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DISSERTATION

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Immune responses to cow's milk allergens: Molecular allergen characterization and epitope mapping

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Contents

Immunantworten auf Kuhmilchproteine: Molekulare Allergencharakterisierung und Epitop-Mapping

Kuhmilchallergie ist die am häufigsten auftretende Lebensmittelallergie innerhalb der ersten Lebensjahre und betrifft ungefähr 2-3% aller Säuglinge und Kleinkinder in Industrieländern. Die meisten Patienten mit Kuhmilchallergie leiden unter Symptomen, die mehr als ein Organ betreffen, wie zum Beispiel kutane, gastrointestinale und respiratorische Symptome und im schlimmsten Fall lebensbedrohliche anaphylaktische Reaktionen. Ungefähr 60% aller allergischen Reaktionen auf Kuhmilch sind IgE-vermittelt und ungefähr 40% sind nicht-IgE-vermittelt. Die Mechanismen bei nicht-IgE-vermittelten Unverträglichkeiten sind nicht genau bekannt und können andere Immunglobuline und/oder zell-vermittelte Wirkungsweisen umfassen.

Der Schwerpunkt dieser Dissertation liegt in der Isolierung von cDNAs, die für Hauptallergene in der Kuhmilch kodieren, sowie in der Expression dieser Allergene in rekombinanter Form in *Escherichia coli*. Dabei wurden zwei Milchproteine, Alpha-Laktalbumin aus der Molkefraktion und AlphaS1-Kasein aus der Kaseinfraktion im Detail charakterisiert. Beide Allergene wurden hinsichtlich ihrer biochemischen Eigenschaften mittels SDS-PAGE, Zirkulardichroismus und Massenspektrometrie analysiert. Ihre IgE Reaktivität wurde durch Immunblotting und ELISA gezeigt und ihre biologische Aktivität durch Degranulation von basophilen Granulozyten bestätigt. Durch die Anwendung von synthetischen Peptiden wurden lineare Epitope auf beiden Allergenen gefunden und es konnte das Vorhandensein von Konformationsepitopen nachgewiesen werden. Diese beiden rekombinanten Allergene können nun für eine Komponenten-spezifische Diagnostik bei Patienten mit Kuhmilchallergie verwendet werden.

Weiters wurden die IgE-reaktiven und biologisch aktiven Allergene zur Entwicklung eines neuen Diagnosesystems verwendet, das aus einem Allergen-Mikroarray und einem Test zur Bestimmung von Mediatorenfreisetzung besteht. Die Mikroarray-Technologie erlaubt die Austestung von vielen verschiedenen Allergenen auf einmal, dies stellt vor allem bei dem Vorhandensein von kleinen Serummengen bei Säuglingen und Kleinkindern einen großen Vorteil dar. Die Kombination mit einem biologischen Test ermöglicht es, Patienten mit Risiko für starke systemische Reaktionen zu identifizieren. Dies ist sinnvoll bei starken Kuhmilchallergikern, die

beim Durchführen von *in vivo* Tests wie Provokationstests an starken Reaktionen leiden könnten.

Ein anderer Scherpunkt dieser Dissertation liegt in der Untersuchung von immunologischen Mechanismen, die durch eventuell nicht-IgE-vermittelte Reaktionen nach Konsum von Kuhmilch auftreten können. Die Bestimmung von IgG_{1-4} Subklassen und IgA Antikörpern gegen gereinigte rekombinante Proteine zeigte, dass Patienten mit nicht-IgE-vermittelter Kuhmilchunverträglichkeit basierend auf der humoralen Immunantwort auf Kuhmilchantigene nicht von Personen ohne Kuhmilchintoleranz unterschieden werden können. Wir sind deshalb zu dem Schluss gekommen, dass andere Antikörper als IgE geringen Einfluss auf Kuhmilchunverträglichkeiten haben.

Zusammenfassend war es möglich, innerhalb dieser Dissertation Hilfsmittel zu entwickeln, die bei der Komponenten-spezifischen Diagnostik von IgE-vermittelter und nicht-IgE-vermittelter Kuhmilchallergie eingesetzt werden können. Dadurch erhält man wertvolle Informationen, die in Zukunft bei der Entwicklung von Strategien zur Behandlung von Kuhmilchallergie verwendet werden können.

Immune responses to cow's milk allergens: Molecular allergen characterization and epitope mapping

Cow's milk allergy is the most prevalent food allergy in the first years of life, affecting about 2 to 3% of infants and young children in developed countries. Most cow's milk allergic patients suffer from symptoms in more than one organ system showing cutaneous, gastrointestinal, respiratory symptoms, and in the worst case anaphylactic reactions. Around 60% of all allergic reactions to cow's milk are IgE-mediated and around 40% are non-IgE-mediated. The mechanisms of the later might involve other immunoglobulins, immune complexes, and/ or cell-mediated mechanisms.

The main aim of this thesis was the isolation of cDNAs coding for major cow's milk allergens and the expression of these allergens in a recombinant form in *Escherichia coli*. The focus was on two milk proteins, alpha-lactalbumin, deriving from the whey fraction, and alphaS1-casein, deriving from the casein fraction. Both allergens were biochemically characterized using SDS-PAGE, circular dichroism analysis, and mass spectrometry. Their IgE reactivity was demonstrated by immunoblotting and ELISA and their allergenic activity was proven using rat basophil leukaemia cells transfected with the human FcεRI. Using synthetic peptides I could map sequential IgE-epitopes on the two allergens and I was able to show in case of both allergens also the presence of conformational IgE epitopes. These two recombinant allergens can be used for component resolved diagnosis of patients suffering from cow's milk allergy.

I further utilized the IgE-reactive and biologically active allergens for the development of a novel diagnostic system, consisting of an allergen microarray and a mediator release assay. The microarray technology has the advantage that a large panel of allergens can be evaluated in parallel especially when only small samples of serum are available as for infants and children. The combination with a biological assay allows identifying cow's milk allergic patients that suffer from severe systemic reactions. This diagnostic system might therefore represent a useful diagnostic tool for highly allergic cow's milk allergic patients where *in vivo* tests can not be applied because of the risk of allergic reactions.

Another part of my thesis was focused on the investigation of immunological mechanisms involved in non-IgE-mediated cow's milk allergic reactions. Determination of IgG_{1-4} subclass and IgA antibody levels to purified recombinant

allergens showed that based on their humoral immune response to cow's milk antigens, persons suffering from non-IgE-mediated cow's milk intolerance can not be discriminated from persons without cow's milk intolerance. We therefore concluded that antibody-mediated forms of hypersensitivity against cow's milk allergens, besides those mediated by IgE, play no or only a minor role in cow's milk intolerance.
In summary, I was able to develop tools for component resolved diagnosis of IgE-mediated and non-IgE-mediated cow's milk allergy and to gain information that might in the future lead to the design of new strategies for treatment of cow's milk allergy.

1. Introduction

1.1. THE HISTORY OF ALLERGY

One of the earliest reports of allergic disease is that of King Menses of Egypt. He was killed by a wasp sting in the year 2641 B.C. (1). It was further reported from ancient history, that Britannicus, the son of the Roman Emperor Claudius, was allergic to horses and "would develop a rash and his eyes swelled to the extent that he could not see where he was going" (2). Lucretius, a Roman philosopher, mentioned that some people had adverse reactions to common substances and said, "what is food to one may be fierce poison to others". According to Shakespeare, King Richard III suffered from strawberry allergy. In 1565, Leonardo Bottallo gave the first modern description of seasonal allergic disorder.

However, the era of allergy began in the 19th century:

In 1819 the British physician John Bostock first accurately described *hay fever* as a disease that affected the upper respiratory tract based on his own symptoms (3). In 1873, Charles Blackley, found the cause of his hay fever by scratching a small amount of grass pollen on his skin. This scratching produced a small hive-like reaction at the test site, his experiment introduced the concept of the *skin test* (4). Charles Richet and Paul Portier introduced the term *anaphylaxis* in 1902 (5), when life threatening responses to medications and protein substances appeared during the course of immunizations. The French immunologist Nicolas Maurice Arthus discovered one year later, that repeated injections of horse serum into rabbits induced edema, which is nowadays known to be a local vasculitis associated with deposition of immune complexes and activation of complement.

In 1906, the Austrian pediatrician Clemens von Pirquet (Fig. 1) noticed that patients had more severe reactions after a second injection of smallpox or horse serum. Together with the Hugarian-born physician Bela Schick, he coined the term *allergy*, from the Greek *allos* meaning "other" and *ergon* meaning "reaction, work" to describe this hypersensitivity reaction (6). Today, allergy is defined as a "hypersensitivity reaction initiated by specific immunologic mechanisms" (7).


MÜNCHENER
MEDIZINISCHE WOCHENSCHRIFT.
ORGAN FÜR A TISCHE ÄRTZE.
No. 29. 24. Juli 1906.
53. Jahrgang.
Originalien
Allergie.
Von C. v. Pirquet.

Fig. 1. The term “allergy” was first published in 1906 by Clemens von Pirquet in the Münchener Medizinische Wochenschrift (8)

The work of Leonard Noon and John Freeman (1911-1914), giving injections of small, gradually increasing amounts of allergens to allergic patients, started the beginning of modern desensitizing immunotherapy. Prausnitz and Kuestner showed in 1921 that an allergic reaction depends on two agents, a serum factor “reagin” and a non-specific tissue component. Almost 50 years later, the Japanise couple Kimishige and Teruko Ishizaka discovered the role of *immunoglobulin E* as the most important mediator in an allergic reaction (9). At the same time the Swedish group of Johannson, Bennich and Wide discovered an atypical myeloma immunoglobulin, which was then identified as *immunoglobulin E* (10).

Two British immunologists Philip Gell and Robert Coombs classified hypersensitivity reactions into four types which is nowadays known as “Gell-Coombs-classification” (Fig. 2) (11).

1.2. GELL AND COOMBS CLASSIFICATION

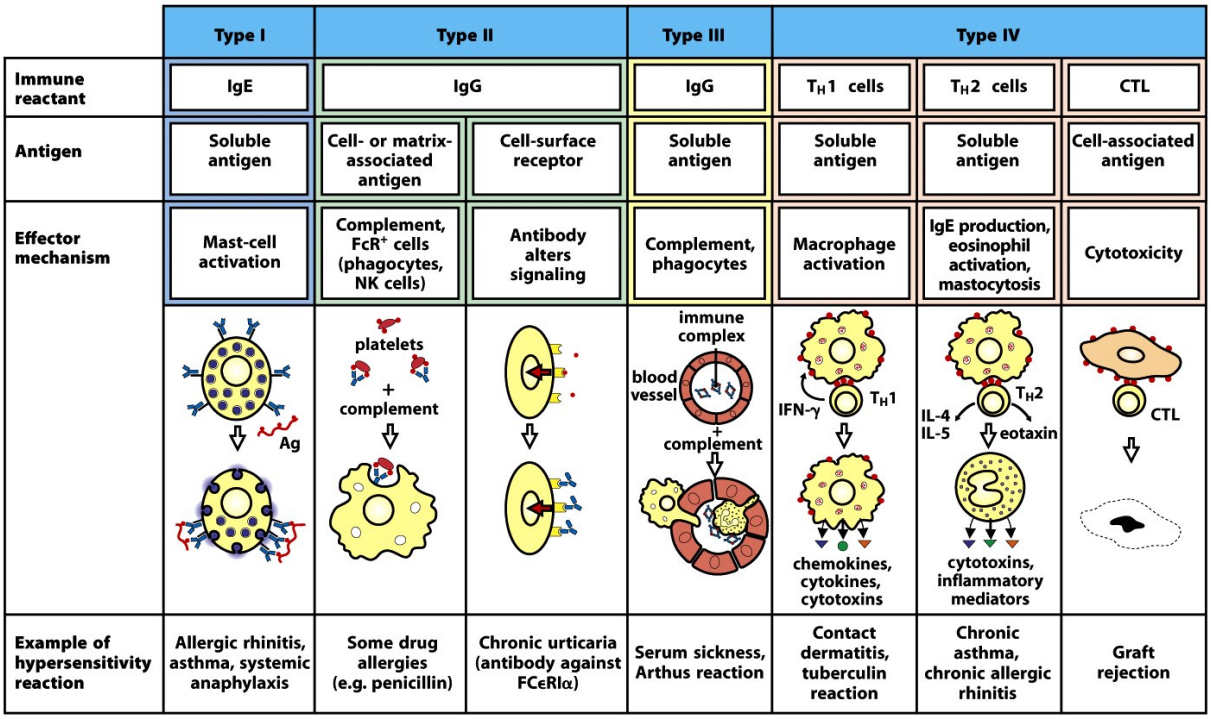

Fig. 2. Classification of hypersensitivity reactions into four types by Gell and Coombs (12)

1.2.1. TYPE I: IMMEDIATE HYPERSENSITIVITY OR ANAPHYLAXIS

Type I allergy, also referred as IgE-mediated allergic reaction, affects around 25% of the population in industrialized countries (13). *Atopy* (from the Greek *atopos* meaning “out of place”) was introduced by Arthur Coka and Robert Cooke in 1923 to describe the hereditary, familial disposition in a person to produce an allergic response to ordinary harmless materials such as pollen, dust, and animal dander and is often used in combination with type I allergy. Clinical manifestations of these reactions are either local as allergic rhinitis and allergic asthma or systemic as anaphylaxis.

The reason for the development of an atopic disease is still not completely clear, several possibilities are discussed nowadays. Genetic predisposition (14, 15) and environmental factors such as air pollution, dietary changes, and exaggerated hygiene may play an important role why harmless antigens are wrongly recognized and induce IgE production (16-18).

The mechanisms of allergic reactions can be summarized in three steps:

1.2.1.1. Sensitization and memory induction

After small amounts of allergen are deposited on a mucosal surface, the allergen is taken up by an antigen-presenting cell (APC) or by an immunoglobulin bound to the surface of a B cell. The APC presents a peptide via the MHC to the T-cell receptor of a naïve T-cell and this can induce the formation of helper 2 (Th2) cells. The differentiation and clonal expansion of Th2 cells go along with the production of cytokines such as interleukins IL-4 and IL-13. These interleukins promote the class switch of B cells to IgE and the establishment of IgE^+memory B-cells (Fig. 3) (19).

1.2.1.2. Immediate phase

Mast cells and basophils that bind IgE via the high affinity IgE receptor (FcεRI) play a major role in the immediate phase. Repeated contact with an allergen leads to a cross-linking of FcεRI-bound IgE and then to a degranulation of mast cells and basophils. Released histamine, besides other vasoactive amines, lipid mediators, chemokines and cytokines can induce immediate symptoms such as tissue swelling, itching or in the worst case decrease of blood pressure (Fig. 3).

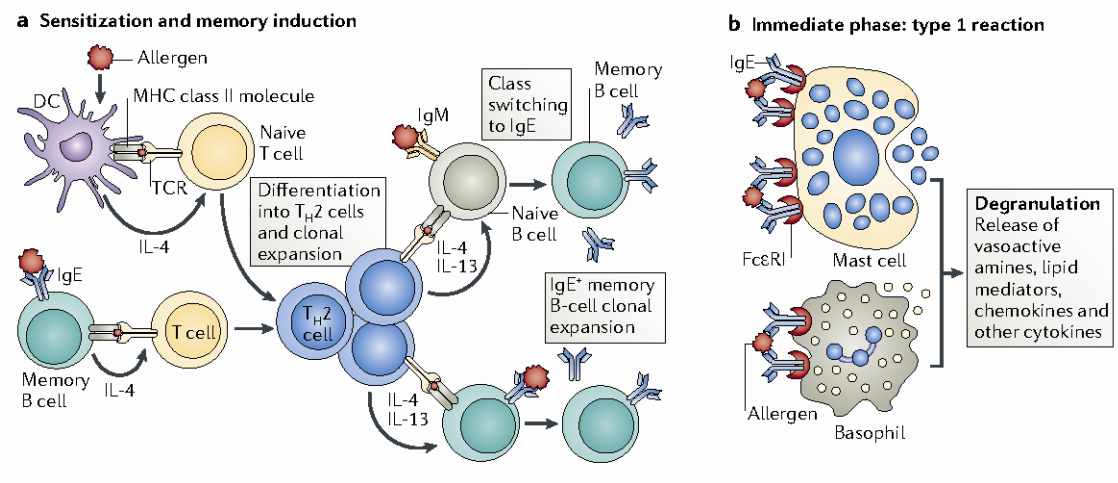

Fig. 3. Sensitization, memory induction and immediate phase of an allergic reaction (19)

1.2.1.3. Late phase: allergic inflammation

In this phase symptoms appear normally after two or more hours and involve the infiltration and activation of Th cells, basophils and eosinophils. These cells release

proinflammatory cytokines (e.g. IL-6, IFN-γ, TNF) and cause symptoms like asthma or atopic dermatitis (Fig. 4) (19).

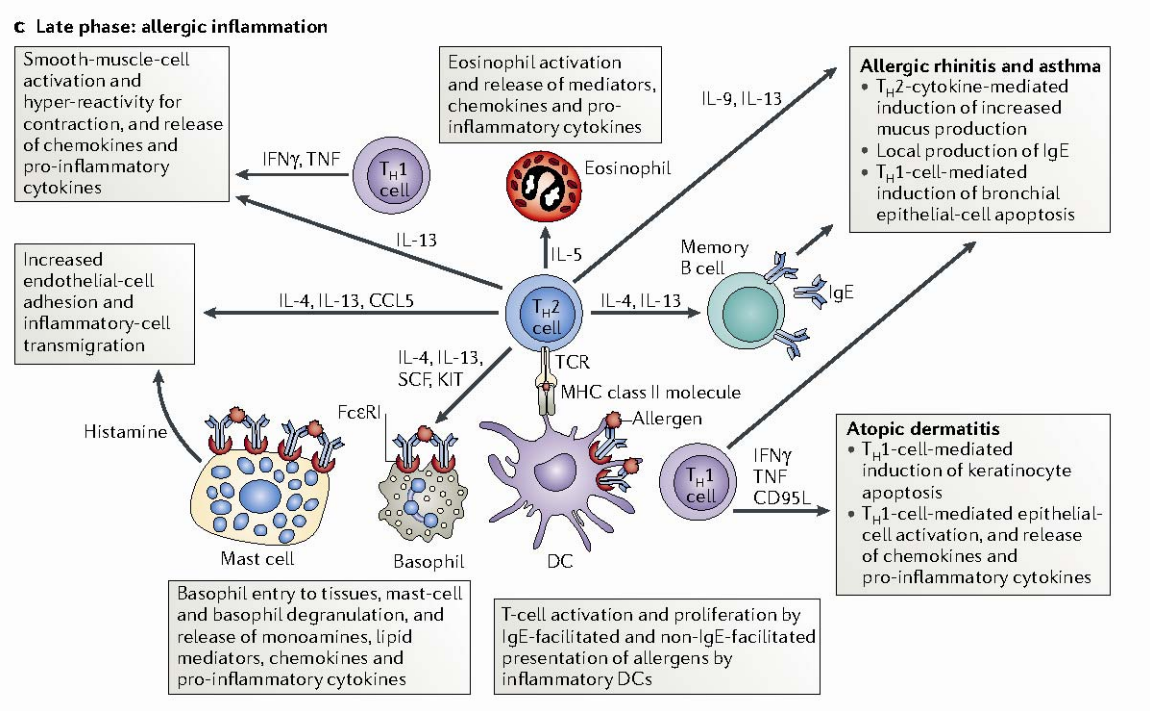

Fig. 4. Late phase of an allergic reaction (19)

1.2.2. TYPE II: ANTIBODY-MEDIATED CYTOTOXIC REACTIONS

Type II reactions, also known as cytotoxic reactions, are antibody-mediated. Cytotoxic antibodies, mainly IgM or IgG, that bind to the surface of target cells, can induce cell damage due to different mechanisms. In the course of an antibody-dependent cell cytotoxicity reaction (ADCC), immunoglobulin-coated target cells attract destructive effector cells (macrophages, neutrophils, eosinophils). In another mechanism the classical pathway of the complement systems gets activated through bound-antibodies, resulting in cell lysis (11).

1.2.3. TYPE III: IMMUNE COMPLEX-MEDIATED REACTIONS

In this type of hypersensitivity soluble antigens react with precipitating antibodies in the tissue and form immune complexes in and around small vessels that cause damage to the cells. Soluble immune complexes can be deposited in the endothelial lining of blood vessel walls in the lung, joints, kidney, and skin. Activation of the complement initiates tissue injury. Macrophages and neutrophils further contribute to this tissue damage (11).

1.2.4. TYPE IV: DELAYED HYPERSENSITIVITY

Contrary to type I-III, this type of hypersensitivity is cell-mediated. The reaction involves antigen-presenting cells that present the antigen to T-lymphocytes which results in proliferation and release of cytokines. Symptoms usually develop within 2-14 days and appear mainly as skin eruptions after exposure to drugs, cosmetics, and environmental chemicals (20, 21). To understand the inflammatory processes during type IV hypersensitivity these reactions have nowadays been grouped into 4 subtypes: type IVa: corresponds to Th1 reactions with mainly IFN-γ, IL-1, and IL-2 production and involves monocytes/macrophages activation; type IVb: corresponds to Th2 responses with cytokine production such as IL-5, IL-4, and IL-13 and activates eosinophils; type IVc: involves cytotoxic cells and production of perforins and granzymes; type IVd: involves T-cells, CD4 and CD8, and neutrophils with cytokine production as IL-8 and GM-CSF (22).

1.3. FOOD ALLERGY

Around 6 to 8% of children younger than 10 years and 1 to 4% of the adult population suffer from food allergies, the “Big-8” food allergens are cow’s milk, hen’s egg, soy, wheat, peanuts, tree nuts, fish, and shellfish (23, 24).

Only a very small number of food proteins are responsible for the allergic reactions compared to the large quantity of proteins that are consumed every day in a normal diet. In young children, mainly milk, eggs, peanuts, soy, and wheat elicit adverse reactions, in adults, peanuts, fish, shellfish, and tree nuts trigger allergic reactions. The food allergens are characterized as water-soluble glycoproteins, ranging from 10 to 70 kDa in size with a high stability against heat, acid, and proteases (25).

Adverse reactions to foods can be classified into true food allergy or non-allergic food intolerances. Intolerances to foods are elicited by different components of the food such as pharmacological ingredients like monosodium glutamate or histamine, non-specific mast cell activation by irritating foods such as strawberries or additives or are elicited by deficiencies in the host like lactase deficiency (26). Symptoms elicited by non-allergic reactions can be flushing, hypotension, urticaria, and in the worst case anaphylactoid reactions (27).

1.4. COW'S MILK ALLERGY

The first observations of adverse reactions to cow's milk were described by Hippocrates (prior to 370 B.C.) as urticaria and gastric upset after cow's milk consumption (28). 500 years later, Galen also described a cow's milk allergic reaction (29). In the beginning of this century adverse reactions to cow's milk were described as "idiosyncrasy" and mainly published in the German literature. It was Wernstedt, in Sweden, who linked such reactions to allergy (30). Since 1950s, more allergic reactions to cow's milk are described, which might be related to the decrease in breast feeding and increase in feeding with cow's milk based formulas (31, 32).

Nowadays, cow's milk allergy is the most prevalent food allergy in the first years of life, affecting about 2 to 3% of infants and young children and 0.5% of purely breastfed infants in developed countries. Cow's milk allergy usually appears after cow's milk or a cow's milk-based formula is introduced into the diet. Cow's milk allergic infants suffer normally from two or more symptoms affecting two or more organ systems (Table I).

