactosidase at 37°C for 3 h, and the cultured *E. coli* were sonicated with a detergent buffer (0.05% sodium deoxycholic acid-1% Triton X-100). After adding Benzonase (Novagen Inc. Madison, WI, USA) on ice and removing the residues by centrifugation at 10 000 × *g* for 10 min, Rβsub was purified by running the supernatant through a GSTrap HP column (GE Healthcare). Cleavage between GST and Rβsub was performed using PreScission protease (GE Healthcare) within the column, and elution was carried out by PBS (pH 7.4). Expression and purification of Rβsub were confirmed by SDS–PAGE under reducing conditions, immunoblotting using rabbit anti-β′-component serum and measuring the N-terminal amino acid sequence. Rβsub thus obtained was concentrated by ultrafiltration and stored at −80°C until required.

Quantitative evaluation of β′-c IgE-binding ability

The inhibiting effect of recombinant β′-c on the reaction between native β′-c (solid-phase antigen) and the specific IgE in patients' sera was evaluated using competitive ELISA to evaluate the IgE-binding ability (9). Carboxymethylation-induced dissociation of the two native β′-c subunits was also examined to investigate the IgE-binding ability of the subunits. The IgE-binding ability of the proteins was evaluated by measuring fluorescence intensity using a β-galactosidase-conjugated rabbit anti-human IgE antibody (American Qualex Manufactures, San Clemente, CA, USA) and 4-methylliferyl-β-D-galactoside with excitation at 365 nm and emission at 450 nm.

Results

Structural information of β′-c and its reactivity to specific IgE in serum of allergic patients

The molecular weight of β′-c purified from yolk protein extract [Fig. 2(1), Lane A] revealed a major 35 kDa and a minor 16 kDa band in SDS–PAGE analysis under non-reducing conditions [Fig. 2(1), Lane B], and the two subunit components (16 kDa and 18 kDa) were confirmed under reducing conditions (lane C). Both subunits were completely separate and collected with the polyacrylamide gel preparative electrophoresis system (Lanes D and E) and were capable

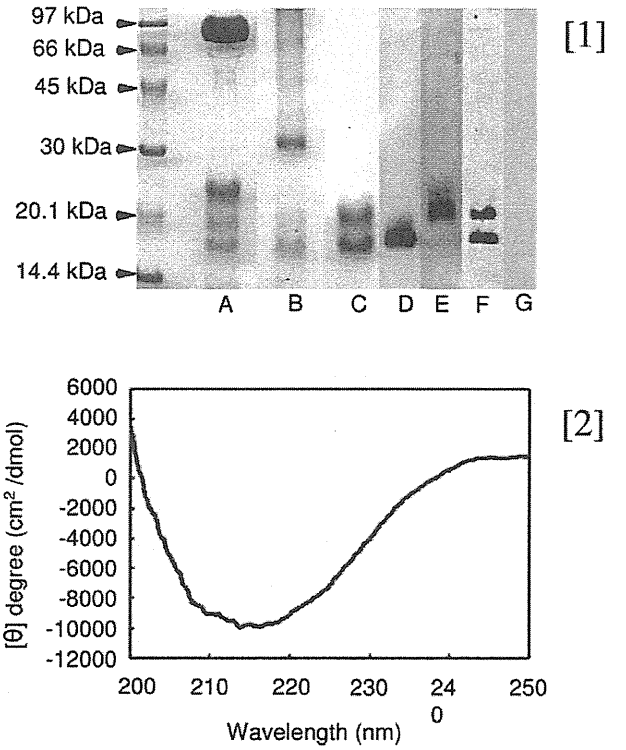

Fig. 2. Molecular characteristics of β′-c. (1) SDS–PAGE patterns of yolk protein and β′-c and their IgE reactivity in immunoblotting. Whole yolk proteins extracted with 0.5 M NaCl (pH 8.0) (A), native β′-c (B, C), 16 kDa and 18 kDa subunits of β′-c (D, E) were electrophoresed. Samples were loaded under reducing (A, C, D, E, F) or non-reducing (B) conditions. β′-c was immunoblotted using patient's (F) and control (G) sera. The 16 kDa and 18 kDa subunits of β′-c were prepared using the preparative SDS–PAGE system in the presence of 2-mercaptoethernol. (2) CD spectrum of native β′-c.

of binding the specific IgE in the serum from the patient allergic to chum salmon (lane F). These results indicate that IgE-binding sites exist in both subunits of β′-c that are covalently cross-linked with disulfide bonds. Both N-terminal amino acid sequences of the β′-c subunits (Lanes D and E) were identical to EVNAVKCSMVGDTLTTFNNR, indicating the similarity of their primary structures.

As shown in Fig. 2(2), the CD spectrum of native β′-c showed only a clear negative peak at 216 nm, suggesting β′-c contains only β-pleated sheet as a regular structure.

As presented in Fig. 3, the MALDI-TOF mass spectrum of native β′-c showed a single peak (M/Z, 36 395) with a small shoulder (M/Z, around 35 200), and two signals were observed in carboxymethylated β′-c (the increment of the molecular mass of each β′-c subunit by carboxymethylation was 290, because they contained five cysteine residues).

cDNA cloning and deduced amino acid sequence of β′-c

cDNA cloning of the partial base sequence of Vg was performed to determine the primary structure of β′-c according to the method shown in Fig. 1. Three 579-bp cDNA fragments were obtained, and their deduced amino acid sequences (Cβ1, Cβ2 and Cβ3) consisted of 170 amino acid residues as shown in Fig. 4. The deduced N-terminal 20 amino acid

sequences (EVNAVKCSMVGDTLTTFNNR) were consistent with those of the purified 16kDa and 18kDa subunits shown in Fig. 5. On the other hand, Cβ1 and Cβ3 differed by a single amino acid at position 151. Seven differences in amino acid residues at positions 88, 90, 94, 101, 103, 104 and 151 were also found between Cβ1 and Cβ2. The sequences have been deposited in the DNA Data Bank of Japan under accession numbers AB474573, AB474574 and AB560769, respectively.

Each of the amino acid sequences had a deduced molecular mass of about 19kDa (Cβ1: 19 108Da; Cβ2: 19 099Da; Cβ3: 19 131Da). The C-terminus of the β′-c subunits could not be clarified by cDNA cloning as β′-c is a cleavage fragment of Vg in fish egg yolk. However, it was apparent that the obtained amino acid sequence contains the 16kDa and 18kDa subunits.

Determination of the amino acid sequence of β′-c subunits

The 16kDa subunit was digested by three kinds of proteases, and the digested peptides were loaded onto reverse-phase HPLC. The 17 peptide peaks (*a–q* in Fig. 5) were collected

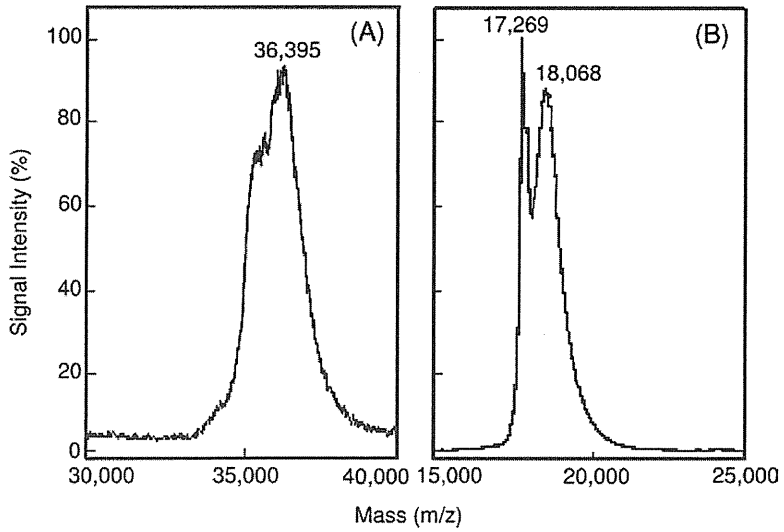

Fig. 3. Molecular mass distribution of β′-c. Native (A) and carboxymethylated β′-cs (B) were applied to the MALDI-TOF system.

Cβ1 / AB474573

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1 bp  GAAGTCAACGCAGTTAAATGTAGCATGGTC
1 aa  E  V  N  A  V  K  C  S  M  V
31 bp GGAGACACATTGACAACATTCAACAACAGG
11 aa G  D  T  L  T  T  F  N  N  R
61 bp AAGTACCCGGTCAATATGCCTCTCTCCTGC
21 aa K  Y  P  V  N  M  P  L  S  C
91 bp TACCAAGTTTTGGCTCAGGATTGCACCATA
31 aa Y  Q  V  L  A  Q  D  C  T  I
121 bp GAGCTCAAATTCATGGTTCTGCTGAAGAAG
41 aa E  L  K  F  M  V  L  L  K  K
151 bp GATCACGCATCTGAACAAAACCACATCAAT
51 aa D  H  A  S  E  Q  N  H  I  N
181 bp GTGAAAATCTCTGACATTGATGTTGACCTG
61 aa V  K  I  S  D  I  D  V  D  L
211 bp TACACTGAGGACCATGGTGTGATGGTGAAG
71 aa Y  T  E  D  H  G  V  M  V  K
241 bp GTCAATGAAATGGAAATTTCCAAAGACAAC
81 aa V  N  E  M  E  I  S  K  D  N
271 bp CTCCCATACACGGACCCCTCAGGTTCTATC
91 aa L  P  Y  T  D  P  S  G  S  I
301 bp ATGATCAAACAGAAGGGTGAAGGCGTGTCT
101 aa M  I  K  Q  K  G  E  G  V  S
331 bp CTCTATGCCAAAAGCCATGGTCTCCAAGAA
111 aa L  Y  A  K  S  H  G  L  Q  E
361 bp GTCTACTTTGATAGCAACTCATGGAAGATT
121 aa V  Y  F  D  S  N  S  W  K  I
391 bp AAAGTTGTGGACTGGATGAAGGGACAGACC
131 aa K  V  V  D  W  M  K  G  Q  T
421 bp TGTGGACTCTGTGGAAAGGCTGATGGCGAA
141 aa C  G  L  C  G  K  A  D  G  E
451 bp AACAGACAGGAGTACCGTACACCCAGTGGC
151 aa N  R  Q  E  Y  R  T  P  S  G
481 bp CGCCTGACCAAGAGCTCAGTCAGCTTTGCC
161 aa R  L  T  K  S  S  V  S  F  A
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Cβ2 / AB474574