Table I. Signs and symptoms of cow's milk allergy (33)

Gastrointestinal	Vomiting, regurgitation
	Abdominal pain
	Colic
	Diarrhoea
	Constipation
	Haemochezia
Skin	Rash
	Atopic eczema
	Urticaria
	Swollen lips
	Angio-oedema
	Pruritis
Respiratory	Rhinitis
	Concuntivits
	Hoarseness, dysphagia
	Wheezing, asthma
General	Food refusal
	Growth retardation
	Iron-deficient anaemia
	Irritability, disturbed sleep
	Apnoea, apparent life-threatening events
	Anaphylaxis

Most cow's milk allergic infants suffer from gastrointestinal symptoms, around 50-70% have cutaneous problems, and about 20-30% will suffer from respiratory

symptoms. Severe symptoms as anaphylactic reactions appear in the worst case. Sixty % of all allergic reactions to cow's milk are IgE-mediated and around 40% are non-IgE-mediated (Fig. 5) (34-36).

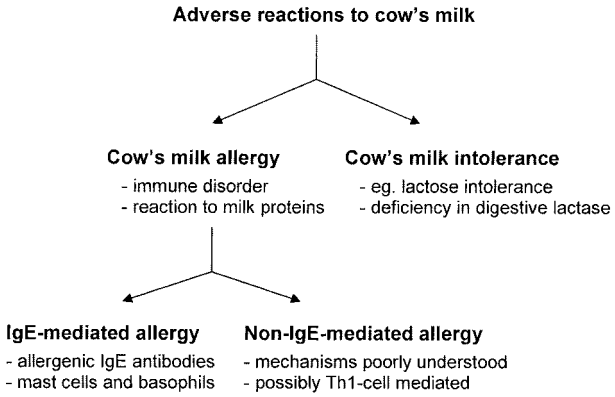

Fig 5. Classification of adverse reactions to cow's milk (24)

The majority of cow's milk allergic infants outgrow their cow's milk allergy, 45 to 50% at 1 year, 60 to 75% at 2 years, and 85 to 90% at 3 years (34). Unfortunately, many of the infants that outgrow cow's milk allergy develop other atopic diseases as asthma, hay fever, or dermatitis to inhalant allergens over the years, also termed "atopic march" (37). It is nowadays assumed that the persistency to cow's milk allergy is related to a different IgE-epitope recognition pattern on the cow's milk allergens (38, 39).

Only 0.1 to 0.5% of adult patients suffer from cow's milk allergy (40). The majority of these patients acquire cow's milk allergy at adult age, in one third cow's milk allergy persists from infancy. These adult patients are very often strongly allergic to cow's milk, most frequently problems are oropharyngeal symptoms and symptoms of the skin. Anaphylactic reactions to cow's milk seem to appear more often in adults than children (41, 42).

1.4.1. COW'S MILK ALLERGENS

Cow's milk contains around 30 to 35g of proteins per liter. Through the acidification of raw skim milk to pH 4.6 at 20°C two fractions are obtained: caseins that contain 80% of total cow's milk proteins and whey that contains around 20% of the proteins (Table II) (43-45).

Table II. Characterization of major cow's milk proteins, adapted after Jost R. (46)

Proteins (Concentration % milk proteins)		Concentration (g/l)	Size (kDa)	Number of amino acid residues per molecule	S-S bridges per molecule	Secondary structures (% of total chain) α-helix	β-sheet	pI
Whey (20%)	β-Lg (10%)	3-4	18.3	162	2 + 1 free SH	+++	15	5.3
(~5g/l)	α-La (5%)	1-1.5	14.2	123	4	++	25	4.8
	Igs (3%)	0.6-1.0	150					
	BSA (1%)	0.1-0.4	66.3	582	17 + 1 free SH	+++	50	4.9-5.1
	Lf (traces)	0.09	80	703	16			8.7
Whole casein (80%)	αS1-cas (32%)	12-15	23.6	199		+	4-15	4.9-5
(~30g/L)	αS2-cas (10%)	3-4	25.2	207	1			5.2-5.4
	β-cas (28%)	9-11	24	209		+	1-10	5.1-5.4
	κ-cas (10%)	3-4	19	169	1	++	14	5.4-5.6

S-S bridge, disulphide bridge; free SH, free cysteine

1.4.1.1. Caseins

This fraction consists of αS1-casein (αS1-cas), αS2-casein (αS2-cas), β-casein (β-cas), and κ-casein (κ-cas). The caseins share little sequence homology but share features as heat resistance, high lability to proteases and exopeptidases and are extensively digested in the gut. The proteins are phosphorylated, the tertiary structure is highly hydrated and flexible. Together the four caseins form aggregates, micelles that are in suspension in the aqueous phase of the milk (45).

1.4.1.2. Whey

This fraction is made up of mainly α-lactalbumin (α-la), β-lactoglobulin (β-lg) but also contains bovine serum albumin (BSA), immunoglobulin (Ig), and lactoferrin (Lf).

Alpha-lactalbumin is a small (14.2 kDa), acidic (pI 4.8) monomeric globular protein of 123 amino residues with a single calcium binding site. The binding of calcium stabilizes the secondary structure of the molecule. Alpha-lactalbumin plays an important role in the biosynthesis of lactose through the interaction with lactose synthase (45).

Beta-lactoglobulin is a dimeric protein, with 162 amino acids per peptide and a total size of 36 kDa. It is the most abundant whey protein in cow's milk but absent in human milk (47). The protein has 2 disulfide bridges that form the typical tertiary structure of a retinol-binding protein that belongs to the lipocalin family. The tertiary structure of lipocalins is made up of a beta-barrel with 8 or 10 antiparallel beta-sheets. The protein has a high resistance against proteases in the gut, therefore large fragments thereof are presented to immune cells (48, 49).

Lactoferrin is an iron-binding glycoprotein with 703 amino acids and a size of 80 kDa that belongs to the transferrin family. Besides its function as a scavenger of free radicals and an antioxidant, it has antimicrobial properties and positive influence on the defence against infections (45).

Bovine serum albumin has 583 amino acids, a weight of 66.4 kDa and contains 17 disulfide bonds (45). BSA is physically and immunologically identical to blood serum albumin, that plays a role in transport, metabolism, distribution of ligands, osmotic pressure of blood and prevention of free radicals (50).

1.4.2. RECOMBINANT COW'S MILK ALLERGENS

Nowadays more than 40 food allergens are available in recombinant form which guarantees standardized quality and also high quantities. For this purpose it is necessary to identify relevant allergen sources, isolate mRNA and synthesize cDNA. The relevant allergen-encoding cDNAs can be isolated from a cDNA library by immunoscreening technology with sera from allergic patients or by a PCR-based approach. In the next step, the allergen-encoding cDNAs are inserted into a vector, which allows the production of pure and stable allergens that contain the same epitopes as natural allergens, using *Escherichia coli* or other expression systems (51). Natural allergen extracts are rarely standardized, so the amounts of allergens can differ from one extract to the other, and the immunological activities are also not defined. Therefore recombinant allergens have a big advantage over natural extracts, they improve diagnosis and facilitate an important progress from extract-based diagnosis to a component-resolved diagnosis. With the usage of recombinant purified proteins possible cross-reactivities and potential severity of the symptoms can be tested (52). Further, recombinant proteins can be modified for immunotherapy so that only T-cell epitopes but no B-cell epitopes are recognized during desensitization (53).

1.5. NON-IGE-MEDIATED FOOD ALLERGY

The immunological mechanisms of non-IgE-mediated food allergy are still unclear and since most of the symptoms are delayed (after several hours or days) there are always difficulties in connecting the allergy eliciting food with the symptoms. Besides, the diagnostic tools for non-IgE-mediated reactions are very limited (54).

Symptoms affect mainly the gastrointestinal tract (diarrhea, indigestion, bloating, loose stool, chronic relapsing reflux, abdominal pain). Pediatric eosinophilic

oesophagitis is the most studied non-IgE-mediated food disorder. It is characterized by eosinophils that infiltrate the oesophagal epithelia in contrast to eosinophilic gastroenteritis where eosinophils are present in the stomach and/ or duodenum (55).

1.6. DIAGNOSIS OF IGE-MEDIATED FOOD ALLERGIES/ COW'S MILK ALLERGY

The diagnosis of food allergies starts with a detailed history and physical examination including possible allergy-eliciting foods, amount of ingested foods, time course of reaction, reaction consistency, and additional factors as intake of drugs, exercise, and stress. In a next step, different *in vitro* and *in vivo* diagnostic tests can be carried out as described below:

1.6.1. IN VITRO DIAGNOSTIC TESTS

1.6.1.1. Determination of serum IgE antibody levels

Measurement of food-specific IgE can be performed with RAST (radioallergosorbent test) or ImmunoCap (Pharmacia, Sweden) testing. Patients' sera are exposed to solid matrix-bound allergens and then detected by a labeled antibody specific for the Fc portion of human IgE. The sensitivity of IgE tests is very high. Since these tests can deliver irrelevant positive results, the clinical history has to be considered for the interpretation of the test results (26, 56).

A new test system, called microarray technology, enables a thorough analyses of several hundreds allergens in one step. Recombinant as well as natural proteins and synthetic peptides are dotted on a carrier surface and tested with very small amounts of patients' sera which is especially important in cow's milk allergy where mainly infants are affected (57, 58). These microarrays add new information to patients' individual sensitization profiles including determination of cross reactive allergens and may facilitate selection of the right allergens for component resolved immunotherapy (CRIT) (59).

1.6.1.2. Basophil mediator release

Different systems are nowadays established to test if allergens capable of binding human IgE also have biological activity in terms of mediator release. Basophils from sensitized patients, IgE-depleted stripped basophils from healthy donors, basophil cell lines or animal cell lines transfected with human IgE high affinity receptor are loaded with patients' sera containing IgE antibodies and are then stimulated with

allergens. Crosslinking of FcεRI-bound IgE induces the release of histamine, which can be measured by radioimmunoassay or enzyme-linked immunosorbent assays. Alternatively basophil activation can also be monitored by measurement of the surface activation markers CD63 and CD203c (60).

1.6.2. IN VIVO DIAGNOSTIC TESTS

1.6.2.1. Skin prick test (SPT)

SPT is a very rapid and cheap possibility to screen patients with suspected food allergy. Glycerinated food extracts or fresh extracts and a saline-glycerin control are applied by the prick or puncture technique. If the patient has IgE antibodies against the food allergen, a wheal at least 3 mm larger than the saline control will appear.

1.6.2.2. Patch testing

Atopy patch testing (APT) is normally used for diagnosis of delayed-type (cell-mediated) allergy (61). Commercial preparations for APT or fresh food allergen sources are applied externally, normally at the back of the patients for up to 24 hours and skin reactions are documented after another 24 to 48 hours (62).

1.6.2.3. Oral food challenge (open or double blind)

Food challenges are very important tools when the diagnosis of cow's milk allergy remains uncertain (33, 63). Patients consume increasing quantities of the suspected food and the challenge is stopped as soon as adverse reactions appear or after a considerable amount has been consumed without reactions. An open food challenge (not double blind or placebo controlled) can be used to confirm negative diagnostic results, in case of positive diagnostic results a double blind, placebo controlled food challenge (DBPCFC) should be applied (27). Since oral food challenges are time consuming, costly, and bear the risk of inducing strong adverse reactions, some studies investigate how predictive specific IgE tests are and if they can replace oral food challenges (64-66).

1.7. TREATMENT OF COW'S MILK ALLERGY

1.7.1. Avoidance of allergens

At the moment, the most frequently recommended treatment of cow's milk allergy is the elimination of cow's milk from the daily nutrition after obtaining a detailed clinical

history and after identification of cow's milk as the allergy-eliciting food component (33). Physicians need to keep the improvement of symptoms, nutritional deficiencies, increase in cost and time in mind when they choose an appropriate diet (67). Management of cow's milk allergy can also include a special diet for nursing mothers and the usage of substitutions based on extensively hydrolyzed cow's milk for cow's milk allergic infants (26, 68, 69).

1.7.2. Food allergy prevention

The European and American recommendations indicate that children with atopic risk should be exclusively breast fed for 4-6 months, followed by delayed introduction of solid foods. There is no confirmation that avoidance of antigens by the mothers during pregnancy or lactation has shown a positive influence on atopic disease prevention (70, 71).

1.7.3. Emergency treatment

This treatment needs to be explained very carefully by the physician and normally includes oral anthistamine for mild reactions affecting the skin or the gut and self-injectable adrenaline for systemic or respiratory reactions (26).

1.7.4. Immunotherapy

Subcutaneous immunotherapy as routinely used for treatment of cat allergy, venom allergy, and several respiratory allergies is very rarely used for the treatment of food allergy where the risk of severe systemic reactions is too high (72, 73). Therefore oral immunotherapy, which induces tolerance by exposing the patient to increasing amounts of the food antigen via the mucosal route, has become the favored therapy (74, 75). Promising improvements in life quality were demonstrated especially in patients with severe and persistent cow's milk allergy (76, 77).

Sublingual immunotherapy, where the antigen is kept under the tongue for some minutes before it is swallowed, is mainly used for treatment of respiratory allergy and very rarely applied for treatment of peanut allergy or cow's milk allergy (78, 79).

Immunomodulatory treatment with anti-IgE molecules has been tested for peanut allergy. However, the safety concerns were very high and the outcome of the study was not so promising (80).

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Aim of the thesis & declaration of the contribution on the manuscripts

The main focus of the work was the molecular characterization and epitope mapping of important allergens and the investigation of immune responses to cow's milk allergens.

Two important cow's milk proteins were analyzed in detail, alpha-lactalbumin that belongs to the whey fraction and alphaS1-casein that belongs to the casein fraction of cow's milk. Based on these studies, tools for better diagnosis of cow's milk allergy were developed. The expression, biochemical characterization, IgE reactivity, and allergenic activity of alpha-lactalbumin are described in **manuscript 1** and of alphaS1-casein in **manuscript 2**.

Manuscript 1: **H. Hochwallner**, U. Schulmeister, I. Swoboda, M. Focke-Tejkl, V. Civaj, N. Balic, M. Nystrand, A. Härlin, J. Thalhamer, S. Scheiblhofer, W. Keller, T. Pavkov, B. Niggemann, S. Quirce, A. Mari, G. Pauli, C. Ebner, N.G. Papadopoulos, U. Herz, E.A.F. van Tol, R. Valenta, and S. Spitzauer. Molecular Characterization, IgE Epitope Mapping, and Assessment of Allergenic Activity of Recombinant Alpha-Lactalbumin, a Major Cow's Milk Allergen. (Submitted)

Contribution:
Expression of recombinant protein, analysis of IgE binding properties, calcium depletion experiments, analysis of data, writing of the manuscript
80 percent

Manuscript 2: U. Schulmeister, **H. Hochwallner**, I. Swoboda, M. Focke-Tejkl, B. Geller, M.Nystrand, A. Härlin, J. Thalhamer, S. Scheiblhofer, W. Keller, B. Niggemann, S. Quirce, C. Ebner, A. Mari, G. Pauli, U. Herz, R. Valenta, and S. Spitzauer. Cloning, Expression, and Mapping of Allergenic Determinants of alphaS1-Casein, a Major Cow's Milk Allergen. *J Immunol.* 2009 Jun 1;182(11):7019-29

Contribution:
Expression and purification of the protein, contribution to the analysis of the data and to the preparation of the manuscript
50 percent

In **manuscript 3** we established an assay that combined IgE-reactivity testing of recombinant milk allergen components by microarray and basophil degranulation and analyzed IgE reactivity as well as allergenic activity to individual cow's milk allergens in a large cohort of cow's milk allergic patients.

Manuscript 3: H. Hochwallner,* U. Schulmeister,* I. Swoboda, B. Geller, M. Nystrand, A. Härlin, J. Thalhamer, S. Scheiblhofer, B. Niggemann, S. Quirce, C. Ebner, A. Mari, G. Pauli, U. Herz, E. A.F. van Tol, R. Valenta, and S. Spitzauer. Microarray and Allergenic Activity Assessment of Milk Allergens. *These authors contributed equally to this work. (Submitted)

Contribution:
Expression and purification of recombinant proteins, analysis of microarray-data and results from the basophil activation tests, writing of the manuscript
70 percent

Finally, in **manuscript 4** we focussed on the investigation of non-IgE-mediated cow's milk hypersensitivities and we tested whether these adverse reactions can be diagnosed based on IgG subclass and IgE measurements to the individual cow's milk allergens.

Manuscript 4: H. Hochwallner, U. Schulmeister, I. Swoboda, M. Kundi, H. Vogelsang, L. Kazemi-Shirazi, S. Quirce, N. Balic, R. Valenta, and S.Spitzauer. Patients suffering from Non-IgE-Mediated Cow's Milk Intolerance cannot be diagnosed based on IgG-Subclass or IgA Responses to Milk Allergens. (Submitted)

Contribution:
Design of study, ELISA analysis, immunoblot experiments, writing of the manuscript
85 percent

2. Molecular Characterization, IgE Epitope Mapping, and Assessment of Allergenic Activity of Recombinant Alpha-Lactalbumin, a Major Cow's Milk Allergen

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Submitted, 2009

Molecular Characterization, IgE Epitope Mapping, and Assessment of Allergenic Activity of Recombinant Alpha-Lactalbumin, a Major Cow's Milk Allergen[1]

Running title: Characterization of Cow's Milk Allergen Alpha-Lactalbumin

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Abstract

Cow's milk allergy is the most common cause of IgE-mediated hypersensitivity reactions in infancy. We isolated a cDNA coding for α-lactalbumin (α-La), a calcium-binding allergen belonging to the whey fraction of cow's milk, from a mammary gland cDNA library. The α-La cDNA coding for a 14.1 kDa protein was expressed as recombinant protein (rα-La) in *Escherichia coli* and was purified to homogeneity. According to circular dichroism analysis the allergen represented a folded protein with a high thermal stability and refolding capacity. Micro-arrayed rα-La reacted with IgE antibodies from 57.6% of cow's milk allergic patients (n=66) and hence represents a major cow's milk allergen. The reduction of IgE reactivity by depletion of protein-bound calcium suggested the presence of conformational IgE epitopes. Using eight synthetic peptides of 19-20 amino acids spanning the complete α-La sequence, sequential IgE epitopes rich in surface-exposed amino acids were identified which clustered at the N- and C-terminus of the protein. A superposition of the peptides onto the three-dimensional structure of α-La revealed a close vicinity of the N- and C-terminal peptides within one surface-exposed patch. rα-La induced strongest degranulation of rat basophil leukaemia cells transfected with human FcεRI when cells were loaded with serum IgE from cow's milk allergic patients who had exhibited gastrointestinal symptoms or severe systemic reactions upon cow's milk exposure. rα-La may be used for the diagnosis of patients suffering from severe allergic reactions to cow's milk and serve as a paradigmatic tool for the development of therapeutic strategies for cow's milk allergy.

Introduction

Food allergies are an important health problem worldwide with increasing incidences and can be responsible for life-threatening anaphylactic reactions (1). Up to 11% of children and approximately 2% of the adult population experience food-induced allergic disorders (2, 3). Cow's milk is the first component introduced into the diet and it is the most common cause of food allergy in young children under five years of age, affecting about 2 to 3% of infants and young children in developed countries (3, 4).

Acute reactions to cow's milk are mediated by activation of mast cells and basophils by IgE-allergen immune complexes (5, 6). In addition, cow's milk allergens and peptides thereof can induce T cell-mediated inflammation (7). Both types of immune responses affect the gut but also other target organs such as the skin and the respiratory tract. Depending on the involved immune mechanisms, they may result in different manifestations, i.e., acute or delayed type reactions (e.g., IgE-mediated: acute gut symptoms, rhinitis, asthma, urticaria, and anaphylactic shock; T-cell-mediated: delayed gut symptoms, chronic asthma, and atopic dermatitis) (8).

The development of allergic sensitization to cow's milk allergens and allergen sensitivity may depend on several factors including exposure, dose, genetic background of the host as well as environmental influences (9-12) and is modulated by immunological control mechanisms such as regulatory T cells and effector cell sensitivity (13, 14).

Cow's milk contains 30 to 35 g of proteins per liter which can be separated using biochemical techniques into a casein fraction that contains 80% of total cow's milk proteins and a whey fraction that contains approximately 20% of the proteins. The casein fraction contains αS1-, αS2-, β-, and κ-casein, the whey fraction comprises α-lactalbumin, β-lactoglobulin, bovine serum albumin, lactoferrin, and immunoglobulins (15). These proteins are known to elicit an allergic immune response but data regarding the frequency of IgE sensitization and the magnitude of the allergic immune response towards the individual allergens show considerable variability or are lacking (16-19).

Several studies have identified αS1-casein as a major cow's milk allergen which induces strong immediate as well as delayed type allergic reactions (19, 20, 21). Despite that αS1-casein represents a class I food allergen, it was found to contain conformational as well as sequential IgE epitopes (19). β-lactoglobulin represents

another important cow's milk allergen which is recognized by approximately 50% of cow's milk allergic patients and for which IgE- as well as T cell epitopes have been studied (22-25). However, for α-La a widely varying prevalence of IgE recognition ranging from 6% to 100% has been reported in the literature (17, 18, 20, 21, 26, 27). α-La plays an important role in the biosynthesis of lactose through the interaction with lactose synthase (28). It is expressed exclusively during lactation in the mammary gland and accounts for 20% of bovine whey, the remaining 80% being mostly β-lactoglobulin (29).

In this paper, we report the isolation of a cDNA coding for α-La, the expression of the recombinant allergen in *Escherichia coli* and its purification to homogeneity. rα-La was characterized regarding its fold by circular dichroism and a mapping of sequential as well as conformational IgE epitopes was performed to localize the major IgE epitopes on the three dimensional structure of the allergen. Using IgE microarray technology and the transfer of serum IgE from cow's milk allergic patients with defined clinical symptoms to rat basophils transfected with the human FcεRI the IgE reactivity and allergenic activity of rα-La was determined and related to clinical manifestations.

Materials and Methods

Isolation of an IgE-reactive cDNA coding for α-La and sequence analysis

The cDNA coding for mature α-La without N-terminal signal sequence and with a C-terminal hexahistidine tag (rα-La) was obtained by PCR-amplification using a cow's mammary gland cDNA library as template (19). PCR amplification of the cDNA was done with the Pfu DNA polymerase system from Fermentas (Fermentas Life Sciences, Vilnius, Lithuania) and the following oligonucleotides (MWG Biotech, Ebersberg, Germany): α-La 5': 5'–GCGGATCCACATATGGAACAGTTAACAAAATG TGAG– 3' (*Nde*I underlined); α-La 3': 5'–CGGAATTCCTGCAGAACTCA*GTGATGAT GATGATGATG*CAACTTCTCACAGAGCCACTGATCCAGC–3' (*Eco*RI underlined; His tag-encoding DNA: italics). The PCR product was gel purified and cloned into the *Nde*I/ *Eco*RI digested expression vector pET 17b (Novagen, Cambridge, UK). The sequence was verified by automated sequencing of both DNA strands (VBC Genomics, Vienna, Austria).

The deduced protein sequence of α-La from cow (Bt) as determined by us and the protein sequences from α-La of other mammalian species which had been obtained from the NIH database (http://www.ncbi.nlm.nih.gov) were aligned manually in Fig. 1 for maximal fit. Potential glycosylation sites were predicted with a NetNGlyc 1.0 Server-Program (http://www.cbs.dtu.dk/services/NetNGlyc/).

Expression and purification of rα-La

For expression of rα-La the *E. coli* strain BL 21 Codon Plus (DE3)-RIPL (Stratagene, La Jolla, CA) was transformed with the α-La-pET 17b plasmid. Bacteria were grown in Luria Broth medium containing 100 mg/l ampicillin (Sigma-Aldrich, Vienna, Austria) and 50 mg/l chloramphenicol (Sigma-Aldrich) at 37°C to an OD (600 nm) of 0.4-0.6. Protein expression was induced by addition of isopropyl-ß-D-thiogalactoside (IPTG, Roche, Mannheim, Germany) to a final concentration of 0.5 mM and growing of the bacteria for additional 3h at 37°C. Bacteria were harvested by centrifugation, bacterial cells were lysed by repeated freeze thaw cycles, and proteins were solubilized in urea buffer by stirring (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8). rα-La was purified under denaturing conditions using Ni-NTA resin affinity columns according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Fractions containing the recombinant allergen were pooled, desalted and the buffer

was exchanged against 10 mM Tris buffer (pH 8.5) with a PD-10 column (Sephadex, GE Healthcare, Uppsala, Sweden). The protein concentration was determined with a Micro BCA Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. The purity was analyzed by SDS-PAGE (30) and Coomassie Brilliant Blue staining (Biorad, Hercules, CA).

To confirm the identity of his-tagged rα-La, the protein was blotted onto nitrocellulose (31) and incubated with an anti-hexahistidine antibody (Histidin-Tag, Dianova, Hamburg, Germany) 1:1000 diluted in PBST over night, and detected with ^{125}I-labeled sheep anti-mouse IgG antibody (GE Healthcare), diluted 1:1000 in PBST.

Matrix-assisted laser desorption and ionization-time of flight mass spectrometry of rα-La

Laser desorption mass spectra were obtained in a linear mode with a time-of-flight Compact MALDI II instrument (Kratos, Manchester, U.K.; piCHEM, R&D, Graz, Austria). The protein was dissolved in 10% acetonitrile, 0.1% trifluoroacetic acid) and α-cyano-4 hydroxy-cinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For the preparation of the sample a 1:2 mixture of protein and matrix solution was deposited onto the target and air-dried.

Circular dichroism analysis of purified rα-La

Circular dichroism (CD) spectra were performed on a Jasco J-810 spectropolarimeter (JASCO Corporation, Tokyo, Japan) fitted with a Jasco PTC-423S/L Peltier type temperature control system. Far-ultraviolet CD spectra were recorded in a 2 mm path-length quartz cuvette (Hellma, Mullheim, Baden, Germany) at a protein concentration of 0.26 mg/ml. Far-UV CD spectra were analyzed from 190 to 260 nm with 1 nm resolution at a scan speed of 50 nm/min and resulted from the average of three scans. All measurements were performed in 1 mM Tris (pH 8.5). Temperature scans were performed according to a step-scan procedure, where the sample was heated from 25°C to 95°C with a heat rate of 2°C/min and cooled back to 25°C at the same rate. Every 5°C continuous wavelength spectra were recorded with the specified parameters. The final spectra were corrected by subtracting the corresponding base line spectrum obtained under identical conditions. Results were expressed as the mean residue ellipticity [Θ] at a given wavelength. For the EGTA

experiments 10 mM EGTA was added to the recombinant protein before CD analysis.
The secondary structure content of rα-La was calculated using the secondary structure estimation program CDSSTR (32).

Patients and biological materials
Sera were obtained from cow's milk allergic patients who had a positive case history of cow's milk allergy, positive skin-prick reactions to cow's milk and/or specific IgE to cow's milk extract as measured by the ImmunoCAP System (Phadia, Uppsala, Sweden). The cow's milk allergic patient group consisted of persons from Austria (n=9), France (n=1), Germany (n=33), Greece (n=2), Italy (n=2), and Spain (n=19). Table I shows the available demographic, clinical and serological data for the adults (age 22-70 years old) and children (age 1 month-16 years old) with IgE reactivity to rα-La. For the anonymous analysis of serum IgE antibodies the permission of the Ethical Committee of the Medical University of Vienna was obtained.
Pasteurized cow's milk containing 3.5% fat was bought at a local market (NÖM, Baden, Austria, batch: 22 550 2:00) and purified natural alpha lactalbumin was purchased from Sigma-Aldrich.

IgE reactivity testing and calcium depletion experiments
For immunoblot analysis 1 µg/gel-slot of rα-La was separated by SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) (30, 31). The nitrocellulose strips were blocked with PBST (PBS, 0.5% v/v Tween 20) and exposed to sera from milk allergic patients diluted 1:20 in PBST over night at 4°C. Bound IgE antibodies were detected with ^{125}I-labelled anti-human IgE antibodies (IBL, Hamburg, Germany), diluted 1:15 in PBST, and visualized by autoradiography using Kodak XOMAT films with intensifying screens (Kodak, Heidelberg, Germany) at -80°C.
IgE reactivities to micro-arrayed whole cow's milk extract, rα-La, nα-La, and α-La-derived peptides were determined as described (33, 19). In brief, milk components and for control purposes, HSA, were spotted onto a capillary-flow membrane attached to an ordinary microscope glass slide and incubated with 30 µl of patients' sera. rα-La, nα-La, and α-La-derived peptides were spotted in three spot replicates after each other in the flow direction with one HSA spot in front of the first spot of each component. Cow's milk extract was spotted later in the flow in six spot

replicates across the flow direction with one HSA spot spotted in front of each CM spot. Bound IgE antibodies were detected with a fluorophore-conjugated anti-IgE antibody at a wavelength of 670 nm. All values were corrected for their proximate HSA values. Values exceeding 125 fluorescence intensities (FI), the highest values obtained with non-allergic individuals, were considered as positive.

IgE reactivity to calcium-depleted rα-La was tested by dot-blotting. Aliquots of 1 µg of rα-La were dotted onto nitrocellulose membranes (Schleicher & Schuell). The nitrocellulose strips were blocked with PBST and exposed to sera from milk allergic patients diluted 1:10 or 1:20 in PBST over night at 4°C in the presence of 5 mM EGTA (ethylene glycol-bis (ß-aminoethylether)-N, N, N', N'-tetraacetic acid, Sigma-Aldrich) or 0.5 mM $CaCl_2$ (calcium chloride dihydrate, MERCK, Darmstadt, Germany). Bound IgE antibodies were detected with ^{125}I-labelled anti-human IgE antibodies as described for IgE immunoblotting.

To compare the IgE binding capacity of rα-La with nα-La, 5 µg of nα-La/ml in sodium carbonate buffer (pH 9.6) were coated onto ELISA plates (Nunc Maxisorb, Roskilde, Denmark) overnight at 4°C. Patients' sera were diluted 1:10 in Tris-buffered saline containing 0.5% v/v Tween 20 (TBST) and were pre-incubated overnight at 4°C with 10 µg/ml of nα-La or rα-La or for control purposes with TBST (not inhibited, NI). On the following day, the plates were washed five times with TBST and blocked with TBST for 2.5h at 37°C. The plates were then incubated with the patients' sera overnight at 4°C. Bound IgE antibodies were detected with a monoclonal anti-human IgE antibody (BD Biosciences, San Jose, CA) 1:1000 diluted in TBST. After incubation overnight at 4°C and five times washing with TBST, bound anti-human IgE was detected with 1:1000 diluted HRP-labelled sheep anti-mouse IgG antibodies (GE Healthcare, Little Chalfont Buckinghamshire, UK). All determinations were carried out in duplicates.

Synthesis of α-La-derived peptides, determination of surface exposed amino acids in the peptide sequences

α-La-derived peptides displayed in Table II and Fig. 1 were synthesized using the Fmoc (9 fluorenyl methoxy carbonyl)-strategy with HBTU [(2-/1H-Benzotriazol-1-yl)1,1,3,3, tetramethyluronium hexafluorophosphat]-activation (0.1 mmol small-scale cycles) on an Applied Biosystems peptide synthesizer Model 433A (Foster City, CA) and purified as described (34).

In order to determine the extent of surface exposure of the amino acid residues in the peptides in the complete α-La, the coordinates of the known bovine alpha-lactalbumin structure were retrieved from the Protein Data Bank (PDB – 1F6S) (35). The solvent accessible surface areas were calculated with the program AREAIMOL (36, 37) by rolling a probe sphere of 1.4 Å radii over the van der Waals surface of the protein. The solvent accessible surface is specified by the centre of the probe sphere. In this way the solvent accessible surface of the complete α-La as well as the individual residues was determined.

Rat basophil leukaemia (RBL) assays

For the assessment of the allergenic activity of rα-La and nα-La, RBL cell mediator release assays were performed as described previously (19, 38). RBL cells (clone RBL-703/21) transfected with the human FcεRI were incubated with sera from cow's milk allergic patients overnight. On the next day the cells were washed, 100 µl of milk components (concentration: 0.3 µg/ml) were added and incubated for 1 hour at 37°C, 7% CO_2 and 95% humidity. Aliquots of the supernatants were mixed with assay solution (0.1 M citric acid or sodium citrate, pH 4.5 + 160 µM 4-methyl umbelliferyl-N-acetyl-β-D-glucosamide) and incubated for 1 hour at 37°C, 7% CO_2 and 95% humidity. Fluorescence was measured with a fluorescence microplate reader (Spectrafluor, Tecan, Salzburg, Austria) and specific release was calculated. Values obtained with buffer alone were subtracted and values exceeding 5% of mediator release were considered as positive.

Results

Expression in E. coli, purification, biochemical, and structural characterization of rα-La

We obtained an α-La-cDNA from which an amino acid sequence could be deduced that was identical to the α-La protein sequence deposited in the NIH database (http://www.ncbi.nlm.nih.gov, accession number: NP_776803). Fig. 1 shows the alignment of the deduced α-La amino acid sequence with α-La amino acid sequences from different mammalian species. α-La contains an N-terminal leader sequence which is cleaved from the mature protein (Fig. 1). A prediction of N-glycosylation sites revealed the presence of 6 potential N-glycosylation sites in the α-La sequence (Fig. 1, underlined: 44NNDS, 56NNKI, 57NKIW, 66NPHS, 71NICN, 102NYWL). The 8 cysteine residues which form disulphide bridges are conserved throughout the species and have been boxed (blue) in Fig. 1. Also the domain forming the calcium-binding loop, residues 79K-88D, is highly conserved among the different species (Fig. 1, yellow box). The amino acids responsible for the binding of Ca^{2+} have been printed in italics. They are identical in all but two sequences. In the rat and in the mouse sequence the aspartic acid shows a conservative exchange to a glutamic acid. Overall, there were strong similarities between the α-La amino acid sequences from cow to man and rodents ranging at approximately 75% identity.

The α-La cDNA coding for the mature protein and a C-terminal hexahistidine tag was expressed in *Escherichia coli* and approximately 6 mg/l culture of the recombinant allergen were purified by nickel affinity chromatography to homogeneity (Fig. 2*A*).

Fig. 2*A* shows the purified protein and the immunoblot in Fig. 2*B* its reactivity with an anti-hexahistidine antibody. MALDI-TOF analysis of purified rα-La resulted in a mass peak of 15.1 kDa (Fig. 2*C*) which corresponds to the molecular weight calculated from the sequence, i.e., 15.14 kDa including the methionine and the C-terminal hexahistidine tag.

The far-ultraviolet CD-spectrum of purified rα-La was recorded at 25°C (Fig. 2*D*, black line). It showed minima at 208 nm and 220 nm and a maximum at 193 nm which are typical for an α-helical protein. The CD spectrum of the natural α-La possessed a more pronounced minimum at 220 nm indicating a better fold of the natural protein (data not shown).

The CD spectrum of rα-La recorded at 95°C was shifted to the left side (i.e., lower wave lengths) indicating an increase of denatured protein, however with the protein

remaining folded. The CD spectrum recorded after cooling to 25°C was almost identical to the spectrum obtained before heating, which suggests that the protein has almost completely folded back to its original conformation. In summary, the spectra observed during the temperature scan and at 95°C point to a high thermal stability of the rα-La.

It has been observed that calcium-chelating agents such as EGTA can destabilize the structure of calcium-binding food allergens (39). However, when we added 10 mM EGTA to rα-La, the CD spectra at 25°C and 95°C were very similar to those recorded without EGTA (data not shown).

Secondary structure analysis performed with the program CDSSTR using the reference database 7 showed that rα-La consists of 8% of α-helices, 32% of β-sheets, 19% of β-strands and 40% of random coils. The normalized root mean standard deviation (NRMSD) value of 0.022 confirmed a good fit between the calculated and the experimentally derived spectra.

rα-La is a major cow's milk allergen according to IgE reactivity

In first experiments, the specific IgE reactivity of the rα-La was demonstrated by immunoblot analysis using serum from a cow's milk allergic patient. Serum IgE from patient A2 reacted specifically with rα-La whereas a control serum from a non-allergic individual (NHS) did not contain IgE specific for rα-La (Fig. 3*A*).

Next, we tested whether the binding of patients' IgE is affected by depletion of protein bound Ca^{2+} using EGTA. Depletion of Ca^{2+} from rα-La led to a reduction of the IgE-reactivity with sera from two of the three rα-La-reactive patients (Fig. 3*B*).

We then investigated whether rα-La contains the IgE epitopes of nα-La by ELISA competition analysis. When tested with sera from four α-La allergic patients we found that rα-La inhibited the binding to nα-La to the same extent as nα-La (Fig. 3*C*).

From most of the cow's milk allergic patients only small amounts of serum were available. We therefore determined the prevalence of IgE recognition for rα-La using microarray technology. We found that 57.6% of the cow's milk allergic patients (n=66) who showed IgE reactivity to cow's milk reacted with the rα-La and 75.8% reacted with nα-La.

Identification of IgE epitopes of α-La

In order to identify IgE-reactive epitopes of α-La we synthesized 8 peptides spanning the α-La sequence (Fig. 1, Table II). The 8 peptides had a length of 19-20 amino acids and overlapped with each other in 5 amino acids. The 8 overlapping peptides were also tested by microarray analysis using sera from the 38 patients with IgE reactivity to rα-La. We found that 9 patients reacted to Lac1, 18 patients to Lac2, 2 patients to Lac4, one patient to Lac5, 2 patients to Lac7, and 3 patients to Lac8 (Fig. 4). In total 22 of the 38 patients with IgE reactivity to rα-La reacted at least with one α-La-derived synthetic peptide.

Interestingly, when we tested also sera from patients without IgE reactivity to complete α-La we found 5 patients who reacted with α-La-derived peptides (i.e., Lac1, Lac2, Lac4, Lac8).

Fig. 5*A*-*D* show the position of the most frequently recognized peptides Lac1 (red), Lac2 (blue), and Lac8 (green) in the ribbon presentation (Fig. 5*A*, 5*C*) and in the molecular surface presentation (Fig. 5*B*, 5*D*). Interestingly, although peptides Lac1 and Lac2 are part of the N-terminal portion of α-La and peptide Lac8 is located at the C-terminal end of α-La, all three peptides appear in close vicinity on the surface of α-La and seem to define an IgE-reactive patch on the protein. Peptides Lac1 and Lac8 contain amino acids which are exposed on the surface of α-La and comprise a high percentage of the α-La surface (Fig. 5*E*, *F*), whereas the other peptides, in particular Lac4, contains less surface-exposed amino acids. The surface calculations of the peptides (Fig. 5*F*) show that Lac1 and Lac8 occupy 24.4% and 25.7% of the total α-La surface, respectively.

rα-La induces specific basophil degranulation

In order to assess the allergenic activity of the purified rα-La, rat basophil leukaemia cells loaded with serum IgE from cow's milk allergic patients (n=59) were stimulated with cow's milk, rα-La, and nα-La. Of the 59 tested patients, 78% had shown IgE reactivity to nα-La and 59.3% exhibited IgE reactivity to rα-La. However, when tested for allergenic activity, basophil degranulation was observed for 11.9% with nα-La and for 23.7% with rα-La. For those patients whose sera induced basophil degranulation with both allergen preparations similar degranulation magnitudes were observed ranging from 6.6-45% (median: 12.22%) for nα-La, and 8.5-53.6% (median: 15.62%) for rα-La. The allergenic activity of nα-La and rα-La was specific because no

degranulation was observed when cells were loaded with sera from non-allergic individuals (n=10) (Fig. 6).

Association of cow's milk-induced symptoms with IgE reactivity and basophil degranulation

Fig. 7 shows the intensities of IgE reactivity and allergenic activity to cow's milk, rα-La, and α-La-derived peptides for cow's milk allergic patients mounting IgE responses against rα-La. The patients had been grouped according to the type and magnitude of their symptoms. Patients were grouped in those without clinically relevant reactions (n=2), with oral allergy syndrome (OAS, n=1), with gastrointestinal symptoms (GI, n=3), with gastrointestinal symptoms and other symptoms (urticaria, atopic dermatitis, eczema, asthma, rhinoconjunctivitis, n=5), with skin symptoms (n=5), with skin and respiratory symptoms (n=5) and those who had severe anaphylactic reactions (n=7) upon cow's milk exposure.

We found a tendency that rα-La-specific IgE levels were highest in the patients who had experienced systemic reactions upon cow's milk exposure followed by those patients with gastrointestinal and other symptoms or with skin and respiratory symptoms. IgE reactivities to α-La-derived synthetic peptides were found in each of the patients groups except the patients who had only skin and respiratory symptoms to cow's milk.

The strongest induction of basophil release with rα-La was observed for sera from those patients who had experienced systemic reactions to cow's milk and to a lesser degree for those with gastrointestinal and other symptoms whereas almost no relevant basophil degranulation was found for patients without reactions or skin reactions to cow's milk (Fig. 7).

Discussion

In this study we isolated a cDNA coding for the cow's milk allergen α-La and characterized the recombinant allergen. Bovine α-La represents a calcium binding protein containing one single calcium-binding domain and exhibits high sequence homology with α-La from several species including man. The high degree of sequence homology of α-La may explain the cross-reactivity of IgE antibodies from cow's milk allergic patients even including the human protein (40, 41). IgE cross-reactivities have also been reported for caseins from different mammalian species in humans allergic to cow's milk (42). We expressed α-La in *Escherichia coli* and demonstrated that the recombinant allergen represents a folded protein which exhibited a remarkable thermal stability. The high stability of the allergen may be explained by the presence of protein-bound calcium which is also a feature of other calcium-binding allergens, including besides respiratory allergens the major fish allergen, parvalbumin (39). α-La as well as fish parvalbumin preserve their allergenic activity even after boiling. For α-La it has been shown that the allergen can interact with low molecular weight organic compounds including phospholipids which may also protect the allergen from digestion (43). Accordingly α-La represents a class I food allergen which may sensitize via the gastrointestinal tract (44). The prevalences of IgE recognition reported for α-La show considerable variability. Wal et al. (20) found IgE reactivity to α-La in 51% of cow's milk allergic patients (n=92) whereas other authors reported recognition frequencies ranging from 6% (Otani, n=17), (Docena, n=80), 25% (Host, n=20), 67% (Gjesing, n=21) to 100% (Baldo, n=6) (17, 18, 21, 26, 27). Goldman et al. described a positive reaction to rα-La in oral challenge tests in 53% of cow's milk allergic patients (n=34) (16).

We determined the IgE recognition frequency of rα-La and nα-La as well as to α-La-derived synthetic peptides by microarray analysis using sera from 66 cow's milk allergic patients and found that 57.6% recognized the recombinant protein and 75.8% the natural protein. The lower recognition frequency of rα-La versus nα-La may be explained by the lack of posttranslational modifications and/or slightly different fold of the recombinant protein. However, when rα-La was compared with nα-La regarding allergenic activity, we found that the recombinant protein induced more often (i.e., 23.7%) degranulation of basophils than nα-La (i.e., 11.9%). This result was unexpected because nα-La had reacted more frequently with serum IgE than rα-La but may be explained by the presence of certain IgE epitopes on nα-La which exhibit

low or no allergenic activity as has been reported for example for hapten-like structures (45, 46) or carbohydrate epitopes (47, 48).

We found that patients who had experienced systemic reactions upon cow's milk exposure contained higher rα-La-specific IgE antibody levels compared to those patients who had experienced milder symptoms which supports earlier observations that patients with more severe symptoms tend to have higher cow's milk-specific IgE levels (49-51). However, it seems impossible to unambiguously identify patients with systemic reactions to cow's milk only on the basis of IgE reactivity. Using the model of rat basophil leukemia cells which had been transfected with the human FcεRI for testing the allergenic activity of rα-La patients with severe systemic reactions and those suffering from gastrointestinal symptoms could be determined. Application of rα-La in RBL assays may therefore contribute to the improvement of *in vitro* diagnostic methods for the identification of patients with severe systemic allergic reactions to cow's milk.

In order to define IgE epitopes of α-La two approaches were pursued. First, we studied whether α-La may contain conformational IgE epitopes by depletion of protein-bound calcium similar as has been done for other calcium-binding allergens (39, 52-56). We could indeed demonstrate that the apo-form (i.e., calcium-depleted form) of rα-La showed a reduced IgE binding capacity which may be explained by an alteration of the protein conformation due to calcium-depletion (57). The existence of conformational epitopes in class I food allergens is quite unexpected but was lately described also for other class I food allergens, namely Cyp c 1 (39) Ara h 2 (58), and αS1-casein (19). The presence of conformational IgE epitopes on food allergens may be explained by sensitization through intact undigested allergen. In this context, it has been described by Moreno et al 2005 (43) that α-La interacts with phosphatidylcholine and hence becomes protected against digestion (59).

Besides conformational epitopes we identified sequential epitopes in α-La using 8 synthetic overlapping peptides spanning the α-La sequence. Interestingly, despite different localization within the α-La sequence, two N-terminal, i.e., Lac1 (1E-G19) and Lac2 (15L-S34) and a C- terminal peptide, i.e., Lac8 (105L-L123) defined an IgE epitope-containing patch on the surface of α-La.

Our IgE epitope mapping data are in good agreement with other studies. Järvinen (22) described four IgE epitopes ranging from amino acid 1E-K16, 13K-W26, 47S-K58, and 93K-N102 in the native bovine α-La. The study of Maynard, Jost and Wal

(60) showed that sequence 17G-K58 and large tryptic peptides sharing this sequence were most strongly and frequently recognized. Adams et al (61) showed that the synthetic peptide ranging from 5K-A18 contains an IgE-binding epitope. Besides the IgE-reactive peptides Lac1, Lac 2, and Lac 8 which formed the major IgE-reactive cluster, we found that patients exhibited also IgE reactivity to Lac4 (45N-D64), Lac5 (60W-K79), and Lac7 (90M-A109).

A similar clustering of IgE epitopes as described for Lac1, Lac 2, and Lac8 has been found for several other respiratory and food allergens such as Bet v 2 (62), Phl p 5 (63), Phl p 1 (64), Phl p 2 (65), Bos d 5 (66) and may be important for efficient cross-linking of IgE antibodies on effector cells and hence determine the degree of allergenic activity of an allergen.

The knowledge of IgE epitopes may be of great relevance for the rational design of allergy vaccines because it allows constructing hypoallergenic allergen derivatives (67) or peptide vaccines (34, 68, 69). Allergy vaccines based on hypoallergenic allergen derivatives of respiratory allergens have already been tested in promising immunotherapy trials in man and are currently developed also for important food allergens (70, 71).

The recombinant α-La defined by us represents an important cow's milk allergen which can be used for the diagnosis of patients suffering from severe cow's milk allergy. Based on the IgE epitope mapping data it may be possible to develop new preventive and therapeutic strategies for cow's milk allergy.