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1 bp  GAAGTCAACGCAGTTAAATGTAGCATGGTC
1 aa  E  V  N  A  V  K  C  S  M  V
31 bp GGAGACACATTGACAACATTCAACAACAGG
11 aa G  D  T  L  T  T  F  N  N  R
61 bp AAGTACCCAGTCAATATGCCTCTCTCCTGC
21 aa K  Y  P  V  N  M  P  L  S  C
91 bp TATCAAGTTTTGGCTCAGGATTGCACCATA
31 aa Y  Q  V  L  A  Q  D  C  T  I
121 bp GAACTCAAATTCATGGTTCTGCTGAAGAAG
41 aa E  L  K  F  M  V  L  L  K  K
151 bp GATCACGCATCTGAACAAAACCACATCAAT
51 aa D  H  A  S  E  Q  N  H  I  N
181 bp GTGAAAATCTCTGACATTGATGTTGACCTG
61 aa V  K  I  S  D  I  D  V  D  L
211 bp TACACTGAGGACCATGGTGTGATGGTGAAG
71 aa Y  T  E  D  H  G  V  M  V  K
241 bp GTCAATGAAATGGAAATTTCCAACGACAAG
81 aa V  N  E  M  E  I  S  N  D  K
271 bp CTCCCATACGAGGACCCCTCAGGTTCTATC
91 aa L  P  Y  E  D  P  S  G  S  I
301 bp AAGATCGGTCGGAAGGGTGAAGGCGTGTCT
101 aa K  I  G  R  K  G  E  G  V  S
331 bp CTCTATGCCAAAAGCCATGGTCTCCAAGAA
111 aa L  Y  A  K  S  H  G  L  Q  E
361 bp GTCTACTTTGATAGCAACTCATGGAAGATT
121 aa V  Y  F  D  S  N  S  W  K  I
391 bp AAAGTTGTGGACTGGATGAAGGGACAGACC
131 aa K  V  V  D  W  M  K  G  Q  T
421 bp TGTGGACTCTGTGGAAAGGCTGATGGCGAA
141 aa C  G  L  C  G  K  A  D  G  E
451 bp CACAGACAGGAGTACCGTACACCCAGTGGC
151 aa H  R  Q  E  Y  R  T  P  S  G
481 bp CGCCTGACCAAGAGCTCAGTCAGCTTTGCC
161 aa R  L  T  K  S  S  V  S  F  A
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Cβ3 / AB560769

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1 bp  GAAGTCAACGCAGTTAAATGTAGCATGGTC
1 aa  E  V  N  A  V  K  C  S  M  V
31 bp GGAGACACATTGACAACATTCAACAACAGG
11 aa G  D  T  L  T  T  F  N  N  R
61 bp AAGTACCCAGTCAATATGCCTCTCTCCTGC
21 aa K  Y  P  V  N  M  P  L  S  C
91 bp TATCAAGTTTTGGCTCAGGATTGCACCATA
31 aa Y  Q  V  L  A  Q  D  C  T  I
121 bp GAACTCAAATTCATGGTTCTGCTGAAGAAG
41 aa E  L  K  F  M  V  L  L  K  K
151 bp GATCACGCATCTGAACAAAACCACATCAAT
51 aa D  H  A  S  E  Q  N  H  I  N
181 bp GTGAAAATCTCTGACATTGATGTTGACCTG
61 aa V  K  I  S  D  I  D  V  D  L
211 bp TACACTGAGGACCATGGTGTGATGGTGAAG
71 aa Y  T  E  D  H  G  V  M  V  K
241 bp GTCAATGAAATGGAAATTTCCAAAGACAAC
81 aa V  N  E  M  E  I  S  K  D  N
271 bp CTCCCATACACGGACCCCTCAGGTTCTATC
91 aa L  P  Y  T  D  P  S  G  S  I
301 bp ATGATCAAACAGAAGGGTGAAGGCGTGTCT
101 aa M  I  K  Q  K  G  E  G  V  S
331 bp CTCTATGCCAAAAGCCATGGTCTCCAAGAA
111 aa L  Y  A  K  S  H  G  L  Q  E
361 bp GTCTACTTTGATAGCAACTCATGGAAGATT
121 aa V  Y  F  D  S  N  S  W  K  I
391 bp AAAGTTGTGGACTGGATGAAGGGACAGACC
131 aa K  V  V  D  W  M  K  G  Q  T
421 bp TGTGGACTCTGTGGAAAGGCTGATGGCGAA
141 aa C  G  L  C  G  K  A  D  G  E
451 bp CACAGACAGGAGTACCGTACACCCAGTGGC
151 aa H  R  Q  E  Y  R  T  P  S  G
481 bp CGCCTGACCAAGAGCTCAGTCAGCTTTGCC
161 aa R  L  T  K  S  S  V  S  F  A
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Fig. 4. cDNA sequences of chum salmon β′-c and deduced amino acid sequences. Accession numbers (DNA Data Bank of Japan nucleotide sequence database) are shown and the deduced amino acid sequences from the cDNAs are represented as Cβ1, Cβ2 and Cβ3. Shaded bases and amino acids in Cβ2 and Cβ3 differed from those of Cβ1.

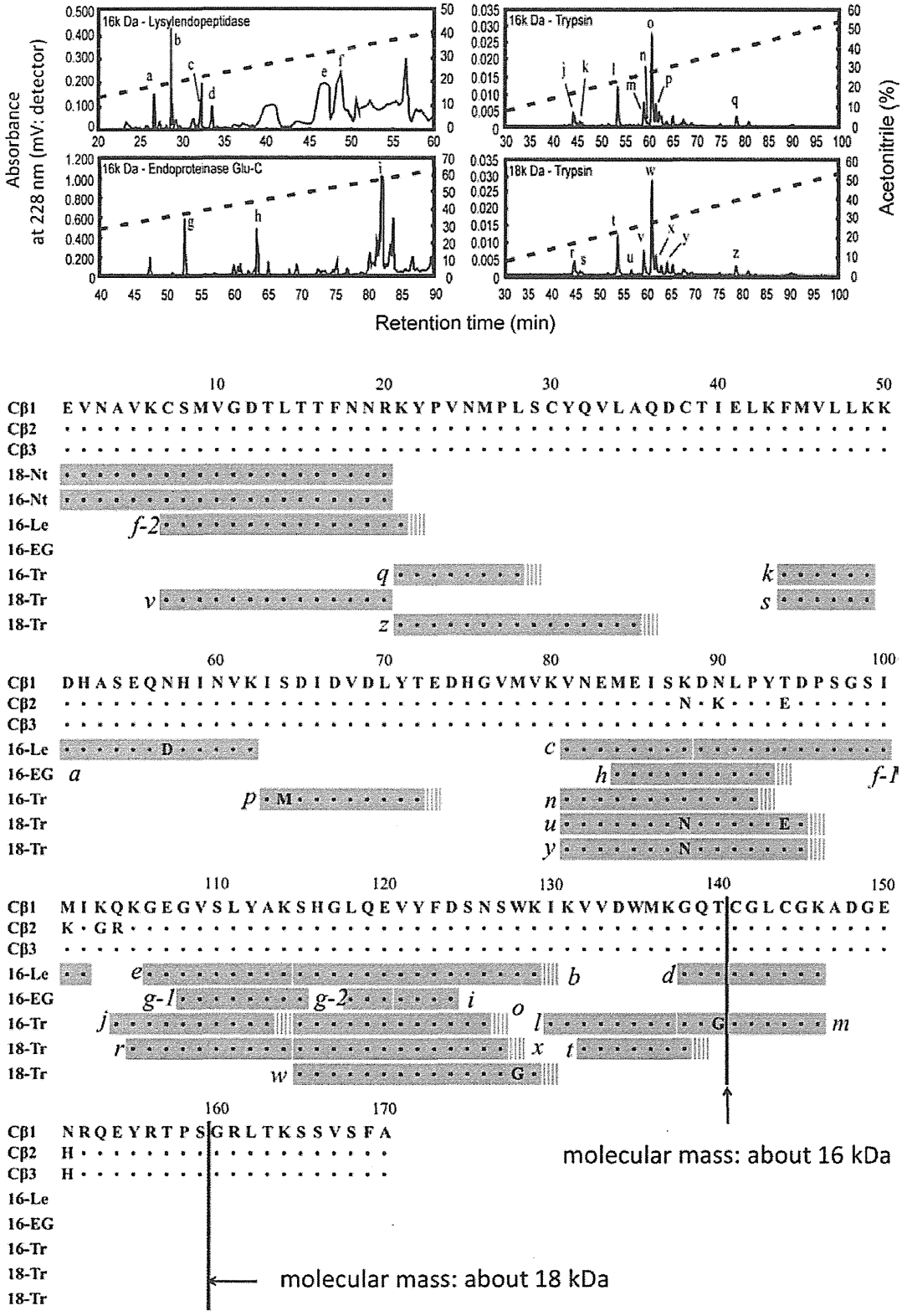

Fig. 5. Peptide mapping of β′-c subunits. Upper figures: HPLC chromatogram of digested β′-c subunits (16 kDa and 18 kDa) by lysyl endopeptidase, endoproteinase Glu-C and trypsin. The solid line represents the elution curve of the digested peptides, and the dotted line is the gradient curve of the acetonitrile concentration. The inserted symbol letters match the peptide sequence shown in the lower figure. Lower figure: positions of digested peptides in the entire amino acid sequence of β′-c subunits. The digested peptides (shadowed) were matched to the predicted amino acid sequences of β′-c. Abbreviations are as follows: 18-Nt, N-terminal of 18 kDa subunit; 16-Nt, N-terminal of 16 kDa subunit; 16-Le, 16 kDa subunit digestion by lysyl endopeptidase; 16-EG, 16 kDa subunit digestion by endoprotease Glu-C; 16-Tr, 16 kDa subunit digestion by trypsin; 18-Tr, 18 kDa subunit digestion by trypsin.

and subjected to amino acid sequential analysis, and their determined N-terminal sequences were found in the deduced amino acid sequences shown in Fig. 4. Consequently, we identified the locations of the 17 peptides in the primary structure of the 16kDa subunit, as shown in Fig. 5, and the 121 amino acid residue sequence was almost consistent with that of Cβs, except for three residues in peptide *a* (57D), peptide *p* (64M) and peptide *m* (140G).

Following tryptic digestion, the peptide chromatogram of the 18kDa subunit resembled that of the 16kDa subunit as shown in Fig. 5 (chromatograms). Additionally, as shown in the peptide mapping, the amino acid sequences of the 18kDa subunit peptides (*r-z*) were almost consistent with those of the peptides from the 16kDa subunit, except for three residues (88N, 94E and 128G), whereas 88N and 94E were consistent with the deduced amino acids from Cβ2. These results indicate the high structural similarity between the 16kDa and 18kDa subunits.

Comparison of the IgE-binding ability between native and recombinant β′-c subunits

The recombinant β′-c subunit (Rβsub) based on Cβ3 was subjected to competitive ELISA using serum from patients allergic to salmon roe (P2–P5 in Table 1), and the IgE-binding ability of Rβsub was compared with that of native β′-c [Fig. 6(A)]. Apparently, Rβsub inhibited the reaction between native β′-c and the specific serum IgEs, and Rβsub completely inhibited the IgE-binding between native β′-c and the specific serum IgEs at the high protein concentrations (>0.01 μg ml^{-1}). Additionally, inhibition effect of Rβsub was not significantly different from that of β′-c at the same protein concentration. Carboxymethylated β′-c (16kDa and 18kDa subunits mixture) also showed the same inhibitory effect as carboxymethylated Rβsub, and the inhibition rate of both carboxymethylated proteins was larger than 80% at 1 μg ml^{-1} [Fig. 6(B)]. These results indicate that the 16kDa and 18kDa subunits have a similar level of IgE-binding ability.

On the other hand, the competitive ELISA-curves of native β′-c and Rβsub [Fig. 6(A)] were shifted to the right side of the figures by carboxymethylation [Fig. 6(B)], and the ELISA-curves of native β′-c were markedly shifted to the right side rather than that of Rbsub except for the case of P2. These changes in the competitive ELISA-curves indicate that the carboxymethylation diminished the IgE-binding ability of the proteins, particularly native β′-c.

Discussion

Structural determination of an allergen is the first step toward understanding its sensitization as most IgEs that induce food allergies bind to allergens by recognizing their specific amino acid sequences. In this work, the primary structure of two subunits of β′-c was investigated by cDNA cloning and peptide mapping. The 16kDa and 18kDa subunits were purified and subjected to the peptide mapping, separately. Consequently, the sequences of 170 amino acid residues were obtained (Fig. 4) and matched with the sequences of 121 residues in the 16kDa subunit and 88 residues in the 18kDa subunit (Fig. 5).

As described above, the DNA sequences encoding major yolk proteins are located in the Vg gene. Since the gene encoding β′-c does not exist independently and the structure of the C-terminal peptide located downstream of β′-c was not identified, the C-terminus of β′-c was not disclosed in this work. However, the entire amino acid sequences of both subunits of native β′-c shown here appear to be covered by

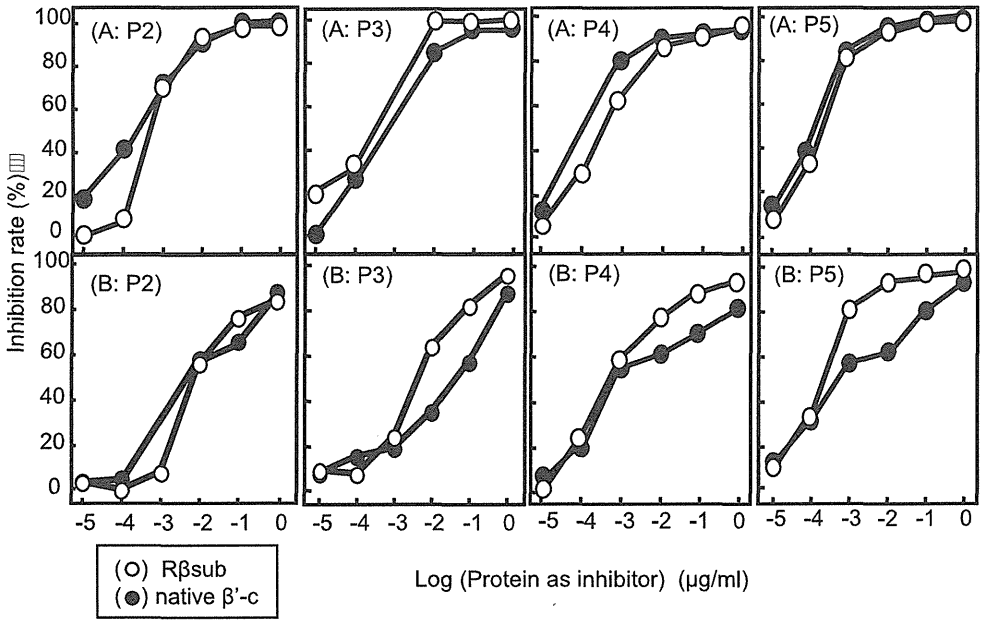

Fig. 6. Inhibitory effect of recombinant β′-c on the reaction between specific IgE and native β′-c. (A) Serum from four patients allergic to salmon roe (P2–P5) were mixed with recombinant (Rβsub) and native β′-cs as inhibitors in a competitive ELISA. (B) The inhibitors were carboxymethylated before examining the competitive ELISA. The ELISA plates were coated with native β′-c.

the deduced amino acid sequences (Cβ1, Cβ2, Cβ3) as their molecular mass was calculated to be 19 kDa.

The authors have successfully detected five kinds of sequential IgE epitopes in β′-c in continuing research (data not shown), and the identified IgE epitopes did not contain the substituted amino acid sequences among the isoforms shown in Fig. 4. Therefore, the allergenicity of 3-isoform recombinants might be identical to each other.

Vg consists of two disulfide-bonded heavy chains, and enzymatic degradation during oocyte growth generates β′-c (8). Although there is no information about cysteine pairs involving in dimerization of β′-c, we suggest that, following cleavage, the fragments of Vg heavy chains, that is the 16 kDa and 18 kDa subunits of β′-c, connect with disulfide bonds as shown in Fig. 2(1) (Lane B, C). HPLC elution patterns of the tryptic-digested peptides derived from the 16 kDa and 18 kDa subunits were quite similar, as shown in Fig. 5. Moreover, the peptides contained in the peaks with identical elution times (o and w, q and z) were consistent, excluding only two amino acid residues between the 16 kDa subunit (121 residues) and the 18 kDa subunit (88 residues). These results clearly indicate that native β′-c is a homodimer protein composed of two isoform subunits. Thus, the 16 kDa subunit appears to be a subfragment of the 18 kDa subunit lacking the C-terminal region.

When the secondary structure of the β′-c subunit was predicted from the deduced amino acid sequence using the computer programs Jpred 3 (26) and PROF-sec (27), approximately half of the entire structure was shown to be composed of β-pleated sheets with no α-helices (Supplementary Figure S1 is available at *International Immunology* Online). Additionally, the CD spectrum of native β′-c [Fig. 2(2)] agreed with these predicted characteristics. Such a high content of β-pleated sheets and a homodimer structure may contribute to the observed high thermal stability of β′-c, in which the water solubility remained unchanged after heating at 98°C for 20 min (data not shown).

Figure 6 showed that the 16 kDa and 18 kDa subunits of native β′-c have an equal IgE-binding ability. Thus, a slight difference in the primary structure between the isoforms and the absence of a C-terminal region in the 16 kDa subunit seems to have no effect on the allergenicity of β′-c. Furthermore, the reaction between native β′-c and the specific IgE was effectively suppressed by carboxymethylated β′-c and Rβsub. This result, although structural IgE epitopes may be reconstructed with refolding of Rβsub, suggests that sequential IgE epitopes are more likely to be involved in sensitization to β′-c in salmon roe allergies than structural IgE epitopes.

On the other hand, the IgE-binding abilities of β′-c and Rβsub were impaired by the carboxymethylation. These changes were presumably caused by loss of structure epitopes with collapse of the subunit structure of native β′-c or by the modification of cysteine residues in sequential epitopes.

It is unclear which of higher order structure or internal structure of β′-c participates in the structural IgE epitopes. Further structural information of native and recombinant proteins before and after carboxymethylation is required to clarify this issue, and these discussions could contribute to identifying IgE epitopes in β′-c.

In oviparous animals including fish and birds, Vg is mainly fragmented to Lv, Pv and specific proteins such as β′-c in teleost fish (7, 8) and 40 kDa glycoprotein in chicken (*Gallus gallus*) (28). However, no amino acid sequence of β′-c clarified in this study was found out in chicken Vg (Accession number: D89547, M18060) (29), indicating that no protein with a similar structure to β′-c exists in hen's egg proteins. Additionally, the patients' sera containing specific IgE with strong reactivity to native β′-c and Rβsub (Fig. 6) showed little IgE reactivity against chicken egg yolk and white proteins as presented in CAP-RAST (Table 1). These results support the previous study (10) that denied the existence of IgE cross-reactivity between fish roe and hen egg.

Since some substitutions of amino acid residues were observed between the deduced amino acid sequences and the digested peptides, as shown in Fig. 5, the two subunits prepared by preparative electrophoresis could contain their own isoforms. Buisine *et al.* (30) reported that the Vg gene of salmonid fish forms a complex Vg gene cluster and that the *Oncorhynchus* group including chum salmon contains 28–30 Vg genes. The reason for the difference in molecular mass between β′-c subunits might be variations in enzyme cleavage sites as a result of the slight difference in the C-terminal amino acid sequences. In MALDI-TOF-MS, one major signal (M/Z, 36,395) with a shoulder part on the low-molecular-weight side was observed in native β′-c. Additionally, in carboxymethylated β′-c, the 18 kDa subunit was shown as a broad and tailing signal (M/Z, 18 068) compared with 16 kDa subunit (M/Z, 17 269). Since only one type of amino acid sequence was observed in the N-terminus of β′-c subunits as shown in Fig. 5, the results of MALDI-TOF-MS suggest that native β′-c contains an 18 kDa subunit having different cleavage sites at the C-terminal side.

In conclusion, β′-c, a major allergen in chum salmon roe, is a homodimer protein composed of two isoform subunits having the same level of IgE-binding ability. These findings are critical data for identifying conformational IgE epitopes and for understanding allergenic cross-reactivity among fish roes. An attempt to identify conformational IgE epitopes is in progress using the structural information obtained in this study.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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References

1 Bledsoe, G. E., Bledsoe, C. D. and Rasco, B. 2003. Caviars and fish roe products. *Crit. Rev. Food Sci. Nutr.* 43:317.

2 Flais, M. J., Kim, S. S., Harris, K. E. and Greenberger, P. A. 2004. Salmon caviar-induced anaphylactic shock. *Allergy Asthma Proc.* 25:233.

3 Untersmayr, E., Focke, M., Kinaciyan, T. *et al.* 2002. Anaphylaxis to Russian Beluga caviar. *J. Allergy Clin. Immunol.* 109:1034.

4 Mäkinen-Kiljunen, S., Kiistala, R. and Varjonen, E. 2003. Severe reactions from roe without concomitant fish allergy. *Ann. Allergy Asthma Immunol.* 91:413.

5 Imamura, T., Kanagawa, Y. and Ebisawa, M. 2008. A survey of patients with self-reported severe food allergies in Japan. *Pediatr. Allergy Immunol.* 19:270.

6 Akiyama, H., Imai, T. and Ebisawa, M. 2011. Japan food allergen labeling regulation–history and evaluation. *Adv. Food Nutr. Res.* 62:139.

7 Hiramatsu, N., Matsubara, T., Weber, G. M., Sullivan, C. V. and Hara, A. 2002. Vitellogenesis in aquatic animals. *Fish Sci.* 68:694.

8 Hiramatsu, N., Cheek, A. O., Sullivan, C. V., Matsubara, T. and Hara, A. 2005. Vitellogenesis and endocrine disruption. In Mommsen, T. P. and Moon, T. W., eds., *Biochemistry and Molecular Biology of Fishes.* Vol. 6, p. 431. Elsevier Science Ltd., Amsterdam, The Netherlands.

9 Shimizu, Y., Nakamura, A., Kishimura, H., Hara, A., Watanabe, K. and Saeki, H. 2009. Major allergen and its IgE cross-reactivity among salmonid fish roe allergy. *J. Agric. Food Chem.* 57:2314.

10 Kondo, Y., Kakami, M., Koyama, H. *et al.* 2005. IgE cross-reactivity between fish roe (salmon, herring and pollock) and chicken egg in patients anaphylactic to salmon roe. *Allergol. Int.* 54:317.