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Figure legends

FIGURE 1. Alignment of α-lactalbumin amino acid sequences from different species. The 8 overlapping synthetic peptides (Lac1-Lac8) are boxed. Cysteine residues are marked in blue, residues that form the calcium-binding loop are marked in a yellow box. Bt, *Bos taurus* (cow, NP_776803); Bg, *Bos grunniens* (yak, AAF06793); Bb, *Bubalus bubalis* (water buffalo, ABG78269); Oa, *Ovis aries* (sheep, CAA29665); Ch, *Capra hircus* (goat, CAA28797); Cf, *Canis lupus familiaris* (dog, BAA95930); Ss, *Sus scrofa* (pig, AAA31060); Mmul, *Macaca mulatta* (rhesus monkey, XP_001102116); Hs, *Homo sapiens* (man, BAC06860); Rn, *Rattus norvegicus* (rat, CAA25150); Mmu, *Mus musculus* (mouse, AAA37208); Ec, *Equus caballus* (horse, LAHO). Dots represent identical amino acids, dashes represent gaps. Potential N-glycosylation sites are underlined.

FIGURE 2. Expression and purification of rα-La. Coomassie brilliant blue-stained SDS-PAGE of a bacterial extract without IPTG-induction (*lane 1*) and one after IPTG-induction (*lane 2*) (*A)*. Urea extraction of rα-La before (*lane 3*) and after (*lane 4*) purification by Ni-NTA resin affinity columns. Western Blot of rα-La incubated with an anti-his tag antibody is shown in *B*. Molecular masses (kDa) are indicated on the left side. *C*, Mass spectrometric analysis of purified rα-La. The mass/charge ratio is shown on the x-axis and the signal intensity is displayed on the y-axis as the percentage of the most intensive signal obtained in the investigated mass range. *D*, Structural characterization and thermal unfolding of rα-La. The figure shows the far ultraviolet circular dichroism (CD) analysis of purified rα-La in 2 mM Tris (pH 8.5). The mean residue ellipticities (Θ, y-axis), recorded at 25°C (continuous line), at 95°C (dashed line) and at 25°C after cooling (dotted line) at given wave lengths are shown (x-axis).

FIGURE 3. *A*, IgE reactivity of blotted rα-La with sera from a cow's milk allergic individual (patient A2) and from a non-allergic person (NHS). *B*, Nitrocellulose-dotted rα-La was exposed to sera from three cow's milk allergic individuals (patient A2, C3, C34) and from a non-allergic individual (NHS) in the presence (+) or absence (-) of calcium. Bound IgE antibodies were detected with ^{125}I-labelled anti-IgE antibodies and visualized by autoradiography. *C*, IgE reactivity to natural α-La after preincubation of sera from four individuals (patients A2, C42, C31, A8) with natural α-

La (naLa) or recombinant α-La (raLa) or with buffer (no inhibitor, NI). IgE reactivity to ELISA plate-bound natural α-La corresponds to optical density values (y-axis).

FIGURE 4. Prevalence (y-axis) of IgE recognition of eight α-La-derived peptides tested with sera from 38 rα-La-allergic patients by microarray analysis.

FIGURE 5. Ribbon (*A, C*) and molecular surface (*B, D*) presentations of α-La. The N- and C-terminus are indicated in *A* and *C*. Peptides Lac1, Lac2, and Lac 8 are colored in red, blue, and green. *E*, Contribution of amino acids (1-123) to the molecular surface in $Å^2$ and percentages of total surface represented by peptides Lac1-Lac8 are shown in *F*.

FIGURE 6. Comparison of basophil degranulation induced by nα-La and rα-La. The percentages of mediator release (y-axis) are displayed for allergic patients and non-allergic controls. The cut off level at 5% is indicated with a horizontal line.

FIGURE 7. Association of cow's milk-induced symptoms with IgE reactivity and basophil degranulation. Patients were grouped according to their symptoms related to cow's milk consumption in the following groups: group 1: no reaction, group 2: oral allergy syndrome (OAS), group 3: gastrointestinal symptoms only (GI only), group 4: gastrointestinal symptoms and other organs affected (GI + others), group 5: skin symptoms only (skin only), group 6: skin symptoms and respiratory symptoms (skin + respiratory), group 7: severe systemic reactions (systemic reactions). Fluorescence intensities (FI) of IgE reactivities to cow's milk (CM), to recombinant alpha-lactalbumin (ra-La) and to eight rα-La-derived synthetic peptides (Lac1-Lac8) measured by microarray analysis after subtraction of HSA values are indicated in different colors. For the mediator release cow's milk (CM) and rα-La were tested. Values higher than 5% of release were considered as positive and are highlighted in grey. y, year; mo, month; nd, not done.

Table I. *Demographic, clinical, and serological characterization of rα-La-positive adults (A2-A8, age: 22y-64y) and children (C1-C57, age: 3mo-16y)*[a]

Patient	Age	Sex	Country	Milk-related symptoms	Other allergies	Total IgE (kU/l)	CM (RAST class)
A2	64y	f	F	U,Sys,GI	no	nd	6
A3	61y	f	A	nk	cat,WF,T,K,P	355	4
A5	42y	f	A	OAS	mite,cat	148	3
A8	22y	f	A	Sys	HE,nuts,pets,PO	3350	6
C1	16y	f	G	NR	HE	433	3
C3	13y	m	I	U,E,V,AS	PO,HE	909	6
C4	13y	m	G	RC,V	HE	866	3
C5	11y	f	I	U, AE, AS	candida	nd	4
C6	10y	f	G	Ap	nd	1432	4
C8	8y	m	G	E,U,AS	HE	2200	4
C9	7y	m	G	U,R,AS	HE	399	4
C11	6y	nk	G	nk	nk	246	3
C17	4y	f	G	Sys	nk	974	6
C19	4y	m	G	nk	nk	1894	3
C20	4y	f	G	Sys	HE	489	5
C21	4y	f	G	nk	nk	116	3
C24	3y	f	G	nk	nk	125	3
C25	3y	m	G	nk	nk	26	3
C27	3y	m	G	Sys	nk	325	4
C28	3y	f	G	Sys	nk	125	3
C29	3y	f	G	nk	nk	201	3
C31	3y	nk	Gr	U, GI	beef,fish,Alt	2000	5
C33	2y	f	S	U(face)	HE	nd	3
C34	2y	f	S	U,AD,V	HE,fish	nd	5
C35	2y	m	A	AD,OB	mite	134	3
C36	2y	f	G	nk	nk	83.4	4
C37	2y	m	G	nk	nk	193	3
C38	2y	m	G	NR	nk	117	3
C40	1y	f	A	AD, OB	HE,soja,nuts	217	3
C42	9mo	nk	Gr	U,Sys	HE,W	1066	6
C43	9mo	f	S	U,GI	no	351	3
C44	7mo	m	S	GI	no	32	3
C45	6mo	m	S	U(face), AD	no	133	3
C49	3mo	f	S	U	nk	1292	3
C50	3mo	f	S	GI	no	29	3
C51	3mo	m	S	U,AE	no	51	3
C54	3mo	f	S	U(face), AD	no	50	3
C57	nk	nk	G	nk	nk	1129	5

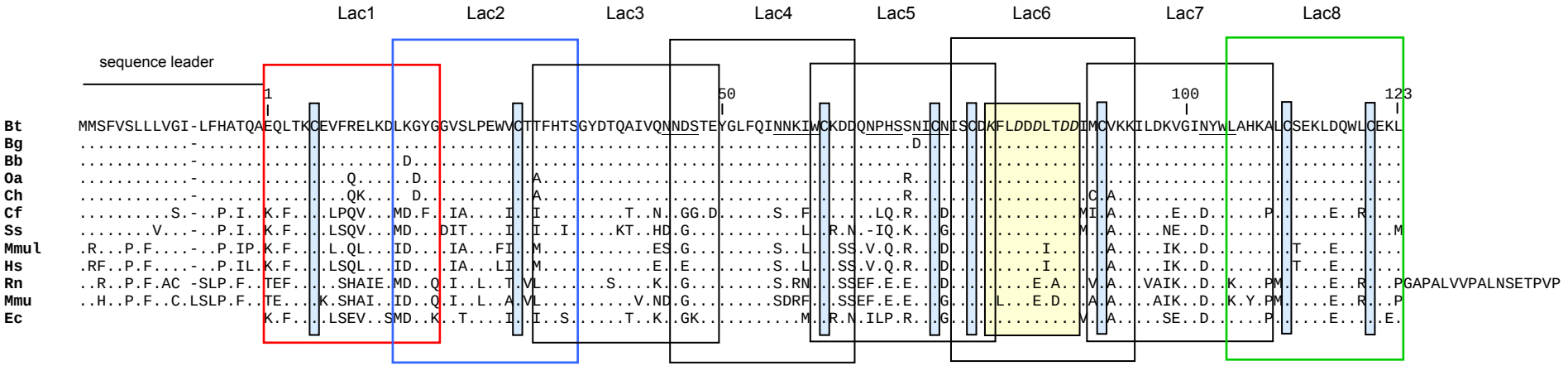
[a]Abbreviations used in the table: y, year; mo, month; f, female; m, male; F, France; A, Austria; G, Germany; Gr, Greece; I, Italy; S, Spain; nk; not known; Symptoms: AD, atopic dermatitis; AE, angioedema; Ap, abdominal pain; AS, asthma; E, eczema; GI, gastrointestinal symptoms; NR, no reaction; OAS, oral allergy syndrome; OB, obstructive bronchitis; R, redness; RC, rhinoconjunctivitis; Sys, systemic reaction; U, urticaria; V, vomiting; Allergen (source): Alt, Alternaria; HE, hen's egg; K, kiwi; P, pork; PO, pollen; T, tomato; W, wheat; WF, wheat flour; kU/l, kilo units per liter; CM, cow's milk; nd, not done.

Table II. *Synthetic α-La-derived peptides*[a]

Peptide	Sequence	Length	pI	MW (Da)
Lac1	EQLTKCEVFRELKDLKGYG	19 aa	6.34	2256.60
Lac2	LKGYGGVSLPEWVCTTFHTS	20 aa	6.74	2182.48
Lac3	TFHTSGYDTQAIVQNNDSTE	20 aa	4.31	2228.27
Lac4	NDSTEYGLFQINNKIWCKDD	20 aa	4.42	2403.60
Lac5	WCKDDQNPHSSNICNISCDK	20 aa	5.3	2307.51
Lac6	ISCDKFLDDDLTDDIMCVKK	20 aa	4.11	2317.67
Lac7	MCVKKILDKVGINYWLAHKA	20 aa	9.52	2330.88
Lac8	LAHKALCSEKLDQWLCEKL	19 aa	6.74	2228.65

[a]Abbreviations used in the table: Lac1-Lac8, α-La-derived peptides 1-8; aa, amino acid; pI, isoelectric point; MW, molecular weight; Da, Dalton.

FIGURE 1


Lac1
Lac2
Lac3
Lac4
Lac5
Lac6
Lac7
Lac8
sequence leader
1
50
100
123
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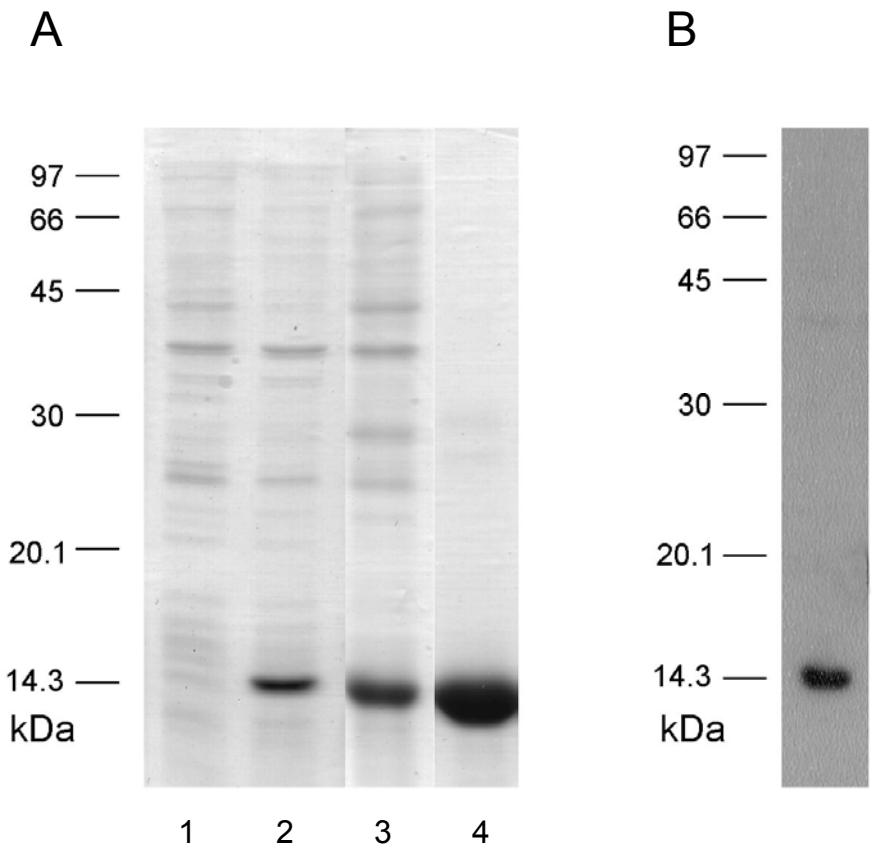
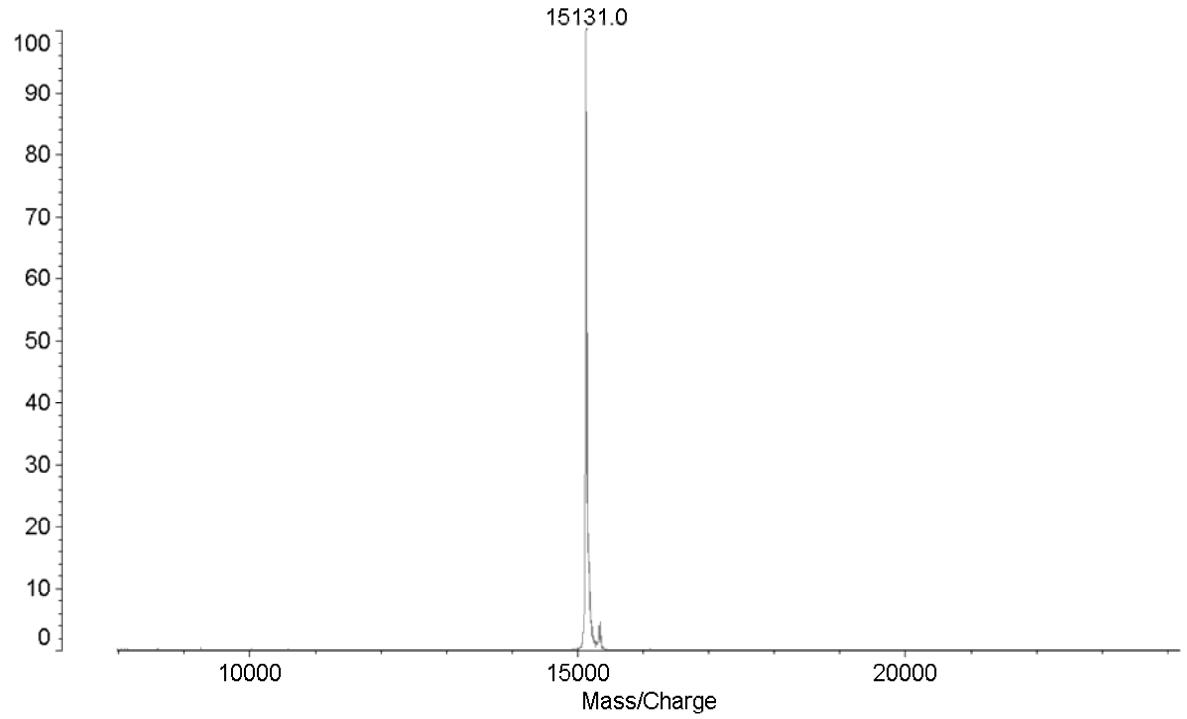
Bg ...........-.....D.....
Bb ...........-.....D.....
Oa ...........-.....Q.....D.....A.....R.....
Ch ...........-.....QK.... D.....A.....R.....C.A.....
Cf .........S.-..P.I..K.F....LPQV...MD.F..IA....I..I.........T..N..GG.D.....S..F.......LQ.R...D.....MI.A.....E..D.....P.....E..R....
Ss ........V...-..P.I..K.F....LSQV...MD...DIT....I..I..I.....KT..HD.G.....L..R.N.-IQ.K...G.....M..A.....NE..D.....M
Mmul .R...P.F....-..P.IP.K.F....L.QL...ID....IA...FI..M.....ES.G.....S..L...SS.V.Q.R...D.....I.....A.....IK..D.....T...E.....
Hs .RF..P.F....-..P.IL.K.F....LSQL...ID....IA...LI..M.....E..E.....S..L...SS.V.Q.R...D.....I.....A.....IK..D.....T...E.....
Rn ..R..P.F.AC -SLP.F..TEF.....SHAIE.MD..Q.I..L..T.VL.....S....K..G.....S.RN...SSEF.E.E...D.....E.A...V.A...VAIK..D..K...PM.....E..R...PGAPALVVPALNSETPVP
Mmu ..H..P.F..C.LSLP.F..TE....K.SHAI..ID..Q.I..L..A.VL.....V.ND.G.....SDRF...SSEF.E.E...G.....L...E.D...A.A....AIK..D..K.Y.PM.....E..R...P
Ec K.F....LSEV..SMD..K..T....I..I..S.....T..K..GK.....M..R.N.ILP.R...G.....M..A.....SE..D.....P.....E.....E.

FIGURE 2



C


15131.0
100
90
80
70
60
50
40
30
20
10
0
10000
15000
20000
Mass/Charge

D

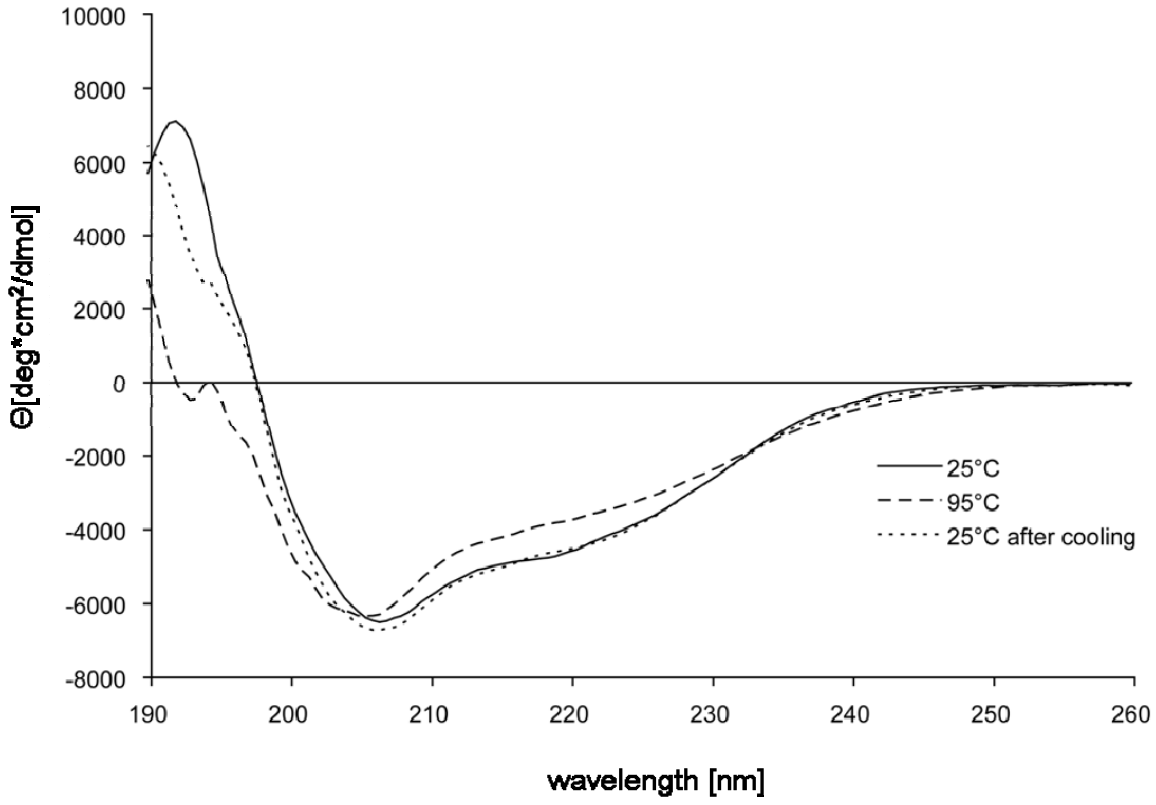
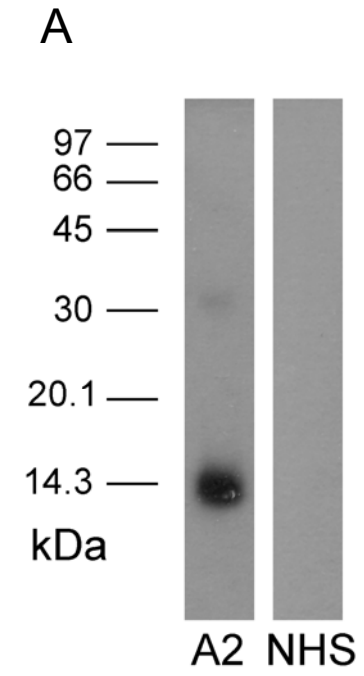
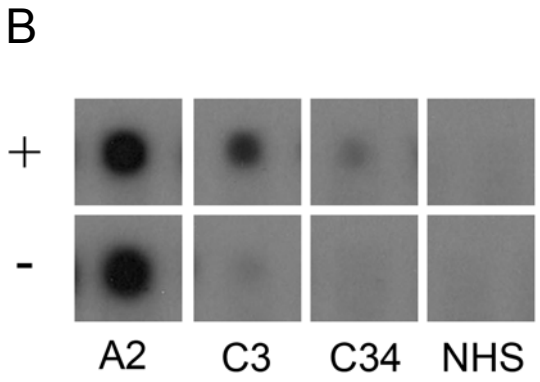

10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
Θ[deg*cm²/dmol]
190
200
210
220
230
240
250
260
wavelength [nm]
25°C
95°C
25°C after cooling