11 Shimizu, Y. and Saeki, H. 2010. Characterization of fish roe allergens. In Shiomi, K. and Saeki, H., eds., *Science of Seafood Allergens*, p. 47. Koseisha-Koseikaku, Tokyo, Japan.

12 Hara, A. 1976. Iron-binding activity of female-specific serum proteins of rainbow trout (*Salmo gairdneri*) and chum salmon (*Oncorhyncus keta*). *Biochim. Biophys. Acta.* 427:549.

13 Hara, A. and Hirai, H. 1978. Comparative studies on immunochemical properties of female-specific serum protein and egg yolk proteins in rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol. B.* 59:339.

14 Matsubara, T., Ohkubo, N., Andoh, T., Sullivan, C. V. and Hara, A. 1999. Two forms of vitellogenin, yielding two distinct lipovitellins, play different roles during oocyte maturation and early development of barfin flounder, *Verasper moseri*, a marine teleost that spawns pelagic eggs. *Dev. Biol.* 213:18.

15 Reith, M., Munholland, J., Kelly, J., Finn, R. N. and Fyhn, H. J. 2001. Lipovitellins derived from two forms of vitellogenin are differentially processed during oocyte maturation in haddock (*Melanogrammus aeglefinus*). *J. Exp. Zool.* 291:58.

16 Mikawa, N., Utoh, T., Horie, N. *et al.* 2006. Cloning and characterization of vitellogenin cDNA from the common Japanese conger (*Conger myriaster*) and vitellogenin gene expression during ovarian development. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 143:404.

17 Sawaguchi, S., Kagawa, H., Ohkubo, N., Hiramatsu, N., Sullivan, C. V. and Matsubara, T. 2006. Molecular characterization of three forms of vitellogenin and their yolk protein products during oocyte growth and maturation in red seabream (*Pagrus major*), a marine teleost spawning pelagic eggs. *Mol. Reprod. Dev.* 73:719.

18 Ohkubo, N., Andoh, T., Mochida, K., Adachi, S., Hara, A. and Matsubara, T. 2004. Deduced primary structure of two forms of vitellogenin in Japanese common goby (*Acanthogobius flavimanus*). *Gen. Comp. Endocrinol.* 137:19.

19 Fujita, S., Shimizu, Y., Kishimura, H., Watanabe, K., Hara, A. and Saeki, H. 2012. In vitro digestion of major allergen in salmon roe and its peptide portion with proteolytic resistance. *Food Chem.* 130:644.

20 Hiramatsu, N. and Hara, A. 1996. Relationship between vitellogenin and its related egg yolk proteins in Sakhalin taimen (*Hucho perryi*). *Comp. Biochem. Physiol. A. Physiol.* 115:243.

21 Gornall, A. G., Bardawill, C. J. and David, M. M. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177:751.

22 Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.

23 Mouchel, N., Trichet, V., Betz, A., Le Pennec, J. P. and Wolff, J. 1996. Characterization of vitellogenin from rainbow trout (*Oncorhynchus mykiss*). *Gene* 174:59.

24 Kanno, G., Kishimura, H., Yamamoto, J. *et al.* 2011. Cold-adapted structural properties of trypsins from walleye pollock (*Theragra chalcogramma*) and Arctic cod (*Boreogadus saida*). *Eur. Food Res. Technol.* 233:963.

25 Crestfield, A. M., Moore, S. and Stein, W. H. 1963. The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* 238:622.

26 Cole, C., Barber, J. D. and Barton, G. J. 2008. The Jpred 3 secondary structure prediction server. *Nucleic Acids Res.* 35:W197. Available at: http://www.compbio.dundee.ac.uk/www-jpred/. [Cited 18 March 2013].

27 Rost, B., Yachdav, G. and Liu, J. 2004. The predict protein server. *Nucleic Acids Res.* 32:W321. Available at: http://www.predictprotein.org/. [Cited 18 March 2013].

28 Yamamura, J., Adachi, T., Aoki, N., Nakajima, H., Nakamura, R. and Matsuda, T. 1995. Precursor-product relationship between chicken vitellogenin and the yolk proteins: the 40 kDa yolk plasma glycoprotein is derived from the C-terminal cysteine-rich domain of vitellogenin II. *Biochim. Biophys. Acta.* 1244:384.

29 Nardelli, D., van het Schip, F. D., Gerber-Huber, S. *et al.* 1987. Comparison of the organization and fine structure of a chicken and a *Xenopus laevis* vitellogenin gene. *J. Biol. Chem.* 262:15377.

30 Buisine, N., Trichet, V. and Wolff, J. 2002. Complex evolution of vitellogenin genes in salmonid fishes. *Mol. Genet. Genomics* 268:535.

Review

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World Allergy Organization Anaphylaxis Guidelines: 2013 Update of the Evidence Base

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Key Words

Anaphylaxis · Systemic allergic reaction · Epinephrine · Adrenaline · Auto-injector · Food allergy · Insect venom allergy · Drug allergy · Latex allergy · Exercise-induced anaphylaxis

Abstract

The World Allergy Organization (WAO) Guidelines for the assessment and management of anaphylaxis are a widely disseminated and used resource for information about anaphylaxis. They focus on patients at risk, triggers, clinical diagnosis, treatment in health care settings, self-treatment in the community, and prevention of recurrences. Their unique strengths include a global perspective informed by prior research on the global availability of essentials for anaphylaxis assessment and management and a global agenda for anaphylaxis research. Additionally, detailed colored illustrations are linked to key concepts in the text [Simons et al.: J Allergy Clin Immunol 2011;127:593.e1–e22]. The recommendations in the original WAO Anaphylaxis Guidelines for management of anaphylaxis in health care settings and community settings were based on evidence published in peer-reviewed, indexed medical journals to the end of 2010. These recommendations remain unchanged and clinically relevant. An update of the evidence base was published in 2012 [Simons et al.: Curr Opin Allergy Clin Immunol 2012;12:389–399]. In 2012 and early 2013, major advances were reported in the following areas: further characterization of patient phenotypes; development of in vitro tests (for some allergens) that help distinguish clinical risk of anaphylaxis from asymptomatic sensitization; epinephrine (adrenaline) research, including studies of a new epinephrine auto-injector for use in community settings, and randomized controlled trials of immunotherapy to prevent food-induced anaphylaxis. Despite these advances, the need for additional prospective studies, including randomized controlled trials of interventions in anaphylaxis is increasingly apparent. This

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2013 Update highlights publications from 2012 and 2013 that further contribute to the evidence base for the recommendations made in the original WAO Anaphylaxis Guidelines. Ideally, it should be used in conjunction with these Guidelines and with the 2012 Guidelines Update.

Introduction

The World Allergy Organization (WAO) Guidelines for the Assessment and Management of Anaphylaxis (subsequently referred to in this publication as the 'WAO Anaphylaxis Guidelines' or 'the Guidelines') were published in early 2011 [1]. The recommendations made in the Guidelines remain unchanged and relevant. In this 2013 Update, a resource intended for use in conjunction with the Guidelines and the 2012 Guidelines Update [2], we highlight major advances in anaphylaxis research published in 2012 and early 2013, thereby strengthening the evidence base for the recommendations made in the Guidelines [1].

Some of the unique aspects of the WAO Anaphylaxis Guidelines are summarized in table 1. These Guidelines were preceded by a survey of the global availability of essentials for the assessment and management of anaphylaxis. They focus on vulnerable patients, risk factors for severe or fatal anaphylaxis, and cofactors that amplify anaphylaxis. They include information on mechanisms and triggers. They emphasize prompt clinical diagnosis and prompt initial treatment that can be carried out even in a low-resource setting, as well as anticipatory long-term management of patients at risk of anaphylaxis recurrence [1].

In 2012, the WAO Anaphylaxis Guidelines and Guidelines-related materials such as posters and pocket cards that promulgate the main concepts of the Guidelines were widely disseminated (table 2). The posters and pocket cards were translated into many different languages [2]. In 2013, a patient information card based on the principles of prompt clinical diagnosis, prompt initial treatment, self-treatment in community settings, and prevention of recurrences was developed and disseminated [1, 3].

Table 1. Unique aspects of the 2011 WAO Anaphylaxis Guidelines

– Preceded by a published survey of global availability of essentials for assessment and management
– Provide a global perspective on anaphylaxis
– Developed in response to absence of global anaphylaxis guidelines[a]
– Developed without corporate funding
– Include evidence-based recommendations
– Cite 150 references, most published from 2006 to 2010 in indexed, peer-reviewed journals
– Include color illustrations linked to the key concepts in the text
– Highlight the role of the allergy/immunology specialist
– Propose a global agenda for anaphylaxis research

[a] ... and absence of national anaphylaxis guidelines in most countries; the WAO Anaphylaxis Guidelines were intended for use not only in countries without guidelines but also as an additional resource in countries with their own national guidelines. Adapted from Simons et al. [1].

Table 2. Dissemination of the WAO Anaphylaxis Guidelines and related materials

– Co-publication (open access) in: *The Journal of Allergy and Clinical Immunology* and in the *World Allergy Organization Journal*
– Posted on the WAO website and on WAO member society websites
– Summary posters and pocket cards translated into many languages[a]
– Presented at meetings worldwide, including plenary sessions at AAAAI, EAACI, and WAO congresses
– Used in undergraduate and postgraduate medical courses
– Used in other specialty areas[b]
– Used in primary care and allied health
– Update of the evidence base published in 2012 in *Current Opinion in Allergy and Clinical Immunology*
– Patient information card developed and disseminated in 2013

AAAAI = American Academy of Allergy Asthma and Immunology; EAACI = European Academy of Allergology and Clinical Immunology. [a] Including Arabic, French, German, Italian, Japanese, Polish, Portuguese, Russian, Spanish, and Turkish. [b] Including sports medicine and the 2012 Olympics (by the Therapeutic Use Exemption Committee).

Epidemiology of Anaphylaxis

Retrospective studies of anaphylaxis have been reported from many countries and a variety of settings, including the community, allergy clinics, emergency departments (ED), hospital wards, and critical care units [1, 2, 4–8]. These studies improve our understanding of anaphylaxis epidemiology and facilitate hypothesis generation. Analysis of standardized clinical data collected from a cohort of 2,012 adults and children with well-defined anaphylaxis is an important step forward and sets the stage for prospective studies [4].

Few epidemiologic studies to date have examined the incidence of anaphylaxis in the general population. Investigators in Spain used electronic medical records from primary care clinics, allergy clinics, ED visits, and hospitalizations, and tracked patients with anaphylaxis across different clinical settings. The incidence rate of 103 episodes per 100,000 person-years was higher than previously reported, with a peak of 314 episodes per 100,000 person-years in the age group 0–4 years [5].

Limited data have been published to date on the epidemiology of anaphylaxis in low- and middle-income countries. Investigators in Turkey used a novel 2-stage approach involving International Classification of Disease (ICD)-10 codes with additional analysis of clinical codes to extract data on patients admitted with a recorded primary diagnosis of anaphylaxis to all 45 hospitals in Istanbul. Overall, 1.95 cases of anaphylaxis per 100,000 person-years were reported, likely an underestimate [6].

In a 5-year retrospective study of all patients seen in the ED of a community hospital, involvement of multiple organ systems or a history of ED visits for anaphylaxis were among the factors contributing to higher admission rates [7].

Anaphylaxis admissions to UK critical care units are increasing year on year, constituting 0.1% of admissions to pediatric units and 0.3% of admissions to adult units. Survival rates are high, at over 90% [8].

Anaphylaxis is sometimes difficult to diagnose postmortem [2]. Brazilian data call attention to the inadequacies of ICD-10 coding for ascertainment of death due to anaphylaxis [8]. Of 498 fatalities, 75% were definitely attributable to anaphylaxis according to established criteria [4]. In order to identify these deaths, the investigators had to consider information from both ICD-10 underlying cause of death fields and ICD-10 contributing cause of death fields. They recommended standardization of coding definitions in order to facilitate international comparisons and trend analyses [9].

Patient Risk Factors

As highlighted in previous WAO Anaphylaxis Guidelines publications [1, 2], for different reasons, infants, teenagers, pregnant women, and the elderly have increased vulnerability to anaphylaxis. Concomitant diseases, such as severe or uncontrolled asthma, cardiovascular disease, and mastocytosis, and concurrent use of some medications increase the risk of severe or fatal anaphylaxis [1, 2].

Different anaphylaxis triggers (elicitors, causes) predominate in different age groups. Among 24,443 adults (mostly nonatopic, 80% female) with a mean age 42 years (range 16–83) admitted to a tertiary health care facility, 516 (2%) were diagnosed with anaphylaxis. Drugs were by far the most common trigger (91% of cases) [10]. In contrast, in two ED studies in adults with an age of 51 ± 16.9 years (mean ± SD) [11] and 44.3 years (interquartile range 32–58) [12], respectively, food triggers were as common as drug triggers, followed by venom triggers.

Most infants and young children with anaphylaxis are atopic and most episodes in this age group are triggered by food. In a retrospective study of 371 infants, children, and teenagers with acute allergic reactions to food, the importance of underlying asthma was confirmed. During anaphylaxis, 72% of those with concomitant asthma had lower airway symptoms, compared with only 49% of those without concomitant asthma ($p < 0.01$) [13].

Reports of fatal anaphylaxis to food have a similar pattern worldwide. In Israel, 4 young patients died after ingesting small amounts of milk (n = 3) or hazelnut (n = 1) to which they had previously experienced allergic reactions. Although all patients had concurrent asthma for which an inhaled bronchodilator had been prescribed, none were on a controller medication [14].

The relationship between mast cell activation disorders and anaphylaxis has been further elucidated [15]. In children with cutaneous mastocytosis, a combination of extensive skin involvement (more than 90% of body surface area) and elevated baseline serum total tryptase concentrations (mean 45.5 ± 5.2 μg/l) predicted severe mast cell mediator-related symptoms and signs requiring hospitalization and in some cases critical care unit admission [16].

The importance of systemic mastocytosis as a risk factor for severe *Hymenoptera* sting-induced anaphylaxis and venom subcutaneous immunotherapy (SCIT)-induced anaphylaxis cannot be overemphasized [1, 2, 17, 18]. The association between drug-induced anaphylaxis and undetected mast cell disease is not as strong; nevertheless, examination for skin signs of mast cell disorders and measurement of baseline tryptase concentrations is recommended in these patients [19]. Elevated baseline tryptase concentrations do not appear to be a risk factor for anaphylaxis from SCIT with airborne allergens [20].

An observational cohort study of patients with *Hymenoptera* venom anaphylaxis confirmed significant contributing factors to be: elevated baseline tryptase concentrations, older age, absence of urticaria or angioedema during anaphylaxis, and symptom onset within 5 min af-

ter a sting; however, in this study, no significant relationship with β-blocker use or angiotensin-converting enzyme (ACE) inhibitor use was identified [18].

In an ED study of anaphylaxis, use of any antihypertensive medication (β-blocker, ACE inhibitor, calcium channel blocker, angiotensin-receptor blocker, or diuretic) was associated with severe episodes involving three or more organ systems, syncope, hypoxia, or hypotension, and increased likelihood of hospitalization. This association occurred independently of age, gender, preexisting lung disease, or suspected trigger [12].

Cofactors, many of which are patient related, are relevant in anaphylaxis [1, 2]. The possibility of cofactor amplification of anaphylaxis should be considered when assessing reactions to foods, nonsteroidal anti-inflammatory drugs (NSAIDs), exercise, and alcohol [21]. Pollinosis has been identified as an external cofactor on the basis of peak hospital admissions for anaphylaxis during the tree pollen season ($p = 0.015$) [13].

Triggers and Mechanisms

Descriptions of new anaphylaxis triggers and improved methods of confirming triggers suggested by the history of the episode have a prominent place in the 2013 Update, as in the 2012 Update [2].

Food

Children who were clinically reactive to peanut (including those with anaphylaxis) had higher specific IgE levels to Ara h 1, Ara h 2, and Ara h 3 than asymptomatic peanut-sensitized children did ($p < 0.00001$). Elevated specific IgE to Ara h 2 was the major contributor to accurate discrimination between clinical reactivity to peanut and asymptomatic sensitization to peanut (99.1% sensitivity, 98.3% specificity, and 1.2% misclassification rate) and had a higher discriminative accuracy than IgE to whole peanut extract ($p = 0.008$) [22].

Short-chain low molecular weight galacto-oligosaccharides with prebiotic effects that are added to some cow's milk formulas have been identified as a new trigger of IgE-mediated anaphylaxis in patients presenting at a median age of 6 years [23].

In tropical climates, orally ingested mites that contaminate wheat flour can trigger anaphylaxis even after cooking (the so-called 'pancake syndrome') and also play a role in food-dependent exercise-induced anaphylaxis [24].

In a prospective study, most patients allergic to red meat were sensitized to gelatin, and a subset was clinically allergic to both red meat and gelatin. The detection of galactose-α 1,3-galactose (α-gal) in gelatin, and correlation between the results of α-gal and gelatin testing, raised the possibility that α-gal-specific IgE might be the target of reactivity to gelatin [25].

In a cross-sectional validation study in 99 fish-tolerant patients and 35 *Anisakis simplex*-allergic patients, in addition to use of commercially available allergens in skin prick tests and the ImmunoCap assay, testing with 5 recombinant *Anisakis* allergens (Ani s 1, Ani s 3, Ani s 5, Ani s 9, and Ani s 10) retained high diagnostic sensitivity and increased diagnostic specificity [26].

Anaphylaxis to food typically occurs after ingestion [1, 2]; however, it can also occur after skin contact with vomited food such as egg and milk [27], or inhalation of minute food particles; for example, sleeping on pillows stuffed with soy products can cause nocturnal anaphylaxis in soy-sensitized patients [28].

In wheat-dependent exercise-induced anaphylaxis, IgE antibodies to recombinant $omega_5$-gliadin are detectable in more than 80% of patients. In their absence, it can be helpful to determine IgE reactivity to other wheat proteins such as α-β-γ-gliadin (especially γ-gliadin) and high molecular weight glutenin [29, 30].

Venoms

True double positivity to bee and vespid venoms is difficult to distinguish from cross-reactivity to these venoms [1]. Only 47% of 76 patients with double positivity to whole bee and wasp (yellow jacket) venoms reacted to recombinant species-specific major allergens (rSSMA) from both of these species. The specificity of IgE to rSSMA was excellent, especially for wasp venom [31]. In another study, component-resolved diagnosis with wasp-specific recombinant allergen components Ves v I and Ves v 5 was a reliable method of diagnosing wasp/yellow jacket allergy [32].

Drugs and Biologic Agents

In a retrospective review, anaphylaxis comprised 6% of 16,157 adverse drug reactions and was reported in patients 7 days to 91 years old. Of these patients, 19% were hospitalized and 3% died. Antibiotics, NSAIDs, antineoplastics/cytotoxic drugs, and immunomodulators were the most common triggers [33].

Proton pump inhibitor administration might increase the risk of developing any drug hypersensitivity. In 161 hospitalized patients, after controlling for confounders, the odds ratio of confirmed drug hypersensitivity was 4.35 (95% CI 2.0–9.45) in those receiving a proton pump inhibitor compared with matched controls. A personal

history of drug allergy and a long hospitalization time were also significant risk factors [34].

Some patients with clinical reactivity to paracetamol (acetaminophen) may have positive immediate skin tests to the drug, indicating involvement of specific IgE; however, negative skin tests do not exclude paracetamol hypersensitivity, which can also be mediated through leukotrienes or other mechanisms [35].

In a 10-year audit of anaphylaxis to muscle relaxants, 20% of 220 patients had positive intradermal tests to the muscle relaxant given during their surgical procedure, most commonly rocuronium or suxamethonium; 65% of those reacting to rocuronium and 29% of those reacting to suxamethonium had cross-reactivity to another muscle relaxant [36].

Biological agents are immunogenic and can cause anaphylaxis [1]. 'Immediate infusion reactions' attributed to rituximab, a common culprit, are usually attributed to cytokine release syndrome; however, rituximab can also be associated with anaphylaxis, rituximab-specific IgE, and rituximab-specific Th2 cells [37].

Other Triggers

In patients with a history of clinical reactivity to latex, latex-specific IgE assays remain useful, although they have a lower sensitivity than previously reported and should not be used for screening the general population [38]. In contrast, in patients with pollinosis who have no history of clinical reactivity to latex, commercially available latex-specific IgE assays are often positive but may not be clinically relevant [39].

Multiple food hypersensitivity was a hallmark of 82 Italian patients with food-dependent, exercise-induced anaphylaxis. When evaluated using skin prick tests, prick-prick tests, and specific IgE levels, including an 89-allergen microarray, 96% were positive to one or more foods and 60% to more than 20 foods; 78% were positive to peach lipid transfer protein Pru p 3 [40].

Rarely, women develop anaphylaxis to human seminal plasma and human prostate-specific antigen [1]. Cross-reactivity of this allergen with the newly identified dog dander allergen Can f 5 appears to be clinically relevant [41].

Based on a PubMed search, 4 genera of helminths are now reported to be associated with anaphylaxis. In addition to *Echinococcus* species and *Anisakis* species, these include *Taenia solium* cysticerosis and *Ascaris* species [42].

Anaphylaxis after skin contact with chemicals such as the persulfate in hair-bleaching products is rare, and the mechanisms have not yet been elucidated [43].

Clinical Diagnosis

Use of validated clinical criteria can be helpful in making the diagnosis of anaphylaxis [1–3]. Based on standardized data collected from 2,012 patients with severe respiratory or cardiovascular involvement in anaphylaxis, symptom profiles support the identification of patient risk factors, because they are impacted by age and comorbid disease in addition to triggers. In elderly patients, cardiovascular symptoms and medication or stinging insect triggers are typical. In young patients, respiratory symptoms, atopy, and food triggers are typical [5].

In a 10-year retrospective study, shock was documented in 41% of 294 patients with anaphylaxis, typically in elderly patients with initial symptoms of syncope, dizziness, and cyanosis after exposure to radiocontrast media or drugs. Patients without shock (59% of the total) tended to be younger, had no initial cardiovascular symptoms, and reported food triggers [44].