FIGURE 3





C

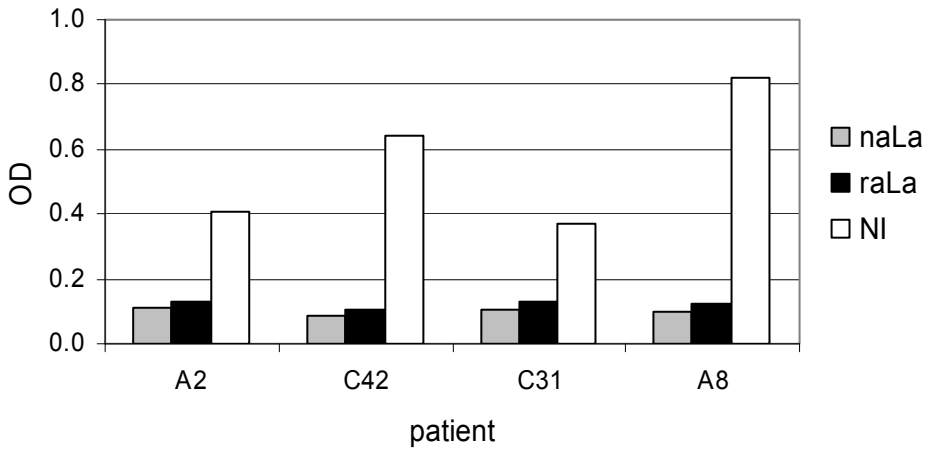


FIGURE 4

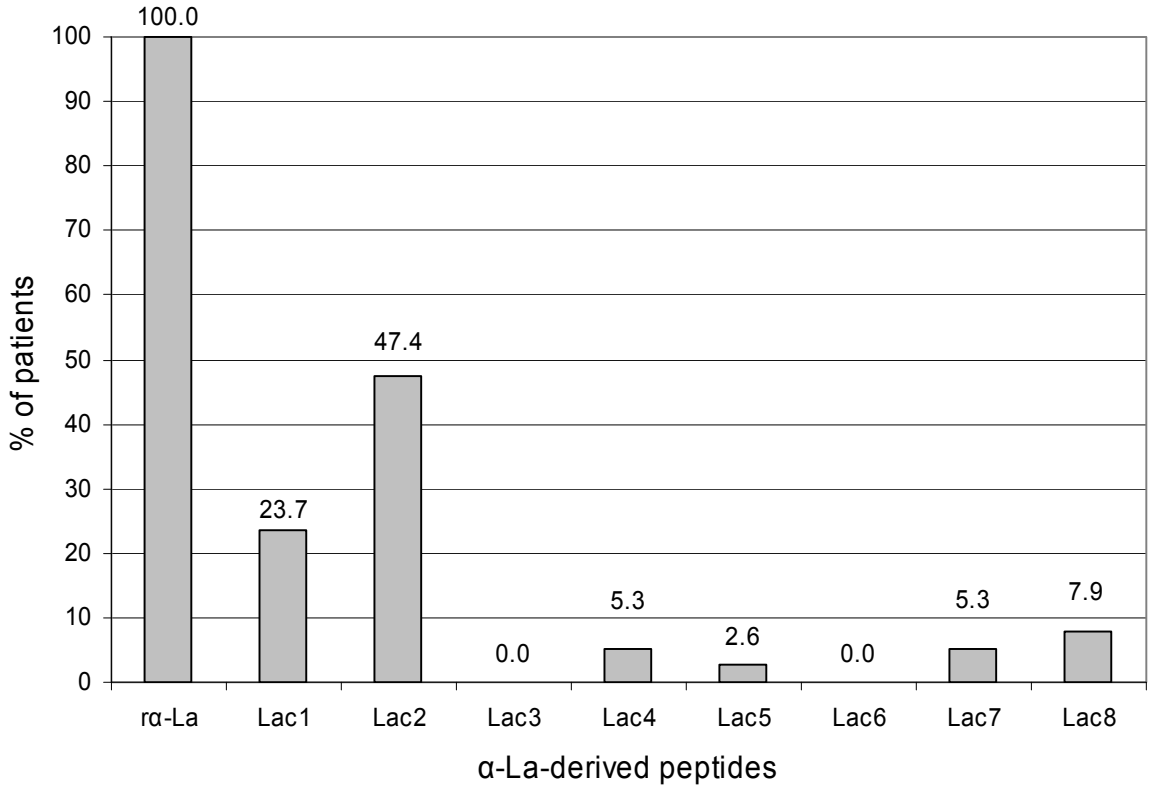
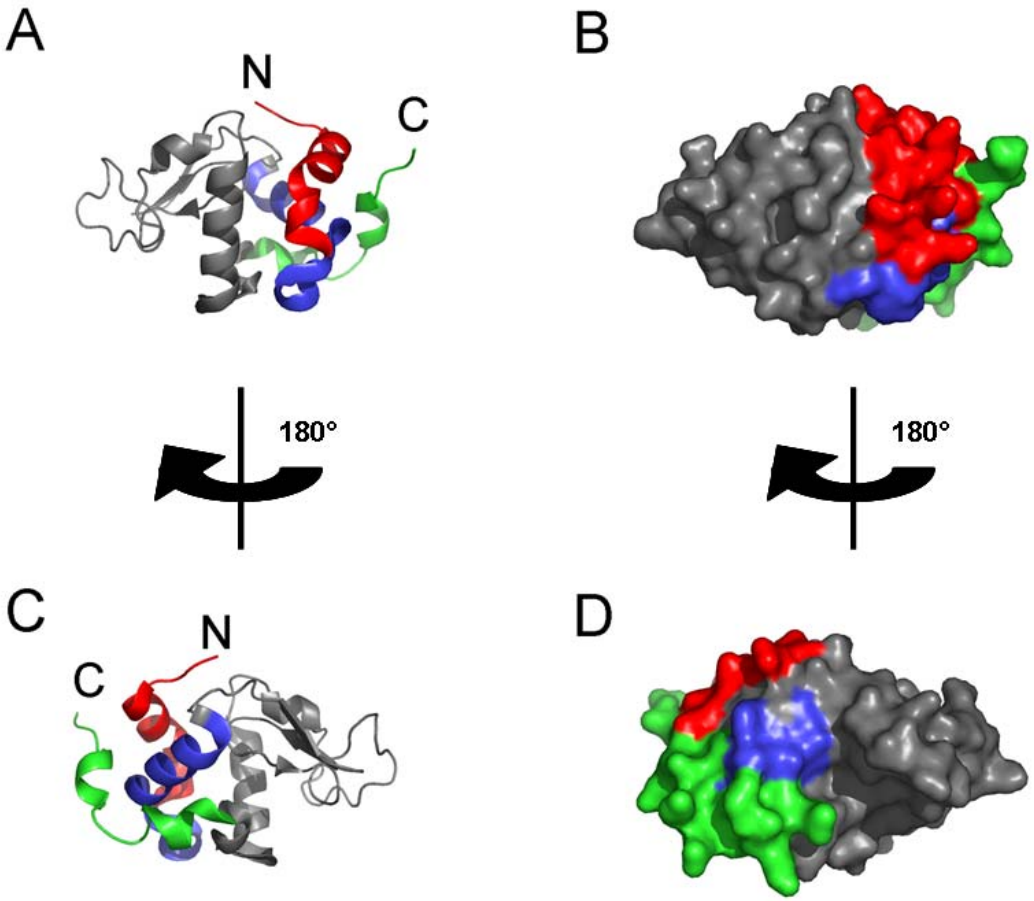
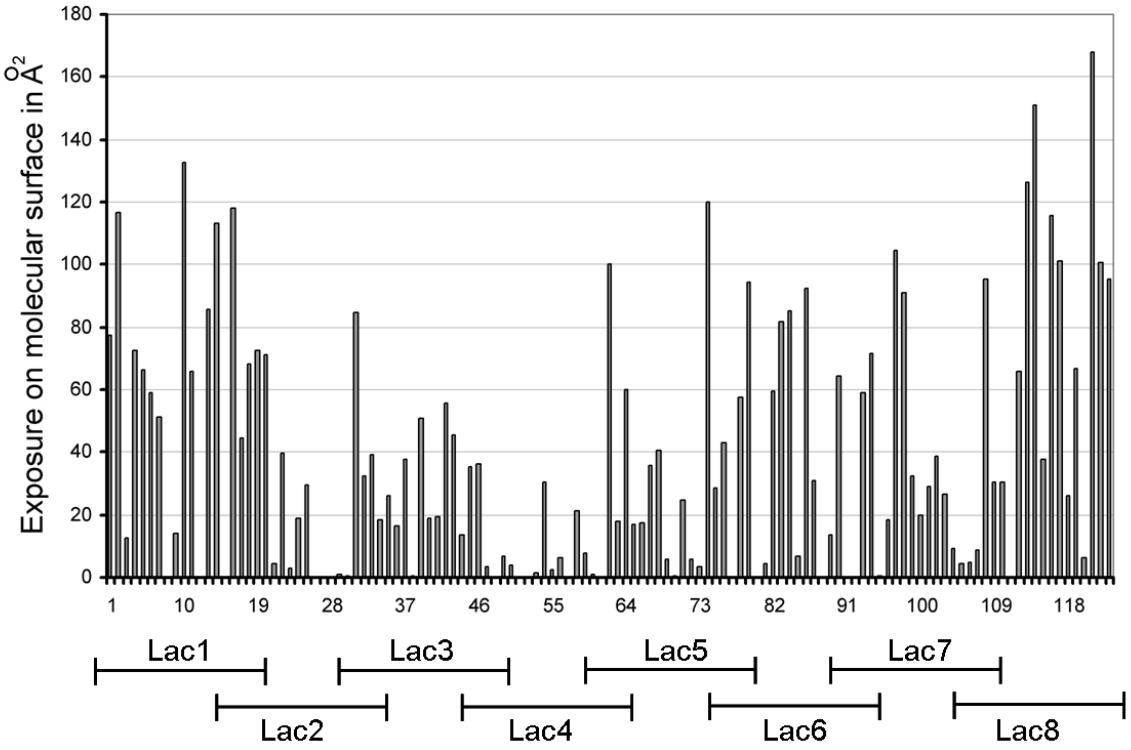

100.0
47.4
23.7
5.3
0.0
2.6
0.0
5.3
7.9
100
90
80
70
60
50
40
30
20
10
0
% of patients
rα-La
Lac1
Lac2
Lac3
Lac4
Lac5
Lac6
Lac7
Lac8
α-La-derived peptides

FIGURE 5



E


Exposure on molecular surface in Å²
180
160
140
120
100
80
60
40
20
0
1
10
19
28
37
46
55
64
73
82
91
100
109
118
Lac1
Lac2
Lac3
Lac4
Lac5
Lac6
Lac7
Lac8

F

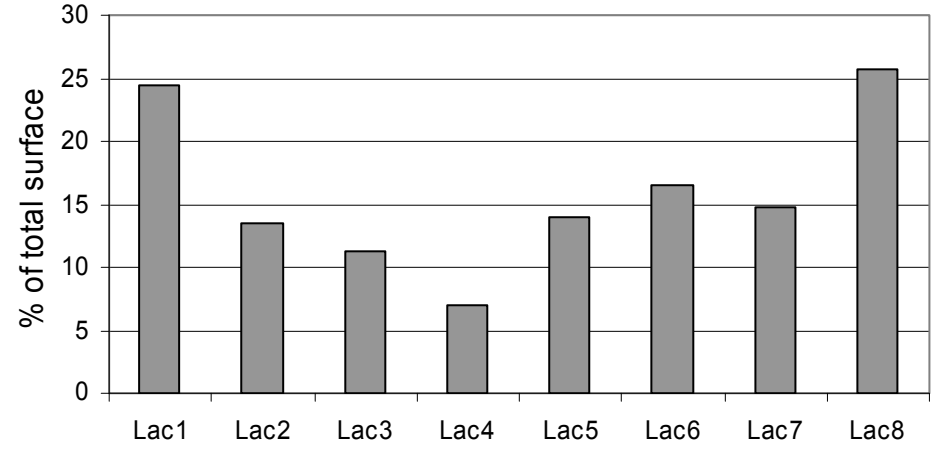

% of total surface
30
25
20
15
10
5
0
Lac1
Lac2
Lac3
Lac4
Lac5
Lac6
Lac7
Lac8

FIGURE 6

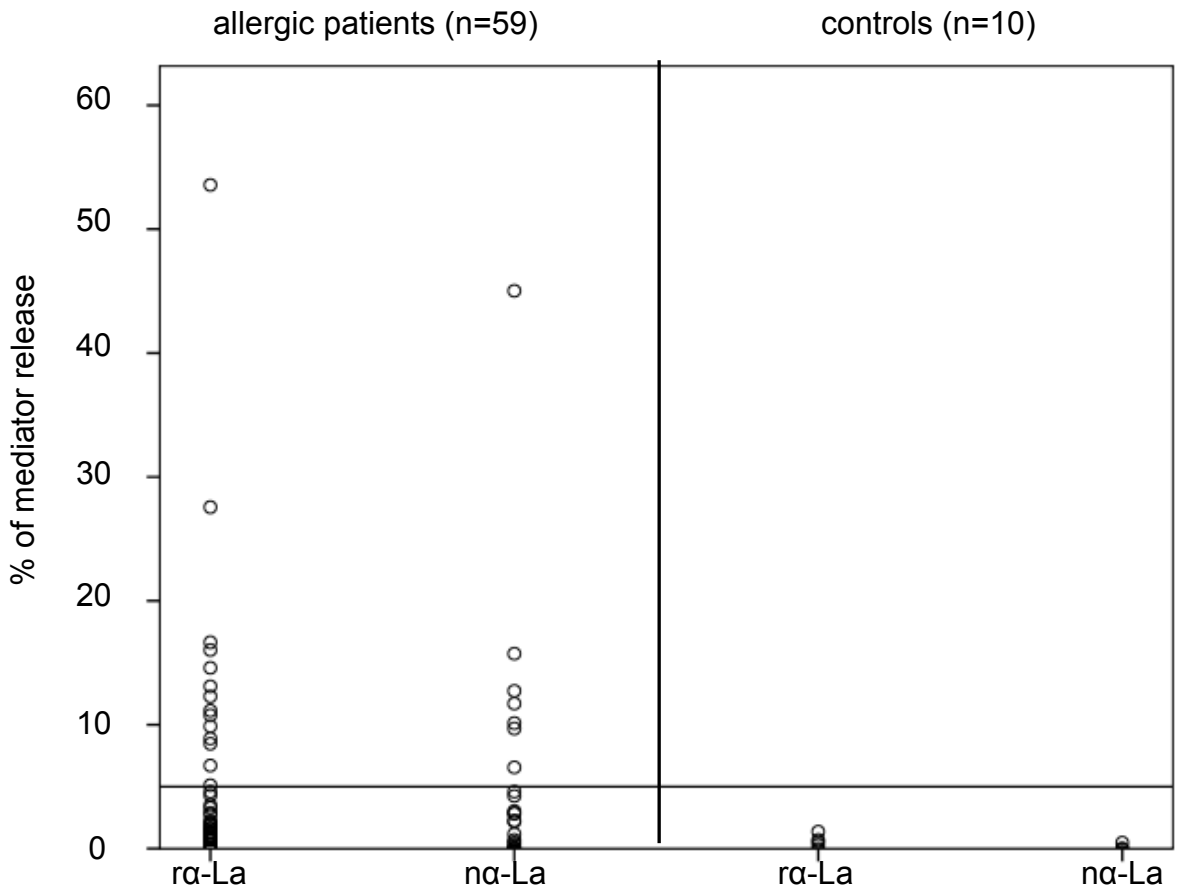

allergic patients (n=59)
controls (n=10)
% of mediator release
60
50
40
30
20
10
0
rα-La
nα-La
rα-La
nα-La

FIGURE 7

Symptoms	Patient	Age	IgE reactivity										Mediator release	
			CM	ra-La	Lac1	Lac2	Lac3	Lac4	Lac5	Lac6	Lac7	Lac8	CM	ra-La
No reaction	C1	16y	5998.5	817.3	-2.3	115.0	-6.0	10.0	-5.0	6.0	0.7	25.0	6.40	-1.00
	C38	2y	3959.3	406.0	58.3	145.0	92.0	111.3	3.0	7.0	6.3	12.3	5.96	1.09
OAS	A5	42y	270.7	153.0	245.0	83.7	22.0	15.7	1.3	-8.7	-9.3	4.3	3.32	-0.07
GI only	C6	10y	16071.8	2631.7	76.7	253.7	-135.7	-95.7	-71.7	-94.3	-38.3	63.7	20.15	12.29
	C44	7mo	1849.0	350.3	34.3	14.7	-7.3	19.3	-9.7	11.0	0.0	-16.3	nd	nd
	C50	3mo	11928.8	1515.7	44.7	44.7	3.7	11.0	6.7	4.7	9.3	14.0	8.83	2.75
GI + others	C3	13y	30760.3	2524.3	-155.0	264.7	-124.7	-111.3	6.7	-3.0	-5.7	2.7	41.51	3.50
	C4	13y	4326.7	1845.0	40.0	66.7	1.3	31.3	25.0	19.3	10.3	20.0	1.92	1.26
	C31	3y	48117.3	7277.3	372.0	1655.0	-392.7	-516.3	89.0	93.0	154.7	114.3	32.22	5.12
	C34	2y	35437.7	599.7	-267.0	1687.7	1.3	19.3	-6.0	32.7	19.7	111.7	55.70	9.87
	C43	9mo	2522.8	1244.3	23.0	35.0	-7.7	17.7	-6.3	3.7	2.0	6.7	16.03	13.10
Skin only	C33	2y	628.7	162.3	9.3	12.0	5.7	27.0	8.3	5.3	0.3	-6.3	-2.41	4.59
	C45	6mo	4345.5	549.0	15.0	26.7	-5.3	5.0	-8.0	2.0	-7.3	0.0	nd	nd
	C49	3mo	7792.3	790.3	79.7	175.7	8.3	45.7	-5.0	27.7	49.0	16.3	1.90	2.24
	C51	3mo	2129.0	469.7	478.3	383.7	-3.0	36.7	7.7	7.7	5.7	18.0	7.25	0.02
	C54	3mo	2444.3	181.7	32.3	35.0	-3.7	144.7	2.3	6.0	5.3	15.0	nd	nd
Skin + respiratory	C5	11y	24790.3	623.7	-209.7	13.3	-97.7	2.0	55.0	-24.3	-2.0	-6.7	6.97	-0.15
	C8	8y	13870.3	1838.0	-91.7	75.7	11.0	34.0	32.3	29.3	-3.3	62.7	7.20	0.39
	C9	7y	14460.3	152.7	41.3	53.7	19.3	57.7	47.7	39.0	38.0	38.0	16.10	-2.01
	C35	2y	5250.7	977.3	10.7	46.3	5.3	12.3	6.7	3.0	-12.0	0.7	2.11	0.73
	C40	1y	449.2	170.0	71.7	66.3	3.3	45.3	23.0	22.0	15.3	41.3	4.01	4.28
Systemic reactions	A2	64y	8535.7	10206.0	2377.7	2263.0	85.3	3834.7	-84.0	16.7	47.3	1080.3	64.00	53.57
	A8	22y	64927.8	16351.7	-233.0	732.3	25.7	-173.0	76.3	10.0	240.7	149.7	13.25	8.47
	C17	4y	46582.5	9896.7	-409.7	465.0	-97.0	-135.7	206.7	-73.0	-13.7	3.7	36.61	27.56
	C20	4y	23448.3	2271.3	967.3	175.0	-11.7	19.0	-18.0	14.0	12.3	9.0	8.75	6.71
	C27	3y	17137.0	2873.3	-8.0	277.0	-5.0	65.0	25.0	34.3	17.0	73.7	36.79	14.59
	C28	3y	3780.7	125.3	39.7	155.7	-2.3	16.3	5.3	15.0	34.7	15.0	14.05	1.63
	C42	9mo	42028.0	8449.3	32.0	127.0	-14.7	12.0	-1.3	8.0	2.3	25.7	19.37	16.65

Microarray (FI)

<125
125-350
350-4850
>4850

Mediator release

0-4.9%
≥5%

3. Cloning, Expression, and Mapping of Allergenic Determinants of αS1-Casein, a Major Cow's Milk Allergen

Ulrike Schulmeister, **Heidrun Hochwallner**, Ines Swoboda, Margarete Focke-Tejkl, Beate Geller, Mats Nystrand, Annika Härlin, Josef Thalhamer, Sandra Scheiblhofer, Walter Keller, Bodo Niggemann, Santiago Quirce, Christoph Ebner, Adriano Mari, Gabrielle Pauli, Udo Herz, Rudolf Valenta, and Susanne Spitzauer

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The Journal of Immunology

Cloning, Expression, and Mapping of Allergenic Determinants of αS1-Casein, a Major Cow's Milk Allergen[1]

Ulrike Schulmeister,* Heidrun Hochwallner,† Ines Swoboda,†‡ Margarete Focke-Tejkl,†‡ Beate Geller,* Mats Nystrand,§ Annika Härlin,§ Josef Thalhamer,¶ Sandra Scheiblhofer,¶ Walter Keller,‖ Bodo Niggemann,# Santiago Quirce, Christoph Ebner,†† Adriano Mari,‡‡ Gabrielle Pauli,§§ Udo Herz,¶¶ Rudolf Valenta,[2]†‡ and Susanne Spitzauer***

Milk is one of the first components introduced into human diet. It also represents one of the first allergen sources, which induces IgE-mediated allergies in childhood ranging from gastrointestinal, skin, and respiratory manifestations to severe life-threatening manifestations, such as anaphylaxis. Here we isolated a cDNA coding for a major cow's milk allergen, αS1-casein, from a bovine mammary gland cDNA library with allergic patients' IgE Abs. Recombinant αS1-casein was expressed in *Escherichia coli*, purified, and characterized by circular dichroism as a folded protein. IgE epitopes of αS1-casein were determined with recombinant fragments and synthetic peptides spanning the αS1-casein sequence using microarrayed components and sera from 66 cow's milk-sensitized patients. The allergenic activity of rαS1-casein and the αS1-casein-derived peptides was determined using rat basophil leukemia cells transfected with human FcεRI, which had been loaded with the patients' serum IgE. Our results demonstrate that rαS1-casein as well as αS1-casein-derived peptides exhibit IgE reactivity, but mainly the intact rαS1-casein induced strong basophil degranulation. These results suggest that primarily intact αS1-casein or larger IgE-reactive portions thereof are responsible for IgE-mediated symptoms of food allergy. Recombinant αS1-casein as well as αS1-casein-derived peptides may be used in clinical studies to further explore pathomechanisms of food allergy as well as for the development of new diagnostic and therapeutic strategies for milk allergy. *The Journal of Immunology*, 2009, 182: 7019–7029.

Immunoglobulin E-mediated allergies belong to the most common forms of immunologically mediated forms of hypersensitivity reactions to food (1). In sensitized individuals dietary intake of food can cause a variety of clinical manifestations reaching from oral allergy syndrome and gastrointestinal symptoms (e.g., vomiting, diarrhea) to skin, respiratory, and severe systemic manifestations such as anaphylactic shock (2–4). The development of food allergy shows a typical course (5). It starts early in childhood mainly against Ags encountered in the initial diet (e.g., cow's milk, eggs) and affects between 4% and 6% of children. Milk is one of the first food components introduced into the diet and therefore represents one of the most important food allergen sources in terms of frequency and severity of allergic manifestations (6–9). The symptoms of cow's milk allergy are due to IgE-mediated activation of mast cells and basophils as well as to activation of allergen-specific T cells, and they comprise a plethora of gastrointestinal, skin, respiratory, and severe systemic manifestations such as death due to anaphylactic shock. Unlike in respiratory allergy, which proceeds untreated from mild (e.g., rhinoconjunctivitis) to severe manifestations (e.g., asthma), many milk-allergic children grow out their allergy, and the induction of tolerance against cow's milk allergens has already been described (10, 11). In this context it has been reported that allergen-specific $CD4^+CD25^+$ regulatory T cells may be involved in the induction of tolerance against cow's milk allergens in children who outgrew cow's milk allergy (12).

Furthermore, it has been suggested that the development of tolerance to cow's milk allergens is associated with a reduction of allergen-specific IgE levels and a reduction of IgE recognition of certain sequential epitopes (13, 14). Reduced sensitivities in the gut and the outcome of oral challenge tests are also reflected by reduced skin sensitivity to food allergens (15, 16).

Cow's milk contains >25 different proteins, but only the whey proteins α-lactalbumin, β-lactoglobulin, BSA, and lactoferrin, as well as the four caseins, have been identified as allergens (17). The casein fraction is composed of αS1-, αS2-, β-, and κ-casein, of which αS1-casein seems to be a major allergen according to IgE and T cell recognition data (18–21).