In infants and children with anaphylaxis, hypotension is an uncommon initial manifestation [1], typically only occurring in severe episodes. During a prospective study of medically-supervised open food challenges in 80 children [median age 5.3 years (range 1.5–16)], a systolic blood pressure decrease greater than 30% was measured in only one child with anaphylaxis symptoms [45].

Uterine breakthrough bleeding and contractions can occur in women with anaphylaxis to honey bee venom, or to SCIT with honey bee venom. This is attributed to melittin, a venom component that interferes with complement cleavage and bradykinin release [46].

Clinical diagnosis of anaphylaxis is based on consideration of the patient's presenting symptoms and signs and on ruling out other sudden-onset multisystem diseases. The differential diagnosis includes common disorders such as acute asthma or acute urticaria [1, 2]. It also includes uncommon disorders in which, as in anaphylaxis, delay in making an accurate diagnosis and initiating appropriate treatment can lead to death. As an example, in fatal attacks of hereditary angioedema due to C1-esterase inhibitor deficiency, the predyspneic phase lasts 3.7 h (range 0–11); however, the dyspneic phase lasts only 41 min (range 2–240) and the loss of consciousness phase lasts only 8.9 min (range 0–20) [47].

Role of Laboratory Tests

Results of laboratory tests performed on blood samples taken during anaphylaxis can be useful in some patients for subsequently confirming the diagnosis [1, 2]. In a prospective study in adults, serum total tryptase con-

centrations were measured sequentially 1–2, 4–6, and 12–24 h after the onset of anaphylaxis symptoms and at baseline (follow-up). In 62% of the patients, initial tryptase levels were elevated (mean 19.3 ± 15.4 μg/l) with positive correlation between grades of severity and tryptase levels ($p < 0.001$, $r = 0.49$) [48].

In early infancy, the normal reference range for baseline tryptase concentrations differs from the normal reference range in older infants, children, and adults. In nonatopic infants under 3 months of age, the median baseline tryptase concentrations were 6.1 ± 3.5 μg/l. In atopic infants under 3 months of age, the median baseline tryptase concentrations were 14.3 ± 10.2 μg/l. Levels gradually decreased during the first year of life, and by age 9–10 months, regardless of atopic status, median levels were 3.9 ± 1.8 μg/l [1, 49].

Transient elevation of platelet-activating factor (PAF) correlates better with anaphylaxis severity than tryptase or histamine concentrations do; however, PAF concentrations return to baseline within 15–20 min [50].

There are still no biomarkers or laboratory tests available for confirmation of the diagnosis of anaphylaxis at the time of presentation, and there are no biomarkers that are elevated regardless of the anaphylaxis trigger or its route of entry. Moreover, local mediator release without elevation of systemic levels of any biomarker might be important in some patients [1].

Management of Anaphylaxis in Health Care Settings

Prompt initial treatment is essential in anaphylaxis. Even a few minutes' delay can lead to hypoxic-ischemic encephalopathy or death. The importance of having a management protocol cannot be over-emphasized because retention of memorized facts and algorithms can be poor in a crisis and there is little or no time to look up information [1, 2].

The time-dependent and concentration-dependent pharmacologic effects of epinephrine (adrenaline) have been confirmed in a new in vitro human vascular smooth muscle cell model, in which early addition of epinephrine proved to be essential for inhibition of PAF-induced PGE_2 release [51]. These findings are consistent with the clinical observation that epinephrine is maximally effective when injected promptly in anaphylaxis [1].

In a retrospective review of 321 ED patients with anaphylaxis treated with epinephrine, need for two or more epinephrine injections did not correlate with obesity or overweight status; however, in an unspecified number of patients in this study, body mass index calculations were based on estimated height and weight rather than directly measured height and weight [52].

H_1-antihistamines are not drugs of choice in *initial* anaphylaxis treatment because they do not relieve life-threatening respiratory symptoms or shock, although they decrease urticaria and itching. The medications and doses used in anaphylaxis are extrapolated from urticaria treatment [1, 2]. Intravenously administered H_1-antihistamines can cause hypotension [53].

An updated Cochrane Database systematic review found no randomized or quasi-randomized controlled trials of glucocorticoid treatment for anaphylaxis, was unable to make definitive recommendations for or against their use, and highlighted the need for a more robust evidence base in this area [54]. Glucocorticoids remain in use for anaphylaxis because they potentially prevent biphasic anaphylaxis; however, medications and dosing are extrapolated from asthma treatment and the onset of action takes several hours. They are not drugs of choice in *initial* anaphylaxis treatment [1].

A discrepancy between anaphylaxis management recommendations in current guidelines and implementation of these recommendations was confirmed in a large ED study, in which only 12% of patients with severe anaphylaxis received epinephrine, although 50% received an antihistamine and 51% received a glucocorticoid. Based on this data, a revised approach to training in anaphylaxis management was proposed [55].

The recommendation in the original WAO Anaphylaxis Guidelines for intravenous fluid resuscitation using the crystalloid normal saline, rather than a colloid, remains current [1]. A Cochrane review of randomized controlled trials of crystalloids versus colloids in thousands of surgical patients requiring volume replacement found that colloid administration did not correlate with increased survival [56].

In anaphylaxis refractory to initial treatment, new interventions are needed. Infusion of methylene blue, a selective inhibitor of the nitric oxide-cyclic guanosine monophosphate pathway, has been successful, especially in patients with distributive shock and profound vasodilation (vasoplegia) [57, 58].

Vulnerable Patients

In the treatment of vulnerable patients such as infants, pregnant women, and the elderly with anaphylaxis, small but important modifications of the management protocol for prompt initial treatment are needed

[1]. As an example, pregnant women with anaphylaxis require not only prompt epinephrine injection but also high-flow supplemental oxygen, positioning on the left side so the gravid uterus does not compress the inferior vena cava and impede venous return to the heart, maintenance of systolic blood pressure at or above 90 mm Hg to ensure adequate placental perfusion, and continuous electronic monitoring of both mother and infant. When cardiopulmonary resuscitation is indicated in a fully gravid woman, continuous chest compressions can be difficult. Emergency cesarean delivery is sometimes necessary [59].

Long-Term Management: Self-Treatment in Community Settings

After successful treatment of anaphylaxis in health care settings, patients should be equipped to treat recurrences that occur despite attempts to avoid trigger exposure in community settings [1, 2] (table 3).

In a prospective longitudinal observational study, advice and written instructions were provided to families of 512 milk- or egg-allergic infants who were age 3–15 months at study entry. During a median follow-up of 36 months, allergic reactions occurred in 53% of the babies. Reactions were associated not only with misreading food labels or food cross-contamination but also with intentional exposure to foods that should have been avoided, and infants being fed by persons other than their parents. Of the 11.4% of infants with anaphylaxis, only 29.9% received epinephrine injections [60].

The annual incidence rate of accidental exposure to peanut in 1,411 at-risk children age 7.1 ± SD 3.9 years was 13% over 1,175 patient-years (95% CI 10.7–14.5). Children with a recent diagnosis, and adolescents, were at increased risk. Only 21% of moderate or severe allergic reactions were treated with epinephrine injections [61].

Patients at risk for anaphylaxis recurrence in community settings should be equipped with one or more epinephrine auto-injectors (EAI) [62]; if auto-injectors are unavailable or unaffordable, alternative (although not preferred) forms of injectable epinephrine should be recommended [1].

In the episodes of fatal anaphylaxis to foods described previously in this Update, the patients who died either had no EAI prescribed or did not have it available during their fatal episode [14].

Patterns of prescribing EAIs for patients at risk of anaphylaxis in community settings vary among allergists. As an example, although many anaphylaxis episodes after allergen SCIT begin after the typical 30-min on-site observation period in the clinic, for patients on SCIT, 13.5% of allergists never prescribe EAIs, 33.3% always prescribe EAIs, and 52.7% risk-stratify [63].

Persistence of acquired skills to treat anaphylaxis in community settings cannot be guaranteed by patient and caregiver training. Carrying EAIs and competency in using them have been documented to decrease with time after instruction [64].

A new EAI designed using human factors engineering principles has a compact (9 × 5 × 1.5 cm) shape and a single safety guard on the same end as the needle. It provides step-by-step audio instructions, a 5-second countdown during injection, and audio and visual confirmation when injection is complete [65]. Method of instruction and auto-injector size, shape, and preference to carry appear to be useful attributes [66].

Recognition of anaphylaxis symptoms and signs can sometimes be difficult, even for health care professionals [1]. In a blinded, cross-sectional online survey of a random sample of emergency medical service personnel, 99% of paramedics correctly identified a classic presentation of anaphylaxis; however, only 3% recognized an atypical presentation of anaphylaxis in a patient with abdominal pain, hypotension, and no skin signs [67].

Progress in anaphylaxis education research continues. An educational curriculum for parents of children with food allergy that includes information on why, when, and how to use EAIs has been validated and is available online at no cost [68].

Pediatric allergists surveyed about when they typically begin to transfer responsibilities for anaphylaxis recognition and EAI use from adults to children and teenagers expected that by age 12–14 years their patients should begin to share these responsibilities. The allergists individualized the timing of transfer based on patient factors such as presence of asthma and absence of cognitive dysfunction [69]. Caregivers of children and teenagers at increased risk of anaphylaxis in community settings expected to begin gradual transfer of responsibilities earlier, to children age <6–11 years [70].

A 24-hour helpline established to provide access to expert management advice for food allergy-related anaphylaxis will potentially facilitate prompt epinephrine administration, improve clinical outcomes, and, when appropriate, provide reassurance [71].

Table 3. Recommendations for prevention and treatment of anaphylaxis recurrences at the time of discharge from the health care setting

Medication
– Self-injectable epinephrine/adrenaline from an auto-injector
– Self-injectable epinephrine from an ampule/syringe or prefilled syringe (alternative but not preferred formulations)
Other aspects of discharge management
– Anaphylaxis emergency action plan (personalized, written)
– Medical identification (e.g. bracelet, wallet card)
– Medical record electronic flag or chart sticker
– Emphasis on the importance of follow-up investigations, preferably by an allergy/immunology specialist [a]
Assessment of sensitization to allergens
– Before discharge from the emergency department, consider measuring allergen-specific IgE levels in serum for assessment of sensitization to relevant allergens ascertained from the history of the anaphylactic episode
– At least 3–4 weeks after the episode, confirm allergen sensitization using skin tests to relevant allergens; if these tests are negative in a patient with a convincing history of anaphylaxis, consider repeating them weeks or months later
– Challenge/provocation tests, e.g. with food or medication, might also be needed in order to assess risk of future anaphylaxis episodes; tests should be conducted only in well-equipped health care settings staffed by trained, experienced professionals
Long-term risk reduction: avoidance and/or immune modulation
– Food-triggered anaphylaxis: strict avoidance of relevant food(s)
– Stinging insect venom-triggered anaphylaxis: avoidance of stinging insects; subcutaneous venom immunotherapy (protects 80–90% of adults and 98% of children against anaphylaxis from future stings)
– Medication-triggered anaphylaxis: avoidance of relevant medications and use of safe substitutes; if indicated, desensitization (using a published protocol) conducted in a health care setting, as described above
– Idiopathic anaphylaxis (anaphylaxis of unknown etiology: continue search for hidden or novel triggers; measure baseline tryptase concentrations to help identify mast cell activation disorders; consider glucocorticoid and H_1-antihistamine prophylaxis for 2–3 months
Optimal management of asthma and other concomitant diseases

[a] Allergy/immunology specialists play a uniquely important role in preparing the patient for self-treatment in the community, confirmation of the etiology of an anaphylactic episode, education regarding allergen avoidance, and immune modulation. Adapted from Simons et al. [1]; please see this reference for details.

Long-Term Management: Prevention of Recurrence

Risk Assessment

Tests to confirm the etiology of an anaphylaxis episode are critically important because the trigger suspected by the patient is not necessarily the true culprit. The confirmed trigger needs to be strictly avoided if future episodes are to be prevented [1, 2] (table 3).

Children with suspected allergy to milk or egg, and a negative skin prick test, require a specific IgE measurement to the suspect allergen, and those with an absent or undetectable IgE level require a skin prick test with the allergen [72]. In appropriately selected patients, medically supervised oral food challenges are needed to confirm or refute clinical reactivity to food [72, 73]; however, standardization of food challenges is needed [73].

For peanut and other well-characterized food allergens, the utility of skin prick tests and specific IgE levels is maximized by considering the degree of positivity of the test results in the context of the reaction history. Component testing that measures IgE binding to specific proteins within the food potentially adds important information to the risk assessment [74].

In a retrospective study, in 98.7% of 478 consecutive patients with a convincing history of anaphylaxis after *Hymenoptera* stings, simultaneous testing with 4 different concentrations of honey bee venom and wasp venom was reported to be tolerated well; 0.6% of patients had allergic reactions and 0.6% had vasovagal reactions [75].

Skin tests to neuromuscular blocking agents were found to have an excellent negative predictive value when used to select an alternative neuromuscular blocking agent for subsequent surgery [76].

After an anaphylaxis episode, it is standard practice to defer skin tests for at least 3–4 weeks [1]. This sometimes presents a problem (e.g. in patients with perioperative anaphylaxis whose surgery cannot be delayed). In a prospective study in 44 patients, skin tests were performed

early (0–4 days after the reaction during anesthesia) and late (4–8 weeks after). The overall agreement between early and late skin tests was 71% ($p = 0.002$). The odds ratio of obtaining a false-negative test 0–4 days after the reaction was 3.3 versus later testing ($p = 0.09$). The investigators noted that while early testing can be useful in some patients, it does not replace later testing [77].

Risk Reduction

In a prospective longitudinal observational study in which 293 children age 3–15 months with clinical reactivity to cow's milk were monitored regularly, 53% became milk tolerant by a median age of 63 months [78]. In some children, however, resolution of clinical reactivity to cow's milk can take years longer, during which they remain at risk of fatal anaphylaxis [2].

Monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 1-α (MIP-1-α) levels are low in children with clinical allergy to cow's milk protein and increase during desensitization to cow's milk [79].

Randomized controlled oral immunotherapy (OIT) or sublingual immunotherapy (SLIT) trials with food allergens such as milk [80], egg [81], or peanut [82] have been conducted in carefully selected patients with well-characterized clinical reactivity and well-defined levels of allergen sensitization. Over many months, milligram doses of allergen are given in OIT; microgram doses are given in SLIT. During treatment, most patients experience symptoms such as itchy mouth and throat, lip swelling, cough, or abdominal pain and up to 20% drop out due to serious adverse effects including anaphylaxis. Temporary desensitization can be achieved and maintained as long as the food is ingested regularly; however, permanent immunologic tolerance has been difficult to demonstrate. New approaches, such as pretreatment with omalizumab followed by combined treatment with omalizumab and food AIT, are promising [83]; however, at this time, food allergen immunotherapy is still not ready for use outside the context of controlled trials approved by research ethics boards [1, 2].

A Cochrane systematic review confirmed that subcutaneous insect venom immunotherapy (VIT) (which *can* lead to immunologic tolerance [1]) effectively prevents future allergic reactions to insect stings and improves the quality of life. The risk of systemic reactions is low, although significant [84]. Rush initiation of VIT with *Hymenoptera* venom is associated with an increased risk of systemic reactions [2]; as an example, ultra-rush initiation (3 visits over 2 weeks) with ant venom increased the risk of objective systemic reactions versus semi-rush initiation (10 visits over 9 weeks) ($p < 0.001$) [85].

In patients with anaphylaxis triggered by a medication that is essential for them, substitution of a medication from a different therapeutic class is recommended [1]. If this is not possible, desensitization to the culprit drug is indicated, using a published protocol, in a hospital setting under medical supervision [1, 2]. Most published literature on drug desensitization pertains to *immediate* hypersensitivity reactions [1, 2]. In the absence of controlled studies or an evidence-based review of desensitization in *delayed* hypersensitivity reactions (defined as onset more than 1 h after drug administration), experts have summarized their experience with regard to indications, selection criteria, contraindications, procedures, risks, and complications of desensitization for these reactions [86]. The definition of *delayed* used in this important paper might require further discussion.

Sugammadex, a cyclodextrin derivative used to reverse the intramuscular blockade produced by rocuronium, encapsulates rocuronium in an inclusion complex and removes it from the neuromuscular junction to the plasma. Sugammadex also rapidly reverses anaphylaxis to rocuronium, possibly by sequestering IgE-bound rocuronium. It is not known whether the rocuronium remains potentially allergenic in inclusion complex form [87].

Vaccines that prevent infectious diseases rarely trigger anaphylaxis [1]. Patients with this history should be evaluated with skin tests to the vaccine and its components. If test results are negative, the vaccine can be administered in usual dose(s) under observation. If test results are positive, it should be administered in graded doses under observation. Patients with egg allergy of any severity (but no history of reacting to the influenza vaccine itself) can receive annual injections of trivalent influenza vaccine with low ovalbumin content, given and observed for 30 min in a setting where anaphylaxis can be recognized and treated promptly. In these patients, although skin testing with the vaccine or dividing the dose is unnecessary, live attenuated influenza vaccines are not recommended [88].

Conclusions

As summarized in this Update, the evidence base for the recommendations for the assessment, management, and prevention of anaphylaxis made in the 2011 WAO Anaphylaxis Guidelines is being strengthened year after year (table 4). Major advances in 2012 and early 2013 were: further characterization of patient phenotypes; development of in vitro tests (for some allergens) that help distinguish clinical risk of anaphylaxis from asymptom-

Table 4. Progress on the global agenda for anaphylaxis research since 2011

Agenda item	Progress	References
Description of patient-specific risk factors; development of instruments to quantify them	++	2, 4, 10–21
Validation of the clinical criteria for diagnosis	++	2, 3
Development of in vitro test(s) to confirm the clinical diagnosis	++	2, 48–50
Development of in vitro tests to distinguish clinical risk of anaphylaxis from asymptomatic sensitization	+++	2, 22, 23, 25, 26, 29-32, 37–41
Epinephrine research [pharmacology, epidemiology, and RCTs (not placebo-controlled) of dose vs. dose and route vs. route]	+++	2, 51, 52, 55, 60–66
RCTs of second-line medications such as H_1-antihistamines or glucocorticoids	±	2, 54
RCTs of immune modulation to prevent anaphylaxis episodes	+++	2, 79–85
Anaphylaxis education	++	1, 2, 64, 68–71

RCT = Randomized controlled trial; ± = minimal; + = some; ++ = good; +++ = excellent.

atic sensitization; epinephrine research including studies of a new EAI for use in community settings, and randomized controlled trials of OIT and SLIT to prevent food-induced anaphylaxis. Despite these advances, additional prospective studies, including randomized controlled trials of interventions, are urgently needed for the diagnosis, treatment, and prevention of anaphylaxis.

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References

1 Simons FER, Ardusso LRF, Bilo MB, El-Gamal YM, Ledford DK, Ring J, Sanchez-Borges M, Senna GE, Sheikh A, Thong BY, for the World Allergy Organization: World Allergy Organization guidelines for the assessment and management of anaphylaxis. J Allergy Clin Immunol 2011;127:593.e1–e22.

2 Simons FER, Ardusso LRF, Bilo MB, Dimov V, Ebisawa M, El-Gamal YM, Ledford DK, Lockey RF, Ring J, Sanchez-Borges M, Senna GE, Sheikh A, Thong BY, Worm M, for the World Allergy Organization: 2012 Update: World Allergy Organization (WAO) guidelines for the assessment and management of anaphylaxis. Curr Opin Allergy Clin Immunol 2012;12:389–399.

3 Sampson HA, Munoz-Furlong A, Campbell RL, Adkinson NF Jr, Bock SA, Branum A, Brown SGA, Camargo CA Jr, Cydulka R, Galli SJ, Gidudu J, Gruchalla RS, Harlor AD Jr, Hepner DL, Lewis LM, Lieberman PL, Metcalfe DD, O'Connor R, Muraro A, Rudman A, Schmitt C, Scherrer D, Simons FER, Thomas S, Wood JP, Decker WW: Second symposium on the definition and management of anaphylaxis: summary report – Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network Symposium. J Allergy Clin Immunol 2006;117:391–397.

4 Worm M, Edenharter G, Rueff F, Scherer K, Pfohler C, Mahler V, Treudler R, Lang R, Nemat K, Koehli A, Niggemann B, Hompes S: Symptom profile and risk factors of anaphylaxis in Central Europe. Allergy 2012;67:691–698.

5 Tejedor Alonso MA, Moro Moro M, Mugica Garcia MV, Esteban Hernandez J, Rosado Ingelmo A, Vila Albelda C, Gomez Traseira C, Cardenas Contreras R, Sanz Sacristan J, Hernandez Merino A: Incidence of anaphylaxis in the city of Alcorcon (Spain): a population-based study. Clin Exp Allergy 2012;42:578–589.

6 Cetinkaya F, Incioglu A, Birinci S, Karaman BE, Dokucu AI, Sheikh A: Hospital admissions for anaphylaxis in Istanbul, Turkey. Allergy 2013;68:128–130.

7 Steele R, Camacho-Halili M, Rosenthal B, Davis-Lorton M, Aquino M, Fonacier L: Anaphylaxis in the community setting: determining risk factors for admission. Ann Allergy Asthma Immunol 2012;109:133–136.

8 Gibbison B, Sheikh A, McShane P, Haddow C, Soar J: Anaphylaxis admissions to UK critical care units between 2005 and 2009. Anaesthesia 2012;67:833–839.

9 Tanno LK, Ganem F, Demoly P, Toscano CM, Bierrenbach AL: Undernotification of anaphylaxis deaths in Brazil due to difficult coding under the ICD-10. Allergy 2012;67:783–789.

10 Gelincik A, Demirturk M, Yilmaz E, Ertek B, Erdogdu D, Colakoglu B, Buyukozturk S: Anaphylaxis in a tertiary adult allergy clinic: a retrospective review of 516 patients. Ann Allergy Asthma Immunol 2013;110:96–100.