Here we constructed an expression cDNA library from bovine mammary glands and used IgE Abs from cow's milk-allergic patients to isolate a cDNA coding for αS1-casein and IgE-reactive αS1-casein fragments. The recombinant αS1-casein allergen was obtained by expression in *Escherichia coli*, purified, and characterized regarding its physicochemical and

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Table I. *Characterization of milk-allergic patients*[a]

Patient	Age	Age Allergy Started	Sex	Country	Milk-Related Symptoms	SPT CM	Challenge	CMA Outgrown	Other Allergies	Total IgE (kU/L)	CM sIgE (kU_A/L)	rαS1-Casein (FI)
1*	61 yr	NK	F	A	NK	Pos	ND	NK	Cat, WF, T, K, P	355	26	21.0
2*	64 yr	38 yr	F	F	U, Sys, GI	Pos	Pos	No	No	1,302	209.6	1,288.0
3*	42 yr	NK	F	A	OAS	Neg	ND	No	Mite, cat	148	15.3	17.5
4*	26 yr	NK	M	G	NK	ND	Pos	NK	NK	3,736	5.34	82.5
5	17 yr	NK	F	G	NR	ND	Neg[d]	NA	NK	926	4.12	29.0
6*	16 yr	NK	F	G	NR	ND	Neg[d]	NA	HE	433	12.4	4513.0
7*	14 yr	NK	F	G	NK	ND	NK	NK	NK	14,525	40.2	12,058.5
8*	13 yr	NK	M	G	RC, V	ND	Pos	NK	HE	866	7.8	210.0
9*	13 yr	11 mo	M	I	U, E, V, AS	Pos	NP[c]	No	PO, HE	909	147.4	12,444.5
10	11 yr	NK	M	G	NK	ND	NK	NK	NK	763	4.99	195.0
11*	11 yr	9 mo	F	I	U, AE, AS	Pos	NP[c]	No	Candida	ND	22.5	8,717.0
12*	10 yr	NK	F	G	Ap	Pos	Pos	NK	ND	1,432	44.8	3,785.5
13	10 yr	NK	F	G	U, V, D	Pos	Pos	NK	ND	653	10.4	893.0
14*	8 yr	NK	M	G	U,I	Pos	Pos[d]	NK	HE	455	3.77	973.0
15*	8 yr	NK	M	G	E, U, AS	Pos	Pos[d]	NK	HE	2,200	49.7	6,165.0
16	8 yr	NK	M	G	U, R, RC	Pos	Pos	NK	HE	1271	84.1	22,097.5
17*	7 yr	NK	M	G	U, R, AS	Pos	Pos[d]	NK	HE	399	17.8	836.5
18*	7 yr	NK	M	G	NK	ND	NK	NK	NK	2,275	20	4,931.5
19	7 yr	NK	M	G	Sys	Pos	Pos[d]	NK	NK	7,480	101	2,435.5
20*	6 yr	NK	NK	G	NK	ND	NK	NK	NK	246	6.09	356.0
21	6 yr	NK	M	G	NK	ND	NK	NK	NK	297	5.95	108.0
22*	5 yr	NK	M	G	NR	ND	Neg[d]	NA	HE	866	4.35	517.5
23	5 yr	NK	F	A	U, AS	Pos	ND	NK	GP, mite, cat, HE, PN	386	16.5	319.0
24	5 yr	NK	M	G	NK	ND	NK	NK	NK	252	27.9	1,595.0
25*	5 yr	NK	M	G	NK	ND	NK	NK	NK	2,760	6.44	1,000.5
26	5 yr	NK	M	G	NK	ND	NK	NK	NK	848	45.2	17.5
27*	4 yr	NK	M	G	U, I	ND	Pos[d]	NK	HE	56.6	6.19	3,658.5
28*	4 yr	NK	F	G	Sys	Pos	Pos[d]	NK	NK	974	98	19,178.0
29*	4 y	NK	M	G	NK	ND	NK	NK	NK	246	6.4	117.5
30*	4 y	NK	M	G	NK	ND	NK	NK	NK	1,894	3.81	87.5
31*	4 y	NK	F	G	Sys	Pos	Pos	NK	HE	489	62.5	8,847.5
32*	4 yr	NK	F	G	NK	ND	NK	NK	NK	116	6.94	943.0
33	3 yr	NK	M	G	U	Pos	Pos	NK	ND	137	24.6	3,986.5
34*	3 yr	NK	M	G	U, AE, R	ND	Pos[d]	NK	HE	59.1	4.13	27.5
35*	3 yr	NK	M	G	U, R, W	Pos	Pos[d]	NK	HE	59.1	4.13	123.0
36	3 yr	NK	M	G	NR	ND	Neg	NA	NK	10.9	5.81	1,025.5
37*	3 yr	NK	F	G	Sys	Pos	Pos[d]	NK	NK	125	11	1,474.0
38*	3 yr	NK	M	G	NK	ND	NK	NK	NK	26	5.19	61.5
39*	3 yr	NK	F	G	NK	ND	NK	NK	NK	1,718	4.15	2,480.0
40*	3 yr	NK	M	G	Sys	ND	ND	NK	NK	325	44.4	5,523.0
41*	3 yr	NK	F	G	NK	ND	NK	NK	NK	125	11	591.5
42*	3 yr	NK	F	G	NK	ND	NK	NK	NK	201	9.66	766.5
43*	3 yr	NK	M	G	NK	ND	NK	NK	NK	67.5	10.7	142.5
44*	2 yr	NK	M	G	RC, U, OAS	Pos	Pos[d]	NK	ND	1,466	82.7	36.5
45*	2 yr	9 mo	F	S	U (face)	Pos	Pos[b]	Yes	HE	ND	3.82	28.0
46*	2 yr	3 mo	F	S	U, AD, V	Pos	Pos[b]	No	HE, fish	ND	68.4	21,518.0
47*	2 yr	NK	M	A	AD, OB	Neg	ND	NK	Mite	134	9.3	620.0
48	2 yr	NK	F	G	NK	ND	NK	NK	NK	53.9	6.71	67.0
49	2 yr	NK	M	G	NK	ND	NK	NK	NK	>100	46.9	7,310.0
50*	2 yr	NK	F	G	NK	ND	NK	NK	NK	83.4	17.7	219.0
51*	2 yr	NK	M	G	NK	ND	NK	NK	NK	193	4.82	132.0
52*	2 yr	NK	M	G	NR	ND	Neg[d]	NA	NK	117	11.8	2,140.5
53	1 yr	NK	F	A	AD	NP[c]	ND	NK	NK	88.5	6.24	99.0
54*	1 yr	NK	F	A	AD, OB	Pos	ND	NK	EW, soja, nuts	217	16.5	118.0
55*	1 yr	5 mo	M	S	U, AE (face)	Pos	Pos[b]	No	HE, fish	13.5	8.05	16.5
56*	9 mo	9 mo	F	S	U, GI	Pos	Pos[b]	No	No	351	16.14	72.0
57	7 mo	7 mo	F	S	U, GI	Pos	Pos[b]	No	No	161	17.3	852.5
58	7 mo	7 mo	F	S	U, GI	Pos	Pos[b]	Yes	No	136	3.11	134.5
59*	4 mo	4 mo	M	S	U (face)	Pos	Pos[b]	No	No	2,000	13	52.5
60*	4 mo	4 mo	F	S	U	Pos	Pos[b]	Yes	No	60	9.97	27.0
61*	4 mo	3 mo	F	S	U (face)	Pos	Pos[b]	Yes	No	18.7	ND	30.5
62*	3 mo	3 mo	F	S	GI	Pos	Pos[b]	Yes	No	29	12.3	450.5
63*	3 mo	3 mo	M	S	U, AE	Pos	Pos[b]	Yes	No	51	16.6	882.5
64*	3 mo	3 mo	F	S	U	Pos	Pos[b]	Yes	NK	1,292	22.4	4,599.5
65	NK	NK	NK	A	NK	ND	NK	NK	NK	179	26.5	1,425.0
66*	NK	NK	NK	G	NK	ND	NK	NK	NK	1,129	66.4	13,629.5

[a] Abbreviations: *, patients tested also for IgE reactivity to Cas1.2–Cas5.6; yr, years; mo, months; F, female; M, male; A, Austria; S, Spain; G, Germany; I, Italy; F, France; CM, cow's milk; SPT, skin-prick test; NA, not applicable; ND, not done; NK, not known; NP, not possible; NR, no reaction; Pos, positive; Neg, negative; FI, fluorescence intensity; kU_A/L, kilounits allergen-specific IgE per liter; kU/L, kilounits per liter; OAS, oral allergy syndrome; AD, atopic dermatitis; AE, angioedema; AS, asthma; Ap, abdominal pain; D, diarrhea; GI, gastrointestinal symptoms; I, itching; OB, obstructive bronchitis; R, redness; RC, rhinoconjunctivitis; Sys, systemic reaction; U, urticaria; V, vomiting; W, wheals; GP, grass pollen; PO, pollen; WF, wheat flour; T, tomato; K, kiwi; P, pork; HE, hen's egg; EW, egg white; PN, peanut.

[b] Open challenge as described by Garcia-Ara et al. (22).

[c] Challenge not possible as the reaction to cow's milk is too strong.

[d] Double-blind placebo-controlled food challenge.

structural properties. IgE-reactive epitopes of αS1-casein were mapped using recombinant fragments and synthetic αS1-casein-derived peptides. The frequency of IgE recognition of αS1-casein and αS1-casein-derived synthetic peptides was determined by microarray technology using sera from cow's milk-allergic patients. Furthermore, the allergenic activity of the intact allergen and αS1-casein-derived peptides was determined using rat basophil leukemia cells expressing the human FcεRI receptor, which had been loaded with allergic patients' sera. Our results indicate that αS1-casein contains several

Table II. *Synthetic αS1-casein-derived peptides*

Peptide	Sequence[a]	Length (aa)	pI	Molecular Mass (kDa)
Cas1[b]	RPKHPIKHQGLPQEVLNENLLRFFVAPFPEV**C**	32	8.22	3.75
Cas1.2	VLNENLLRFFVAPFPEVFGKEKVNELSKDIGS	32	5.01	3.64
Cas2	FGKEKVNELSKDIGSESTEDQAMEDIKQMEAES**C**	33	4.18	3.70
Cas2.3	EDQAMEDIKQMEAESISSSEEIVPNSVEQK	30	3.89	3.38
Cas3	ISSSEEIVPNSVEQKHIQKEDVPSERYLGYEQLLR**C**	36	4.80	4.21
Cas3.4	VPSERYLGYLEQLLRLKKYKVPQLEIVPNS	30	9.31	3.57
Cas4	**C**LKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKE	34	8.14	3.96
Cas4.5	RLHSMKEGIHAQQKEPMIGVNQELAYFYPE	30	6.07	3.55
Cas5	**C**PMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYV	35	4.14	4.24
Cas5.6	FYQLDAYPSGAWYYVPLGTQYTDAPSFSDI	30	3.42	3.44
Cas6	PLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW**C**	33	3.92	3.60

[a] Cysteine residues added to facilitate coupling are indicated in boldface letters.
[b] Cas indicates αS1-casein-derived peptide.

sequential IgE epitopes, but the isolated peptides were less potent than the complete allergen in triggering effector cell degranulation.

Materials and Methods

Patients and biological materials

Sera from children (n = 62; age 3 mo to 17 years; 27 females and 32 males, 2 unknown) and adults (n = 4; age 26–64 years; three women, one man) containing cow's milk-specific IgE Abs (CAP-FEIA; Phadia) were obtained from Austria (n = 7), Germany (n = 44), Italy (n = 2), Spain (n = 12), and France (n = 1). Table I summarizes the available demographic, clinical, and serological characteristics of the patients. Information regarding symptoms after consumption of cow's milk was available for 42 out of the 66 patients and for 16 patients it was known whether they had persistent cow's milk allergy or if they had become tolerant (persistent, n = 9; tolerant, n = 7).

Fourteen patients underwent double-blind challenge and 12 had received an open challenge as described by Garcia-Ara et al. (22). For 15 patients we knew the exact age when their cow's milk allergy started. Only patient no. 2 became allergic when she was adult.

The study was approved by the Ethics Committee of the Medical University of Vienna. Natural α-casein fractions containing a mixture of αS1- and αS2-casein were purchased from Sigma-Aldrich, and cow's

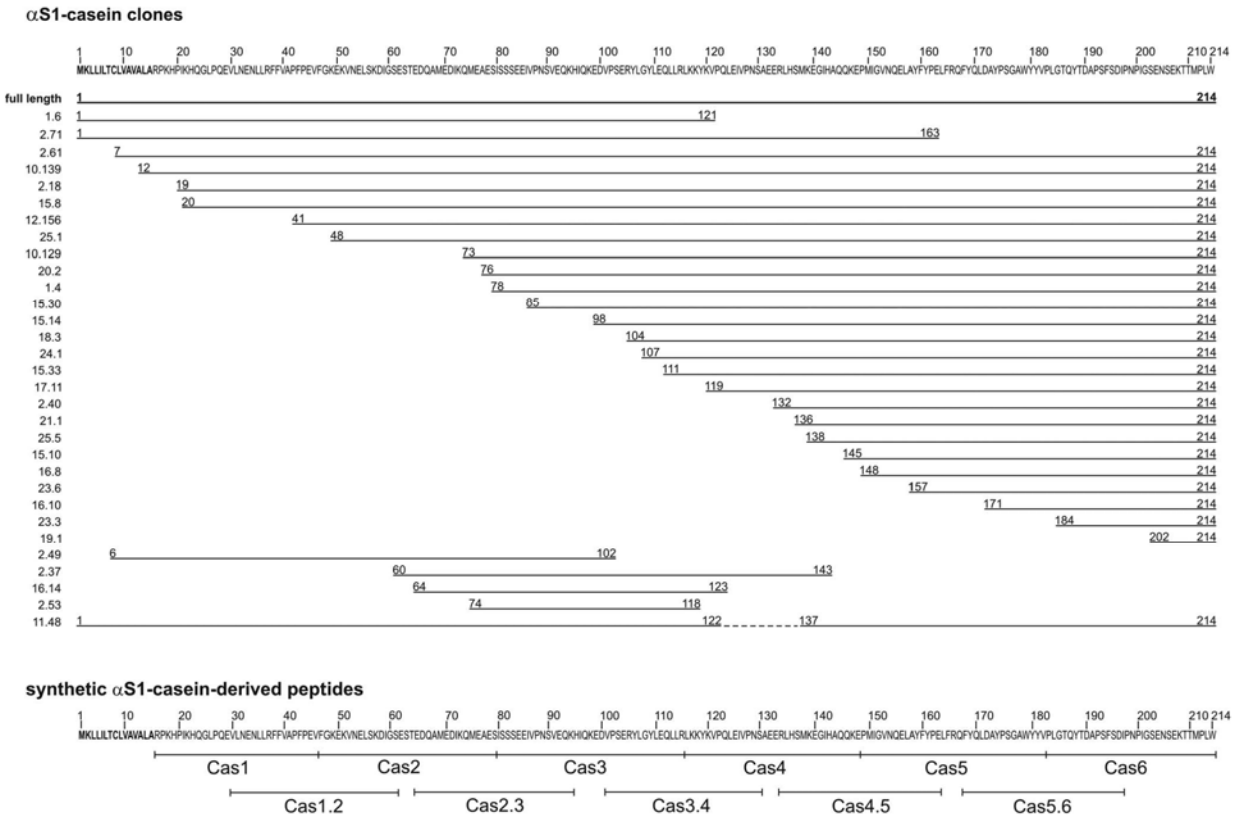

FIGURE 1. IgE-reactive recombinant αS1-casein fragments identified by screening of an expression cDNA library from bovine mammary glands. At the top the deduced amino acid sequence of αS1-casein (aa 1–214) is shown in the single letter code. Twenty-eight full-length cDNAs with identical sequence and 31 cDNAs coding for recombinant fragments (left margin, clone numbers) are shown. The cDNA of clone 11.48 codes for a protein variant with a deletion (broken line). At the bottom the location of synthetic αS1-casein peptides used for epitope mapping is shown. The DNA and deduced amino acid sequence of the αS1-casein clones were submitted to GenBank under the accession nos. EU221551–EU221581.

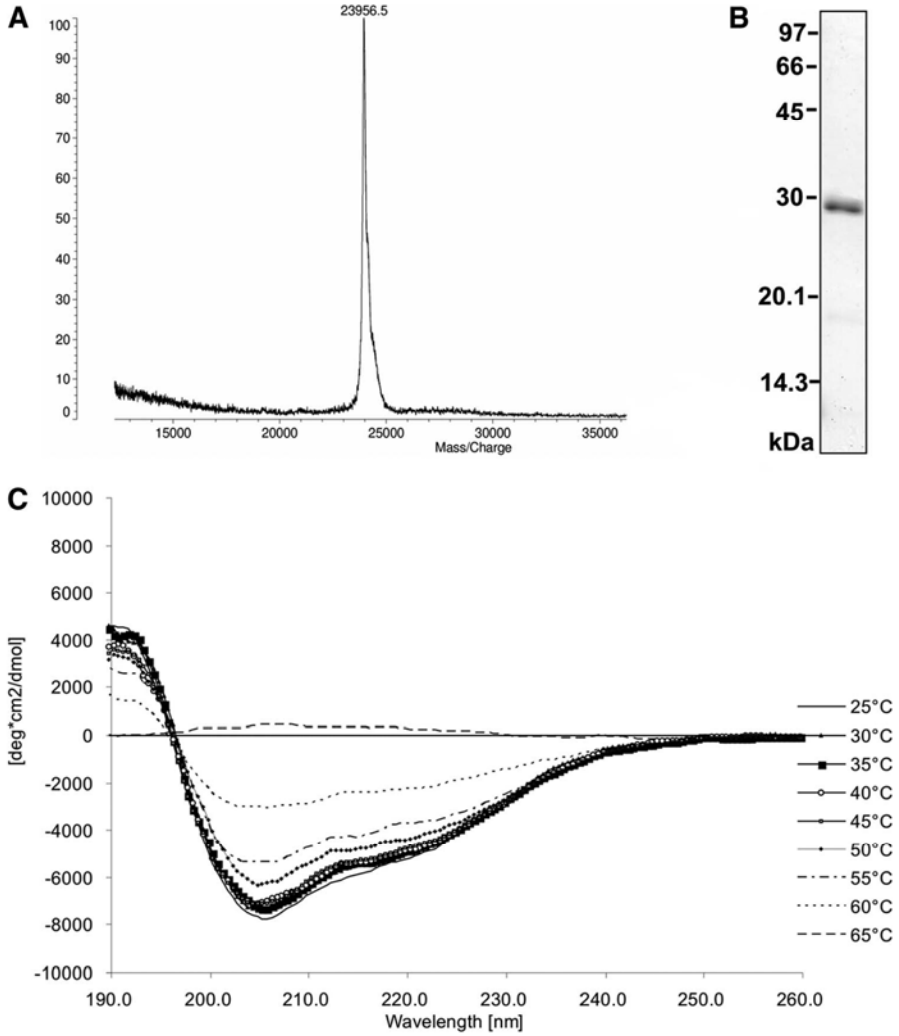

FIGURE 2. Biochemical and structural characterization of purified rαS1-casein. *A*, Mass spectrometric analysis of purified rαS1-casein. The mass/charge ratio is shown on the *x*-axis and the signal intensity is displayed on the *y*-axis as the percentage of the most intensive signal obtained in the investigated mass range. A Coomassie brilliant blue-stained SDS-PAGE containing purified rαS1-casein is shown in *B*, where molecular masses are indicated in kDa in the left margin. Thermal unfolding of rαS1-casein recorded by circular dichroism is displayed in *C*. The observed ellipticity (Θ, *y*-axis) was monitored from 190 to 260 nm at different temperatures (25–65°C).

milk (NÖM; 3.6% fat, pasteurized, batch 324103:43) was bought at the local market.

Construction and IgE screening of a bovine mammary gland cDNA library

Total RNA was isolated from bovine mammary glands obtained from a lactating cow using the NucleoSpin RNA L kit (Macherey-Nagel). Polyadenylated mRNA was separated using the NucleoTrap mRNA Mini kit (Macherey-Nagel). cDNA was obtained using oligo(dT)15 primers and the cDNA Synthesis System (Roche) following the manufacturer's instructions. The double-stranded cDNA was methylated, ligated to *Eco*RI linkers, digested with *Eco*RI, and inserted into dephosphorylated λgt11 *Eco*RI-cut arms and packed into λ phages using the Lambda gt11/*Eco*R I/CIAP-Treated/Gigapack III cloning kit (Stratagene). The expression library (162,000 phages) was screened with a pool of sera from three cow's milk-allergic patients with a broad IgE reactivity profile to cow's milk allergens, and bound IgE Abs were detected with ^{125}I-labeled anti-IgE Abs (IBL International). Membranes were exposed to x-ray films (Kodak). Phage DNA of positive clones was amplified using λgt11 forward primer and λgt11 reverse primer (Stratagene), and the amplified PCR products were subjected to automated sequencing (VBC Genomics). The obtained nucleotide sequences were compared with the sequences deposited in the National Institutes of Health database (www.ncbi.nlm.nih.gov/BLAST/) and submitted to GenBank under the accession nos. EU221551–EU221581.

Expression and purification of full-length recombinant αS1-casein

The cDNA coding for the mature αS1-casein without N-terminal signal sequence and with C-terminal hexahistidine tag (rαS1-casein) was obtained by PCR amplification using the PFU DNA polymerase system from Fermentas Life Sciences and the following oligonucleotides (MWG Biotech): αS1-casein 5′, 5′-GCG GAT CCA CAT ATG AGG CCT AAA CAT CCT ATC AAG-3′ (*Nde*I underlined); αS1-casein 3′, 5′-CG GAA TTC CTG CAG AAC TCA *GTG ATG ATG ATG ATG ATG* CCA CAG TGG CAT AGT AGT CTT TTC-3′ (*Eco*RI underlined; hexahistidine-encoding sequence is in italics); and the cDNA from the mammary gland cDNA library was used as a template. The PCR product was gel purified and cloned into the *Nde*I/*Eco*RI-digested expression vector pET17b (Novagen). The sequence was verified by automated sequencing (VBC Genomics).

E. coli strain BL21 (DE3) (Novagen) was transformed with the αS1-casein-pET17b construct. Protein expression was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM. Recombinant proteins accumulated in the inclusion body fractions. Bacterial cells were lysed, and proteins were solubilized in urea buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl (pH 8)) and purified by Ni^{2+}-affinity chromatography (Qiagen). Purified rαS1-casein was dialyzed against 10 mM sodium acetate (pH 5), and the protein concentration was determined with the Bio-Rad DC protein assay (Bio-Rad Laboratories) using BSA as a standard. The purity was assessed by SDS-PAGE (23) and Coomassie staining (24).

Synthesis of αS1-casein-derived peptides

Peptides displayed in Table II were synthesized using Fmoc (9-fluorenylmethoxycarbonyl) strategy with HBTU ((2-(1*H*-benzotriazol-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphat) activation (0.1 mmol small-scale cycles) on an Applied Biosystems peptide synthesizer model 433A and purified as described (25).

The peptides Cas1, Cas2, Cas3, Cas4, Cas5, and Cas6 covered segments of αS1-casein, which according to surface prediction (26) contained sequences with high accessibility for Abs. In a second attempt for more detailed epitope mapping, peptides Cas1.2, 2.3, 3.4, 4.5, and 5.6 were made to overlap with the first six peptides (Table II).