11 Beyer K, Eckermann O, Hompes S, Grabenhenrich L, Worm M: Anaphylaxis in an emergency setting – elicitors, therapy and incidence of severe allergic reactions. Allergy 2012;67:1451–1456.

12 Lee S, Hess EP, Nestler DM, Bellamkonda Athmaram VR, Bellolio MF, Decker WW, Li JTC, Hagan JB, Manivannan V, Vukov SC, Campbell RL: Antihypertensive medication use is associated with increased organ system involvement and hospitalization in emergency department patients with anaphylaxis. J Allergy Clin Immunol 2013;131:1103–1108.

13 Vetander M, Helander D, Flodstrom C, Ostblom E, Alfven T, Ly DH, Hedlin G, Lilja G, Nilsson C, Wickman M: Anaphylaxis and reactions to foods in children – a population-based case study of emergency department visits. Clin Exp Allergy 2012;42:568–577.

14 Levy MB, Goldberg MR, Nachshon L, Tabachnik E, Katz Y: Lessons from cases of mortality due to food allergy in Israel: cow's milk protein should be considered a potentially fatal allergen. Isr Med Assoc J 2012;14:29–33.

15 Valent P: Mast cell activation syndromes: definition and classification. Allergy 2013;68:417–424.
16 Alvarez-Twose I, Vano-Galvan S, Sanchez-Munoz L, Morgado JM, Matito A, Torrelo A, Jaen P, Schwartz LB, Orfao A, Escribano L: Increased serum baseline tryptase levels and extensive skin involvement are predictors for the severity of mast cell activation episodes in children with mastocytosis. Allergy 2012;67:813–821.
17 Alvarez-Twose I, Bonadonna P, Matito A, Zanotti R, Gonzalez-de-Olano D, Sanchez-Munoz L, Morgado JM, Orfao A, Escribano L: Systemic mastocytosis as a risk factor for severe Hymenoptera sting-induced anaphylaxis. J Allergy Clin Immunol 2013;131:614–615.
18 Stoevesandt J, Hain J, Kerstan A, Trautmann A: Over- and underestimated parameters in severe Hymenoptera venom-induced anaphylaxis: cardiovascular medication and absence of urticaria/angioedema. J Allergy Clin Immunol 2012;130:698–704.
19 Brockow K, Bonadonna P: Drug allergy in mast cell disease. Curr Opin Allergy Clin Immunol 2012;12:354–360.
20 Asero R, Farioli L, Pastorello EA: Baseline serum tryptase levels and adverse reactions to injection specific immunotherapy with airborne allergens: is there a relationship? Int Arch Allergy Immunol 2012;158:276–280.
21 Cardona V, Luengo O, Garriga T, Labrador-Horrillo M, Sala-Cunill A, Izquierdo A, Soto L, Guilarte M: Co-factor-enhanced food allergy. Allergy 2012;67:1316–1318.
22 Hong X, Caruso D, Kumar R, Liu R, Liu X, Wang G, Pongracic JA, Wang X: IgE, but not IgG4, antibodies to Ara h 2 distinguish peanut allergy from asymptomatic peanut sensitization. Allergy 2012;67:1538–1546.
23 Chiang WC, Huang Chiung-H, Llanora GV, Gerez I, Goh SH, Shek LPC, Nauta AJ, Van Doorn WA, Bindels J, Ulfman LH, Knipping K, Delsing DJ, Knol EF, Lee BW: Anaphylaxis to cow's milk formula containing short-chain galacto-oligosaccharide. J Allergy Clin Immunol 2012;130:1361–1367.
24 Sanchez-Borges M, Suarez Chacon R, Capriles-Hulett A, Caballero-Fonseca F, Fernandez-Caldas E: Anaphylaxis from ingestion of mites: pancake anaphylaxis. J Allergy Clin Immunol 2013;131:31–35.
25 Mullins RJ, James H, Platts-Mills TAE, Commins S: Relationship between red meat allergy and sensitization to gelatin and galactose-alpha-1,3-galactose. J Allergy Clin Immunol 2012;129:1334–1342.e1.
26 Caballero ML, Umpierrez A, Perez-Pinar T, Moneo I, de Burgos C, Asturias JA, Rodriguez-Perez R: *Anisakis simplex* recombinant allergens increase diagnosis specificity preserving high sensitivity. Int Arch Allergy Immunol 2012;158:232–240.
27 Chernin LR, Swender DA, Hostoffer RW, Tcheurekdjian H: Anaphylaxis secondary to contact with a vomited food allergen. Ann Allergy Asthma Immunol 2012;109:279.
28 Armentia A, Pineda F, Martin B, San Miguel A, Gil FJM, Puente Y, de Lecea C, Palacios R: Anaphylaxis caused by hidden soybean allergens in pillows. J Allergy Clin Immunol 2013;131:228–230.
29 Hofmann SC, Fischer J, Eriksson C, Bengtsson Gref O, Biedermann T, Jakob T: IgE detection to alpha/beta/gamma-gliadin and its clinical relevance in wheat-dependent exercise-induced anaphylaxis. Allergy 2012;67:1457–1460.
30 Takahashi H, Matsuo H, Chinuki Y, Kohno K, Tanaka A, Maruyama N, Morita E: Recombinant high molecular weight-glutenin subunit-specific IgE detection is useful in identifying wheat-dependent exercise-induced anaphylaxis complementary to recombinant omega-5 gliadin-specific IgE test. Clin Exp Allergy 2012;42:1293–1298.
31 Muller U, Schmid-Grendelmeier P, Hausmann O, Helbling A: IgE to recombinant allergens Api m 1, Ves v 1, and Ves v 5 distinguish double sensitization from crossreaction in venom allergy. Allergy 2012;67:1069–1073.
32 Ebo DG, Faber M, Sabato V, Leysen J, Bridts CH, De Clerck LS: Component-resolved diagnosis of wasp (yellow jacket) venom allergy. Clin Exp Allergy 2013;43:255–261.
33 Ribeiro-Vaz I, Marques J, Demoly P, Polonia J, Gomes ER: Drug-induced anaphylaxis: a decade review of reporting to the Portuguese Pharmacovigilance Authority. Eur J Clin Pharmacol 2013;69:673–681.
34 Ramirez E, Cabanas R, Laserna LS, Fiandor A, Tong H, Prior N, Calderon O, Medrano N, Bobolea I, Frias J, Quirce S: Proton pump inhibitors are associated with hypersensitivity reactions to drugs in hospitalized patients: a nested case-control in a retrospective cohort study. Clin Exp Allergy 2013;43:344–352.
35 Rutkowski K, Nasser SM, Ewan PW: Paracetamol hypersensitivity: clinical features, mechanism and role of specific IgE. Int Arch Allergy Immunol 2012;159:60–64.
36 Brereton A, Russell WJ: Anaphylaxis to muscle relaxants: an audit of ten years of allergy testing at the Royal Adelaide Hospital. Anaesth Intensive Care 2012;40:861–866.
37 Vultaggio A, Matucci A, Nencini F, Pratesi S, Petroni G, Cammelli D, Alterini R, Rigacci L, Romagnani S, Maggi E: Drug-specific Th2 cells and IgE antibodies in a patient with anaphylaxis to rituximab. Int Arch Allergy Immunol 2012;159:321–326.
38 Accetta Pedersen DJ, Klancnik M, Elms N, Wang ML, Hoffmann RG, Kurup VP, Kelly KJ: Analysis of available diagnostic tests for latex sensitization in an at-risk population. Ann Allergy Asthma Immunol 2012;108:94–97.
39 Unsel M, Mete N, Ardeniz O, Sin A, Gulbahar O, Kokuludag A: Diagnostic value of specific IgE analysis in latex allergy. Int Arch Allergy Immunol 2012;158:281–287.
40 Romano A, Scala E, Rumi G, Gaeta F, Caruso C, Alonzi C, Maggioletti M, Ferrara R, Palazzo P, Palmieri V, Zeppilli P, Mari A: Lipid transfer proteins: the most frequent sensitizer in Italian subjects with food-dependent exercise-induced anaphylaxis. Clin Exp Allergy 2012;42:1643–1653.
41 Basagana M, Bartolome B, Pastor-Vargas C, Mattsson L, Lidholm J, Labrador-Horrillo M: Involvement of Can f 5 in a case of human seminal plasma allergy. Int Arch Allergy Immunol 2012;159:143–146.
42 Minciullo PL, Cascio A, David A, Pernice LM, Calapai G, Gangemi S: Anaphylaxis caused by helminths: review of the literature. Eur Rev Med Pharmacol Sci 2012;16:1513–1518.
43 Hoekstra M, van der Heide S, Coenraads PJ, Schuttelaar MLA: Anaphylaxis and severe systemic reactions caused by skin contact with persulfates in hair-bleaching products. Contact Dermatitis 2012;66:317–322.
44 Park HJ, Kim SH: Factors associated with shock in anaphylaxis. Am J Emerg Med 2012;30:1674–1678.
45 Caffarelli C, Rico S, Rinaldi L, Povesi Dascola C, Terzi C, Bernasconi S: Blood pressure monitoring in children undergoing food challenge: association with anaphylaxis. Ann Allergy Asthma Immunol 2012;108:285–286.
46 Mingomataj EC, Bakiri AH: Episodic hemorrhage during honeybee venom anaphylaxis: potential mechanisms. J Investig Allergol Clin Immunol 2012;22:237–244.
47 Bork K, Hardt J, Witzke G: Fatal laryngeal attacks and mortality in hereditary angioedema due to C1-INH deficiency. J Allergy Clin Immunol 2012;130:692–697.
48 Sala-Cunill A, Cardona V, Labrador-Horrillo M, Luengo O, Esteso O, Garriga T, Vicario M, Guilarte M: Usefulness and limitations of sequential serum tryptase for the diagnosis of anaphylaxis in 102 patients. Int Arch Allergy Immunol 2013;160:192–199.
49 Belhocine W, Ibrahim Z, Grandne V, Buffat C, Robert P, Gras D, Cleach I, Bongrand P, Carayon P, Vitte J: Total serum tryptase levels are higher in young infants. Pediatr Allergy Immunol 2011;22:600–607.
50 Vadas P, Perelman B, Liss G: Platelet-activating factor, histamine, and tryptase levels in human anaphylaxis. J Allergy Clin Immunol 2013;131:144–149.
51 Vadas P, Perelman B: Effect of epinephrine on platelet-activating factor-stimulated human vascular smooth muscle cells. J Allergy Clin Immunol 2012;129:1329–1333.
52 Rudders SA, Geyer BC, Banerji A, Phipatanakul W, Clark S, Camargo CA Jr: Obesity is not a risk factor for repeat epinephrine use in the treatment of anaphylaxis. J Allergy Clin Immunol 2012;130:1216–1218.
53 Ellis BC, Brown SGA: Parenteral antihistamines cause hypotension in anaphylaxis. Emerg Med Australas 2013;25:92–93.
54 Choo KJL, Simons FER, Sheikh A: Glucocorticoids for the treatment of anaphylaxis. Cochrane Database Syst Rev 2012;4:CD007596.