MALDI-TOF mass spectrometry of rαS1-casein

Laser desorption mass spectra were acquired in a linear mode with a TOF Compact MALDI II instrument (Kratos and piCHEM). Samples were dissolved in 10% acetonitrile (0.1% trifluoroacetic acid) and α-cyano-4 hydroxycinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

Circular dichroism (CD)[3] analysis of purified rαS1-casein

CD spectra were conducted on a Jasco J-810 spectropolarimeter fitted with a Jasco PTC-423S/L Peltier type temperature control system. Far-UV CD spectra were measured in a 2-mm pathlength quartz cuvette (Hellma) at a protein concentration of 0.1 mg/ml. Spectra were recorded from 190 to 260 nm with 1-nm resolution at a scan speed of 50 nm/min and resulted from averaging three scans. All measurements were performed in 1 mM sodium acetate (pH 5). The final spectra were corrected by subtracting the corresponding baseline spectrum obtained under identical conditions. Results were expressed as the mean residue ellipticity (Θ) at a given wavelength. For thermal denaturation, experiments of rαS1-casein spectra were recorded at gradually increasing temperature (5°C steps) from 25°C to 95°C with a heat rate of 2°C/min.

IgE reactivity by immunoblot, dot blot, and microarray

For immunoblot analysis, 2 μg/cm gel of rαS1-casein was separated by SDS-PAGE and blotted onto nitrocellulose (27). For dot blot analysis 1 μg of the protein was dotted onto nitrocellulose. Blots were incubated with sera from milk-allergic patients diluted 1/20 in PBST (PBS, 0.5% (v/v) Tween 20). Bound IgE Abs were detected with 1/15 diluted ^{125}I-labeled anti-human IgE Abs (IBL International).

IgE reactivity to microarrayed whole cow's milk extract, rαS1-casein, a natural α-casein fraction, as well as to synthetic α-casein-derived peptides was determined as described in Nystrand (28). Whole milk extracts, recombinant and purified natural, as well as recombinant cow's milk proteins were spotted with a Nano-Plotter NP2 (Gesellschaft für Silizium-Mikrosysteme) onto capillary-flow membrane that was attached to ordinary microscope glass slides. The spotted microarrays were pre-wetted with 30 μl of assay buffer, incubated with 25 μl of undiluted sera of milk-allergic patients, washed with 30 μl of assay buffer and assayed with 20 μl of detecting Ab. Finally, the membrane was washed with 2 × 30 μl of assay buffer. Bound IgE Abs were detected with a fluorescence-conjugated anti-IgE Ab and fluorescence intensities (FI) were measured at a wavelength of 670 nm. Cut-off level was set to a FI of 400 according to values gained with human serum albumin.

ELISA competition experiments

For the IgE competition experiments, rαS1-casein in sodium carbonate buffer (pH 9.6) was coated onto ELISA plates (Nunc Maxisorb) at a concentration of 5 μg/ml overnight at 4°C. Patients' sera diluted 1/5 in Tris-buffered saline containing 0.5% (v/v) Tween 20 (TBST) were preincubated overnight at 4°C either with 10 μg/ml rαS1-casein or with 10 μg/ml each of the synthetic αS1-casein-derived peptides (Table II) (see Fig. 5*A*, Cas1–Cas6, and *B*, Cas1–Cas6 plus Cas1.2–5.6) (Mix) or, for control purposes, with TBST (not inhibited, NI). On the next day, the plates were washed five times with TBST and blocked with TBST at 37°C for 2.5 h. Preincubated patients' sera were exposed to ELISA plate-bound rαS1-casein overnight. Bound IgE Abs were detected with a 1/1000 diluted monoclonal anti-human IgE Ab (BD Biosciences) and subsequently with a 1/1000 diluted HRP-labeled sheep anti-mouse IgG Ab (GE Healthcare). All determinations were conducted as duplicates.

[3] Abbreviations used in this paper: CD, circular dichroism; kU_A/L, kilounits allergen-specific IgE per liter; RBL, rat basophil leukemia.

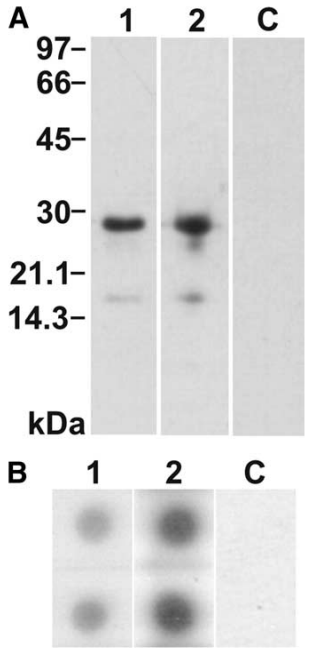

FIGURE 3. IgE binding of rαS1-casein. IgE reactivity of nitrocellulose-blotted (denatured) rαS1-casein by immunoblot (*A*) and dot blot analysis (*B*) under native conditions. Nitrocellulose-bound rαS1-casein was incubated with the sera from two cow's milk-allergic patients (*lanes 1* and *2*) and a nonallergic individual (*lane C*), and bound IgE Abs were detected with ^{125}I-labeled anti-IgE Abs. Molecular masses are indicated in kDa.

Statistical analysis

Differences regarding IgE reactivity and induction of rat basophil leukemia (RBL) release among the patients with persistent vs transient cow's milk allergy were analyzed with the Mann-Whitney *U* test and were considered significant when p was <0.05 and have a trend toward significance when $p < 0.1$.

RBL assays

RBL cells (clone RBL-703/21) transfected with the human FcεRI receptor (29) were cultured in RPMI 1640 medium supplemented with 10% FCS, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, 100 μM 2-ME, and 1% penicillin/streptomycin.

Cells were resuspended in culture medium (2×10^6 cells/ml), and 50 μl of this solution per well of a 96-well flat bottom microplate was pipetted. Sera from cow's milk-allergic patients were diluted 1/10 in culture medium and 50 μl thereof was added to the cells incubated overnight at 37°C, 7% CO_2, 95% humidity.

Medium was removed and the plates were washed three times with 200 μl/well Tyrode's buffer containing 0.1% (w/v) BSA or HSA, respectively. Then 100 μl of the milk components (concentration, 0.3 μg/ml) dissolved in Tyrode's buffer containing 50% D_2O and 0.1% (w/v) BSA/HSA was added to the cells and incubated for 1 h at 37°C, 7% CO_2, 95% humidity. Spontaneous release was determined by lysing cells in 1% Triton X-100 in Tyrode's buffer. Fifty-microliter aliquots of the supernatant were transferred to a fresh plate, mixed with 50 μl of assay solution (0.1 M citric acid or sodium citrate (pH 4.5) plus 160 μM 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide), and incubated at 37°C, 7% CO_2, 95% humidity for 1 h. The reaction was stopped by adding 100 μl of glycine buffer (200 mM glycine, 200 mM NaCl (pH 10.7)) to each well. Fluorescence was measured at λ_{Ex} of 360 nm and λ_{Em} of 465 nm (where Ex is excitation and Em is emission) in a fluorescence microplate reader. Specific release was calculated using the formula: $[(Fl_S - Fl_{Sp}):(Fl_Z - Fl_{Sp})] \times 100$, where Fl_S is the fluorescence of the sample, Fl_{Sp} is the fluorescence of the spontaneous release, and Fl_Z is the fluorescence of the total release. Values that exceeded 5% of the total release were considered as positive. Values obtained with buffer alone were subtracted.

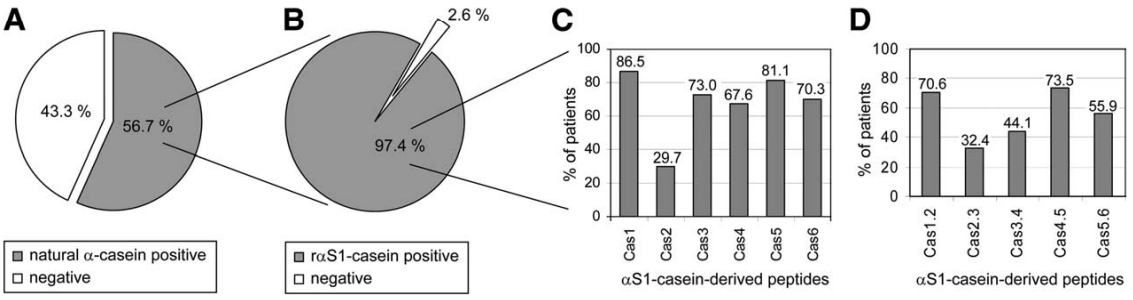

FIGURE 4. Frequency of IgE recognition of purified natural α-casein, rαS1-casein, and rαS1-casein-derived synthetic peptides. Circle diagrams (*A*) show the percentage of cow's milk-allergic patients ($n = 66$) that recognize natural α-casein and (*B*) the percentage of sera positive with natural α-casein ($n = 38$) recognizing rαS1-casein, respectively. The frequency of IgE recognition (*y*-axis) of αS1-casein-derived peptides (*x*-axis: Cas1–Cas6 in *C*; Cas1.2.–Cas5.6 in *D*) in rαS1-casein-positive patients ($n = 37$ for *C*; $n = 34$ for *D*) is shown in the form of bar diagrams.

Results

Isolation and characterization of cDNAs coding for IgE-reactive αS1-casein and αS1-casein fragments

A cow's milk expression cDNA library was constructed using mammary gland tissue from a lactating cow and screened with the sera from cow's milk-allergic patients. The sequence analysis of IgE-reactive cDNA clones revealed that 59 independent cDNA clones contained cDNAs coding for αS1-casein (31 fragments and 28 full-length clones). The DNA sequences of the complete clones coding for αS1-casein were identical, and the deduced amino acid sequence of αS1-casein is displayed in Fig. 1. In the deduced amino acid sequence of αS1-casein a hydrophobic leader peptide, which is cleaved from the mature protein, is shown in boldface letters. IgE-reactive recombinant αS1-casein fragments of sizes ranging from 12 to 207 aa with a distribution over the whole molecule were identified (Fig. 1). Interestingly, a cDNA clone coding for a deletion variant of αS1-casein was identified (Fig. 1, clone 11.48), which possibly originates from a loop formation during reverse transcription and cDNA synthesis. Eleven αS1-casein-derived peptides spanning the complete sequence of the mature αS1-casein have been chemically synthesized (Fig. 1, *bottom*, and Table II).

Expression, purification, and biochemical analysis of rαS1-casein

A recombinant allergen corresponding to the mature αS1-casein allergen was obtained by expression of the αS1-casein cDNA without the hydrophobic leader sequence in *E. coli*. A C-terminal hexahistidine tag was added to facilitate the purification of rαS1-casein. Approximately 1.6 mg/L culture of rαS1-casein could be purified by nickel affinity chromatography. The calculated molecular mass of 23.9 kDa was confirmed by mass spectrometry (Fig. 2*A*), suggesting that the authentic protein had been produced. Consistent with earlier reports, purified rαS1-casein showed a higher molecular mass in SDS-PAGE than expected according to mass spectrometry (Fig. 2*B*) (30).

The far-UV CD spectrum of purified recombinant αS1-casein recorded at 25°C (Fig. 2*C*, black line) with a minimum at 205 and 218 nm indicated a folded protein that contains a considerable amount of secondary structure (β-strands, β-turns, and random coiled structure). Thermal unfolding of rαS1-casein was monitored at gradually increasing temperature (5°C steps) in a temperature range from 25°C to 95°C (Fig. 2*C*). During the heating process the allergen started to precipitate at ~60°C, but the remaining soluble fraction of rαS1-casein still showed features of the folded protein. After cooling to 25°C it was possible to resuspend the protein, which showed again a considerable amount of secondary structure as observed for the untreated rαS1-casein (data not shown).

rαS1-casein is a major cow's milk allergen according to IgE reactivity

The IgE binding capacity of rαS1-casein was evaluated first by immunoblot and dot blot analysis under denaturing and native conditions, respectively (Fig. 3). Sera from two cow's milk-allergic patients (Fig. 3, *lanes 1* and *2*) but not serum from the nonallergic individual (Fig. 3, *lane C*) showed comparable IgE reactivity with the denatured rαS1-casein and with the native protein (Fig. 3*B*).

A detailed analysis of the IgE reactivity of rαS1-casein was performed with sera from 66 cow's milk-allergic patients (Table I) using microarrayed components. IgE reactivity to cow's milk extract, a natural α-casein fraction, and rαS1-casein was analyzed, and the percentages of reactive sera are displayed in Fig. 4, *A* and *B*. We found that 56.7% of the patients showed IgE reactivity to the natural α-casein fraction containing αS1- and αS2-casein (Fig. 4*A*). The vast majority (97.4%) of these patients showed IgE reactivity to rαS1-casein (Fig. 4*B*).

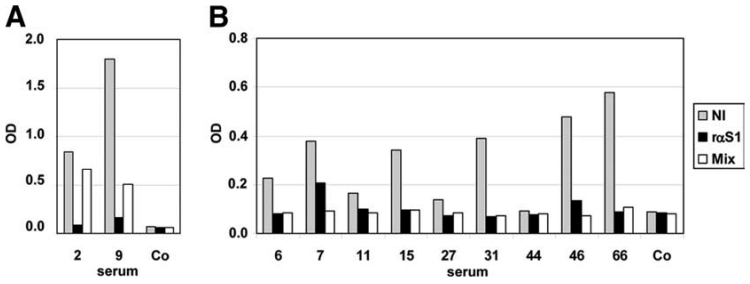

FIGURE 5. Importance of sequential IgE epitopes on αS1-casein. *A*, Sera from two cow's milk-allergic patients (nos. 2 and 9) and a nonallergic person (Co) were preadsorbed with rαS1-casein (rαS1), a mix of αS1-casein-derived peptides (Cas1–Cas6), or without inhibitor (NI). *B*, Sera from an additional nine IgE-positive patients (nos. 6, 7, 11, 15, 27, 31, 44, 46, 66) were preadsorbed with all available peptides (Cas1–Cas6 plus Cas1.2–Cas5.6). IgE levels to ELISA plate-bound rαS1-casein are displayed as OD values (*y*-axis).

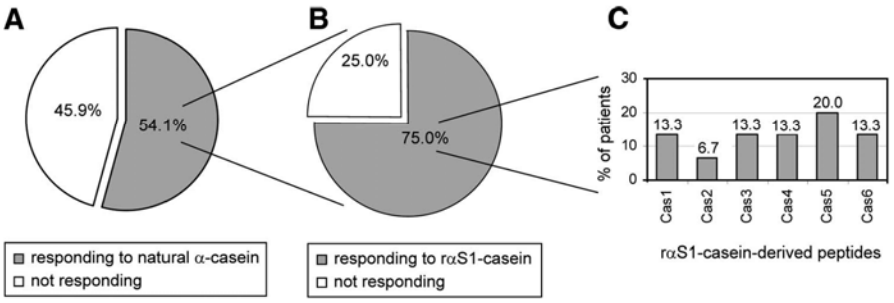

FIGURE 6. Frequency of degranulation induced in RBLs loaded with cow's milk-allergic patients' serum IgE with natural α-casein, rαS1-casein, and rαS1-casein-derived synthetic peptides. Circle diagrams (*A*) show the percentage of sera from cow's milk-allergic patients ($n = 37$) that after loading to RBL cells induce degranulation in response to natural α-casein. *B*, Percentage of positive sera from *A* ($n = 20$), which induced degranulation in response to rαS1-casein. The frequency (*y*-axis) of degranulation in response to αS1-casein-derived peptides (*x*-axis, Cas1–Cas6) in positive patients from *B* ($n = 15$) is shown in *C*.

Most of our patients were children displaying high as well as low cow's milk allergen-specific IgE levels, but we found no evidence that this would affect IgE reactivity to αS1-casein. For example, patient no. 52 in Table I had 11.8 kilounits of allergen-specific IgE per liter (kU_A/L) cow's milk-specific IgE and 2140.5 FI specific for rαS1-casein, whereas patient no. 56 had 16.14 kU_A/L cow's milk-specific IgE and only 72 FI rαS1-casein-specific IgE.

Sequential IgE epitopes of rαS1-casein are distributed over the whole molecule

An in-depth analysis of IgE reactivity to microarrayed αS1-casein-derived peptides (Cas1–Cas6) showed that the IgE reactivity in the rαS1-casein-positive sera varied from 29.7% (Cas2) to 86.5% (Cas1) of the tested sera (Fig. 4*C*). Since each of the αS1-casein-derived peptides was recognized by IgE Abs, it appeared that IgE epitopes are distributed over the complete allergen. We therefore synthesized an additional five peptides (Cas1.2, Cas2.3, Cas3.4, Cas4.5, and Cas5.6), which overlap with the original six peptides and tested them for IgE reactivity with sera from 49 patients from whom serum samples were available (Table I, patient numbers with asterisks). The frequencies of IgE recognition of Cas1.2–Cas5.6 among the 34 patients positive with rαS1-casein ($n = 34$) are shown in Fig. 4*D* (Cas1.2, 70.6%; Cas2.3, 32.4%; Cas3.4, 44.1%; Cas4.5, 73.5%; Cas5.6, 55.9%), demonstrating that sequential IgE epitopes are indeed spread over the allergen. Interestingly, we found nine patients who showed IgE reactivity to αS1-casein-derived peptides but not to complete rαS1-casein. Seven of these patients reacted with the peptide Cas3, suggesting that this region may represent a cryptic epitope.

Using an ELISA competition experiment, we studied if rαS1-casein may also contain conformational IgE epitopes. For this purpose sera from two cow's milk-allergic patients with IgE reactivity to the peptides were preadsorbed with the αS1-casein-derived peptides and the complete rαS1-casein allergen. IgE reactivity to rαS1-casein was strongly inhibited by the peptides in one patient but not in the other (Fig. 5*A*). Serum from a nonallergic person did not show IgE reactivity to rαS1-casein (Fig. 5*A*). We then preincubated sera from an additional nine patients with a mix containing each of the 11 casein-derived peptides (Table II), yielding a similar inhibition of IgE binding to rαS1-casein as compared with inhibition with the complete rαS1-casein (Fig. 5*B*).

Natural, recombinant αS1-casein and αS1-casein-derived synthetic peptides induce basophil degranulation only with sera from a subgroup of cow's milk-allergic patients

To assess the allergenic activity of the purified recombinant and synthetic milk components, RBL cells loaded with the same sera that had been tested for IgE reactivity were stimulated with the natural α-casein fraction, rαS1-casein, or the αS1-casein-derived synthetic peptides (Cas1–Cas6). We found that 54.1% of the sera that were positive in RBL assay with cow's milk ($n = 37$) also triggered RBL release upon exposure to the natural α-casein fraction (Fig. 6*A*). Most (i.e., 75%) of the latter sera also induced RBL release upon contact with rαS1-casein (Fig. 6*B*). Even the αS1-casein-derived synthetic peptides (Cas1–Cas6) induced basophil degranulation (Fig. 6*C*), but the extent of degranulation observed with the peptides was rather low (Fig. 7) and degranulation was observed only with 12 of the sera containing rαS1-casein-reactive IgE. Besides sera with IgE reactivity to rαS1-casein, Fig. 7 includes also sera that had reacted only with cryptic αS1-casein-derived peptides. It demonstrates that peptide-induced degranulation was mild because most sera induced <20% of mediator release. Only two sera gave a >20% release in conjunction with peptides (Cas4,

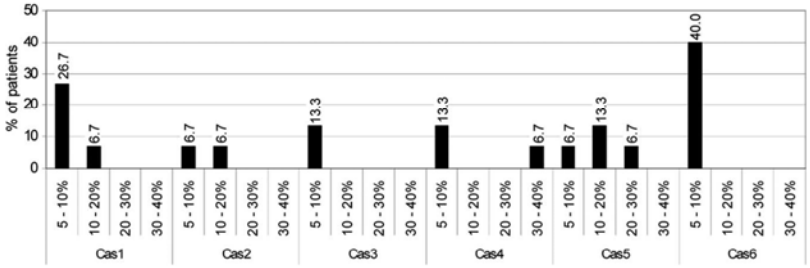

FIGURE 7. Percentages of αS1-casein-reactive sera ($n = 15$) giving a degranulation reaction with αS1-casein-derived peptides. The percentages of sera giving a degranulation reaction of defined magnitudes (*x*-axis, percentages of total release) with αS1-casein-derived peptides are displayed for those sera that had induced degranulation to rαS1-casein.

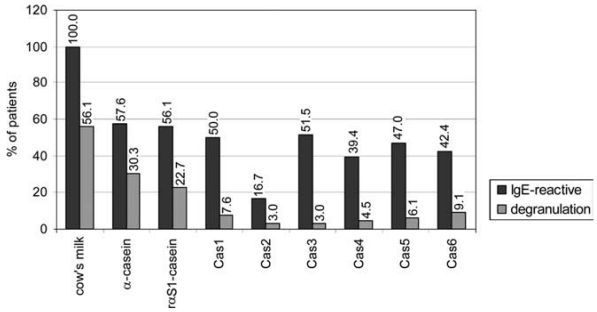

FIGURE 8. Comparison of the frequencies of IgE recognition and degranulation responses. The percentages of patients (n = 66) showing IgE reactivity to cow's milk, an α-casein fraction, rαS1-casein, as well as to synthetic αS1-casein-derived peptides (Cas1–Cas6) are represented by black bars and the percentages of sera giving a positive degranulation reaction in RBL assays are shown by gray bars.

Cas5). In fact, Fig. 8 shows that only a relatively low percentage of all tested sera (3.0–9.1%) caused degranulation in response to peptide exposure. The complete allergens were more potent in inducing RBL degranulation. We found that 30.3% of sera induced degranulation to the natural α-casein fraction and 22% to rαS1-casein (Fig. 8).

The additionally synthesized peptides Cas1.2–5.6 could not be tested in the RBL assays due to lack of sera.

Association of cow's milk-induced symptoms with IgE reactivity and basophil degranulation

In Table I demographic, clinical, and serological data of the 66 persons with cow's milk-specific IgE Abs are displayed. Fig. 9*A* shows the IgE reactivity and basophil degranulation due to cow's milk-, α-casein-, rαS1-casein-, and αS1-casein-derived peptides for 42 patients with known clinical symptoms. These patients were grouped according to their symptoms (group 1, no reaction; group 2, only gastrointestinal symptoms; group 3, gastrointestinal symptoms and other symptoms; group 4, only skin symptoms; group 5, skin and respiratory symptoms; group 6, severe, systemic reactions).

We found that individuals with cow's milk-specific IgE but no symptoms (n = 5) showed almost no mediator release upon stimulation with rαS1-casein, whereas almost all patients with severe systemic reactions (n = 6) showed mediator release after stimulation with rαS1-casein. In agreement with earlier studies we found that persons without reactions had much lower cow's milk-specific IgE levels (mean IgE level, 7.62 kUA/L) than did patients with systemic reactions (mean IgE level, 88.08 kUA/L) (Table I). The latter group also exhibited more frequent and stronger IgE reactivity to αS1-casein-derived peptides. αS1-casein was a major allergen for patients from most of the groups (i.e., groups 1–3, 5, and 6), but only 5 out of 13 patients with only skin symptoms showed IgE reactivity to this allergen.

Seven of our patients suffered from transient and nine from persistent cow's milk allergy (Fig. 9*B*). Peptide Cas2 was the only peptide that was recognized by serum IgE of patients in the persistent group but not from transient cow's milk-allergic patients (Fig. 9*B*). Interestingly, this peptide contains the sequence (aa 39–48) that has been identified earlier (14, 19). Patients who had not outgrown their cow's milk allergy showed more frequent and more intense IgE reactivity and mediator release to αS1-casein and αS1-casein-derived peptides than did patients who had outgrown their milk allergy. A statistically significant difference between these two groups was found for cow's milk-, natural α-casein-, and rαS1-casein-induced mediator release (p = 0.031, 0.043 and 0.031, respectively), and for IgE reactivity we observed a trend toward significance ($p < 0.1$) in the cases of Cas2 and Cas6.

IgE reactivities to Cas1.2–Cas5.6 are also indicated for the different patients groups (Fig. 9) but could not be determined for each of the patients due to limited availability of sera.

Discussion

Many of the most common respiratory allergens are available as recombinant proteins for basic immunological studies, diagnostic purposes, and allergen-specific therapeutic approaches. In contrast, the molecular structures of a considerably lower number of food allergens have been revealed, and fewer recombinant food allergens resembling the structural and immunological properties of the natural counterparts are available. The recombinant αS1-casein reported in our study is the first recombinant cow's milk allergen that shows IgE reactivity and allergenic activity. The cDNA coding for αS1-casein has been isolated from a cDNA expression library constructed from cow's mammary glands with IgE Abs from cow's milk-allergic patients. The amino acid sequence deduced from the αS1-casein-encoding cDNA was identical with those that were earlier described in the context of dairy research. rαS1-casein was expressed in *E. coli* and could be purified as a folded protein that resembled a primarily β-fold structure as determined by CD spectroscopy. The rαS1-casein remained folded up to temperatures of 55°C and even after cooking could be resuspended as a folded protein. Accordingly, rαS1-casein was recognized by IgE Abs in its nondenatured form as well as after boiling and denaturing SDS-PAGE followed by immunoblotting. When we tested 66 cow's milk-sensitized patients (children, adults) from different European populations we found that 56.1% showed IgE reactivity to rαS1-casein, which therefore represents a major cow's milk allergen. None of the patients without IgE reactivity to natural α-casein showed IgE reactivity to rαS1-casein. Only one serum giving a positive reaction in the RBL assay with rαS1-casein failed to react with the natural casein preparation. It thus seems that rαS1-casein is highly specific for picking up αS1-casein-reactive patients. A potential advantage of rαS1-casein over natural α-casein preparations, which may also contain αS2-casein, could be that it allows to discriminate αS1-casein from αS2-casein-reactive patients.

αS1-casein is considered as a true food allergen, also classified as class I food allergen, which is comparable to the major fish allergen parvalbumin (31), the major peanut allergens (Ara h 1, Ara h 2, Ara h 3) (32), and the major shrimp allergen (33), and it can induce severe and life-threatening anaphylactic reactions. Interestingly, we found that >50% of patients with gastrointestinal symptoms, skin and respiratory symptoms, or severe systemic reactions, but fewer patients with only skin symptoms, showed IgE reactivity to rαS1-casein (Fig. 9*A*).

Most of the true food allergens have been reported to contain sequential IgE epitopes. In fact, using recombinant αS1-casein fragments as well as synthetic αS1-casein-derived peptides we were able to identify several continuous (i.e., sequential) IgE epitopes spanning almost the complete αS1-casein sequence. The most frequently recognized αS1-casein-derived synthetic peptides were Cas1 and Cas5, which were recognized by IgE Abs of 86.5% and 81.1% of the rαS1-casein-reactive patients. This finding is in agreement with earlier studies that identified regions covered by Cas1 and Cas5 as major B cell epitopes (18, 19, 34). Interestingly, Cas3 was recognized also by patients without IgE reactivity to complete rαS1-casein or the natural α-casein fraction, suggesting that this region contains a cryptic epitope that becomes accessible

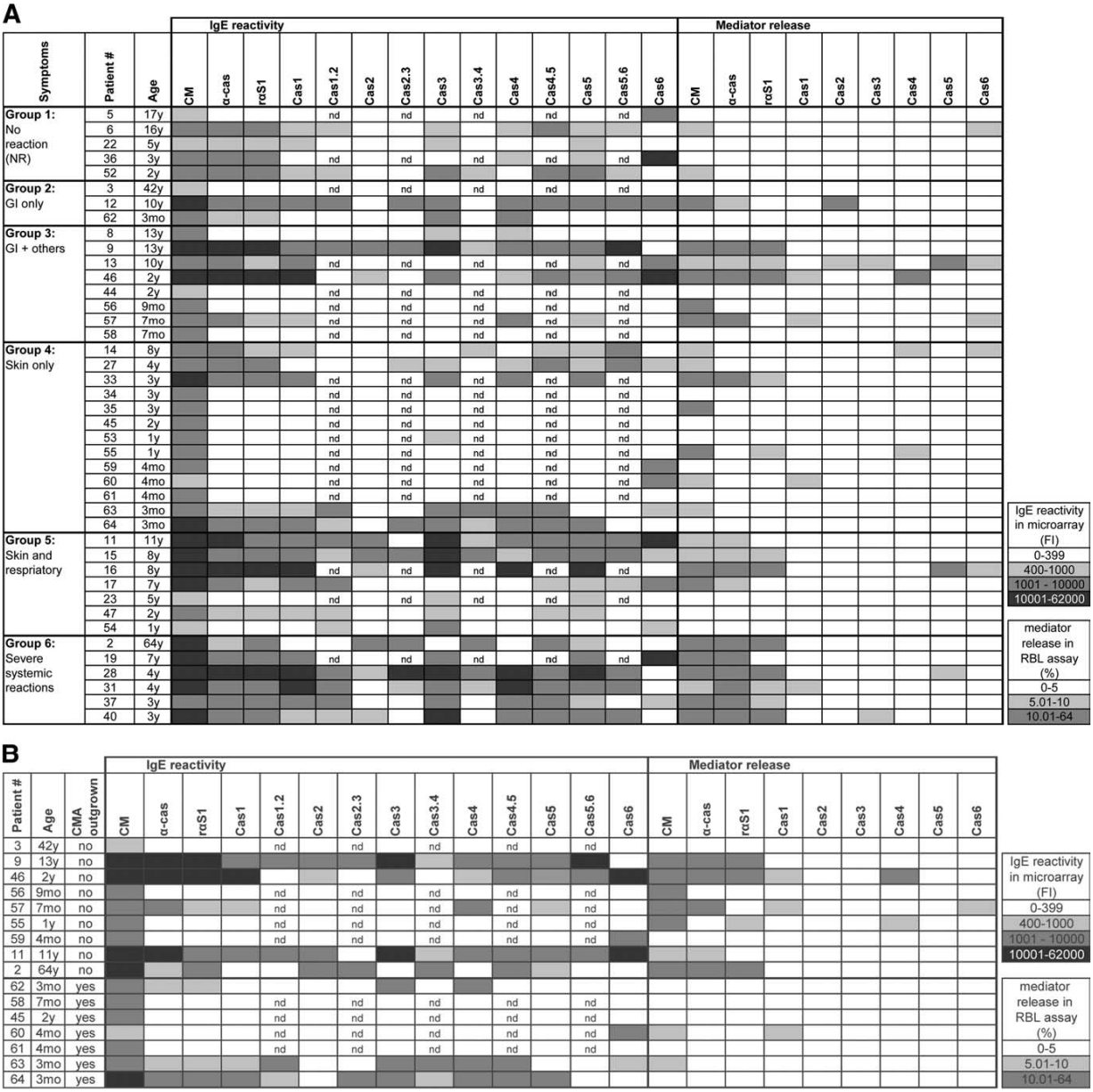

FIGURE 9. *A*, Association of milk-related symptoms with IgE reactivity and basophil degranulation. Patients were grouped according to their milk-related symptoms (group 1, no reaction; group 2, gastrointestinal symptoms only (GI only); group 3, gastrointestinal symptoms and symptoms in other organ systems (GI + others); group 4, skin symptoms only (skin only); group 5, skin and respiratory symptoms (skin + respiratory); group 6, severe systemic reactions. Intensities of IgE reactivities to microarrayed components (cow's milk, CM; natural α-casein, α-cas; recombinant αS1-casein, rαS1-casein; αS1-casein-derived synthetic peptides, Cas1–Cas6; Cas1.2–Cas5.6) as well as the induction of degranulation by these components are indicated. *B*, IgE reactivity and basophil degranulation in persons with persistent and transient cow's milk allergy. The intensities of IgE reactivity and basophil degranulation in response to cow's milk (CM), natural α-casein (α-cas), recombinant αS1-casein (rαS1-casein), and αS1-casein-derived synthetic peptides (Cas1–Cas6, Cas1.2–Cas5.6) are shown for each patient.

to IgE Abs after digestion of αS1-casein. In total, we found that 6 of the 66 sera contained IgE Abs to αS1-casein-derived peptides and induced RBL degranulation in response to peptides, although they did not react with natural α-casein, indicating the presence of cryptic epitopes that may be revealed after degradation of the allergen in the gastrointestinal tract.

Using IgE competition studies in which we preadsorbed sera from rαS1-casein-reactive patients with the synthetic αS1-casein-derived peptides, we found that in 10 out of 11 patients the peptide mix inhibited IgE reactivity to rαS1-casein as well as the complete rαS1-casein, indicating that most of the patients recognize sequential IgE epitopes.

Differences in recognition of specific epitopes have been described previously between patients with transient and persistent cow's milk allergy (14, 19, 35, 36). It appeared that patients with persistent cow's milk allergy showed more frequent and more intense IgE reactivity and mediator release to rαS1-casein and αS1-casein-derived peptides than did patients who had outgrown milk

allergy. Peptide Cas2, which contains an earlier described sequence (14, 19, 34), was the only peptide that was recognized by serum IgE of patients in the persistent group but not from transient cow's milk-allergic patients.

Since the synthetic αS1-casein-derived peptides were rather small (<37 aa), we asked the question whether these IgE-reactive fragments contain a sufficient number of IgE epitopes to induce cross-linking of mast cell or basophil-bound IgE Abs. In fact, the number of IgE epitopes on an allergen is important for its allergenic activity (37) and relates directly to the capacity of a given structure to induce immediate inflammatory-type reactions in patients (38). To evaluate the allergenic activity of rαS1-casein and the αS1-casein-derived synthetic peptides, we used basophil degranulation experiments. RBL cells that had been transfected with the human FcεRI were loaded with the very same sera that had been tested for IgE reactivity with the complete recombinant allergen and the synthetic αS1-casein-derived peptides. Approximately half of the sera containing IgE Abs to rαS1-casein or to the natural α-casein fraction induced basophil degranulation upon exposure to the proteins, but only very few sera containing IgE Abs to the αS1-casein-derived peptides induced degranulation in response to the peptides. This result indicates that the αS1-casein-derived peptides induce only weak degranulation compared with the complete allergens, most likely due to the lack of a sufficient number of IgE epitopes. It may thus be assumed that primarily the intact αS1-casein or larger fragments thereof induce IgE-mediated symptoms of cow's milk allergy, whereas the peptides appeared less potent. Our results further suggest that not all cow's milk-allergic patients show vigorous basophil degranulation with the intact allergen, although they did exhibit IgE Ab reactivity. Similar observations have been made for respiratory allergens where discrepancies between the IgE reactivities of allergens and their ability to induce basophil degranulation and in vivo allergic inflammation have been found (39). These findings may explain why the occurrence of clinical manifestations of cow's milk allergy does not always correlate with the presence of IgE Abs specific for milk allergens (40). In fact, one of the major diagnostic problems regarding cow's milk allergy is that the presence of IgE does necessarily imply clinical manifestations, and expensive, labor-intensive, and potentially hazardous double-blind, placebo-controlled food challenges are required.

We found that five patients (nos. 5, 6, 22, 36, and 52) without clinical symptoms after cow's milk consumption showed lower IgE levels to cow's milk and failed to mount relevant degranulation when RBLs were loaded with their serum and exposed to α-casein, rαS1-casein, and αS1-casein-derived peptides. In contrast, patients with severe systemic reactions showed strong degranulation. It is therefore possible that the RBL degranulation test may become useful as a diagnostic tool to discriminate patients without clinical symptoms. Unfortunately, all of the patients analyzed in our study exhibited IgE reactivity also to other cow's milk allergens besides αS1-casein. We therefore cannot answer the question whether patients lacking a substantial basophil degranulation in response to rαS1-casein exposure will lack clinical symptoms of cow's milk allergy. Another important factor that needs consideration if one would like to use the RBL assays for the potential assessment of the clinical sensitivity of patients is that allergens are subjected to degradation in the gastrointestinal tract, which may lead to the destruction of IgE epitopes or even to the unmasking of cryptic epitopes. It may therefore be necessary to simulate this process before using the RBL assay for the assessment of allergenic activity. Finally, it must be considered that it is also difficult to decipher whether the clinical symptoms recorded for patients are exclusively IgE-mediated or T cell-related, as has been reported for respiratory allergy to synthetic Fel d 1 peptides (41).

However, the availability of purified rαS1-casein and αS1-casein-derived peptides should allow to further dissect the pathomechanisms of milk-induced food allergy in clinical studies. Furthermore, these molecules may allow the development of diagnostic tests and of novel therapeutic strategies for milk allergy.

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Disclosures

R.V. serves as a consultant for Phadia (Uppsala, Sweden) and Biomay (Vienna, Austria). The other authors have no financial conflicts of interest.

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4. Microarray and allergenic activity assessment of milk allergens

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Microarray and allergenic activity assessment of milk allergens

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ABSTRACT

Background: Cow's milk is one of the most common causes of food allergy affecting approximately 2.5% of infants in the first years of their life. However, only limited information regarding the allergenic activity of individual cow's milk allergens exists.

Objective: To analyze the frequency of IgE reactivity and to determine the allergenic activity of individual cow's milk allergens.

Methods: A nitrocellulose-based microarray based on purified natural and recombinant cow's milk allergens was used to determine IgE reactivity profiles using sera from 78 cow's milk-sensitized individuals of varying ages. The allergenic activity of the individual allergens was tested using patients' sera for loading rat basophil leukaemia cells (RBL) expressing the alpha-chain of the human receptor FcεRI.

Results: Using the microarray and the RBL assay, cow's milk allergens were assessed for frequency of IgE recognition and allergenic activity. Moreover, the RBL assay allowed distinguishing individuals without or with mild clinical reactions from those with severe systemic or gastrointestinal symptoms as well as persons who grew out cow's milk allergy from those who did not.

Conclusion: Component-resolved testing using milk allergen microarrays and RBL assays seems to provide useful additional diagnostic information and may represent a basis for future forms of prophylactic and therapeutic strategies for cow's milk allergy.

Clinical Implications:

Combined IgE-reactivity testing of recombinant milk allergen components by microarray and basophil degranulation may allow identifying patients with severe and persistent forms of cow's milk allergy.

Capsule summary:

Recombinant cow's milk allergens may be useful for microarray-based IgE reactivity- and basophil degranulation testing to identify patients suffering from severe cow's milk allergy.

Keywords: Cow's milk allergy, recombinant allergen, microarray, basophil degranulation, diagnosis.

Abbreviations:

CMA: cow's milk allergy
CMP: cow's milk protein
GM: goat's milk
CM: cow's milk
SM: sheep's milk
HM: human milk
MM: mare's milk
Lf: lactoferrin
rLf: recombinant lactoferrin
BLGA: beta-lactoglobulin variant A
BLGB: beta-lactoglobulin variant B
rBLG: recombinant beta-lactoglobulin
parv: parvalbumin
BSA: bovine serum albumin
SSA: sheep serum albumin
HSA: human serum albumin
rBSAF1: recombinant BSA fragment 1
rBSAF2: recombinant BSA fragment 2
rBSAF3: recombinant BSA fragment 3
hALA: human alpha-lactalbumin
ALA: alpha-lactalbumin
rALA: recombinant alpha-lactalbumin
GC: goat casein fraction
CC: cow casein fraction
SC: sheep casein fraction
KC: kappa-casein
rKC: recombinant kappa-casein
BC: beta-casein
rBC: recombinant beta-casein
AC: alpha-casein
raS1C: recombinant alphaS1-casein
raS2C: recombinant alphaS2-casein

INTRODUCTION

Cow's milk is one of the most common causes of food allergy in the first years of life with around 2.5% of infants showing adverse reactions to cow's milk.[1-3] Symptoms of cow's milk allergy (CMA) range from mild to severe reactions and involve the skin, respiratory tract, gastrointestinal tract and in the worst case appear as life-threatening systemic reactions. The diagnosis of cow's milk allergy as well as the assessment and prediction of the severity of symptoms is particularly difficult for infants because only small amounts of serum can be obtained for serological diagnosis and it is often impossible to perform provocation tests.[4, 5] Due to the difficulties of obtaining sufficient serum samples and the lack of highly pure allergens, data regarding the prevalence of immunoglobulin E (IgE) recognition of individual cow's milk allergens are incomplete and sometimes controversial.[6] Furthermore, there is little information regarding the allergenic activities of individual cow's milk allergens.

Cow's milk contains more than 25 different proteins of which eight have been characterized as allergens. They include alpha-lactalbumin (Bos d 4), beta-lactoglobulin (Bos d 5), BSA (Bos d 6), and lactoferrin from the whey fraction, alphaS1-casein (Bos d 8 alphaS1), alphaS2-casein (Bos d 8 alphaS2), beta-casein (Bos d 8 beta), and kappa-casein (Bos d 8 kappa) from the casein fraction (Bos d 8).[6] Here we report the establishment of a miniaturized chip assay which contains highly pure recombinant cow's milk allergens and natural milk allergen preparations for the analysis of the IgE reactivity profiles of cow's milk allergic patients of various age groups including infants, children, and adults. The milk allergen array allowed us detecting the allergen profiles and frequencies of IgE reactivities towards a panel of nine recombinant cow's milk allergens/allergen fragments with as little as 25 µl of serum. In order to assess the allergenic activity of the individual allergen components a basophil degranulation test was developed which is based on RBL cells expressing the human FcεRI. These cells could be loaded with small volumes of serum and allowed us testing the allergenic activity of the individual allergen components.

METHODS

Clinical description of patients

Sera from cow's milk allergic children and adults (Table I) were obtained from Europe (Austria: n=12; Germany: n=47; Italy: n=2; Spain: n=16; France: n=1). Patients were

selected according to a positive case history, positive skin-prick reactions, and/or determination of specific IgE to cow's milk extract using the ImmunoCAP System (Phadia, Uppsala, Sweden). Within the study population three age groups were defined (3 month-5 years, n=53; 6-14 years, n=16; 16-70 years, n=9) (Table I). For 51 patients detailed clinical information regarding milk-related symptoms were available (Table I). Results from cow's milk challenge tests were available of 38 patients. The analysis of the serum samples was performed after patients were made anonymous with approval of the Ethics Committee of the Medical University of Vienna.

Natural and recombinant allergens, antibodies

Purified natural milk proteins (alpha-casein, AC; beta-casein, BC; kappa-casein, KC; alpha-lactalbumin, ALA; human alpha-lactalbumin, hALA; beta-lactoglobulin variant A, BLGA; beta-lactoglobulin variant B, BLGB; bovine serum albumin, BSA; sheep serum albumin, SSA; human serum albumin, HSA; lactoferrin, Lf) and casein fractions (goat casein fraction, GC; cow casein fraction, CC; sheep casein fraction, SC) were purchased from Sigma-Aldrich (Vienna, Austria). Milk samples from cow (CM), sheep (SM), goat (GM), and mare (MM) were bought at a local market and stored at -20°C until analysis. Human milk (HM) samples were obtained from a lactating woman.[7]

cDNAs coding for mature alpha-lactalbumin (rALA), beta-lactoglobulin (rBLG), alphaS1-(raS1C), alphaS2- (raS2C), beta- (rBC), and kappa-casein (rKC) and for three fragments spanning the whole sequence of bovine serum albumin (rBSAF1: amino acids (aa) 1-199, rBSAF2: aa 200-389, rBSAF3: aa 390-590) without N-terminal signal sequences and with C-terminal hexahistidine tags were obtained from a mammary gland cDNA library by IgE immunoscreening or PCR amplification.[8] Recombinant proteins were expressed in the *Escherichia coli* strain BL21 Codon Plus (DE3)-RIPL (Stratagene, La Jolla, CA) and purified using Ni-NTA resin affinity columns (QIAGEN, Hilden, Germany). Recombinant parvalbumin (parv) was produced as reported.[9]

Rabbit anti-sera were raised against each of the purified recombinant milk allergens (Charles, River, Kissleg, Germany). The purity of natural cow's milk allergens was tested by ELISA using the rabbit anti-sera raised against the recombinant cow's milk allergens diluted 1:5000 in Tris-buffered saline. Bound IgG antibodies were detected

with a 1:1000 diluted HRP-labelled anti-rabbit IgG antibody (GE Healthcare, Little Chalfont Buckinghamshire, UK). All determinations were carried out in duplicates and are expressed as mean values.

Allergen microarray analysis

For measurement of IgE reactivity, 0.1-0.15 ng/spot of whole milk extracts, casein fractions, and purified as well as recombinant cow's milk proteins were spotted with a Nano-Plotter NP2 (Gesellschaft für Silizium-Mikrosysteme mbH, Großerkmannsdorf, Germany) onto a capillary flow membrane that was fixed to an ordinary microscope glass slide as described by N ystrand.[10] After spotting, the microarrays were pre-wetted with 30 µl of assay buffer (phosphate buffer, pH 7.5), then incubated with 25 µl of undiluted sera, washed with 30 µl of assay buffer and assayed with 20 µl of detecting antibody. Finally, the microarrays were washed two times with 30 µl of assay buffer and bound IgE antibodies were detected with a fluorophore-conjugated anti-IgE antibody as fluorescence intensities (FI), measured at a wavelength of 670 nm (GenePix 4000B Axon Instruments; Molecular Devices, Sunnyvale, CA). The cut off level was set to FI = 400 based on values gained with spotted human serum albumin that was used as a negative control.

Rat basophil leukaemia (RBL) assays

The allergenic activity of the milk components was tested using RBL cells expressing the human receptor FcεRI. RBL cells were loaded with patients sera and exposed to the allergen preparations.[8, 11]

RESULTS

Establishment and characterization of an allergen chip containing micro-arrayed milk components

In total, 27 milk samples (dots 1-9, 11, 12, 14-29) and for control purposes HSA (dots 13) and the unrelated fish allergen, recombinant parvalbumin (dots 10), were spotted in duplicates on a nitrocellulose membrane as shown in the application scheme in Fig 1, *A*. Purified IgE (dots 0) was spotted as a position marker. The correct immobilization of the allergens on the nitrocellulose chip was checked at a wavelength of 635 nm (Fig 1, *B*). The use of a fluorescence-labelled detection antibody allowed determining the fluorescence intensities of the anti-IgE labelling at a wavelength of 670 nm. According to the color intensities of the fluorescence signals the specific IgE concentrations of the samples could be ranked (i.e., white > red > orange>yellow>green>light blue>dark blue) and semi-quantitative results were obtained.

In pilot experiments we compared IgE reactivity profiles by chip analysis and by ELISA for certain sera, of which larger volumes were available, and obtained comparable results with both tests (data not shown).

We then tested sera from 78 cow's milk-sensitized individuals, which we had divided into three age groups (Table I), for IgE reactivity using the microarrays. Figure 1, *C* shows representative images of microarray results obtained with sera from three cow's milk allergic patients and with the serum from a non-allergic individual. Serum IgE from patient B68, a 13-years-old boy with persistent cow's milk allergy who suffered from urticaria, eczema, vomiting, and asthma after milk consumption, reacted with most of the allergens except BSA and lactoferrin. The serum did not react with sheep serum albumin, parvalbumin, and HSA (Fig 1, *C*). Similar results were obtained with the serum from patient B63, a 9-years-old girl, who suffered mainly from gastrointestinal symptoms and with the serum from patient A18, a 22-months-old girl, who suffered from urticaria, atopic dermatitis, and vomiting (Fig 1, *C*). Both sera showed IgE reactivity to most of the spotted components. The IgE reactivity profiles depicted in Fig 1, *C* have been obtained with small serum volumes of approximately 25 µl. The specificity of the method was demonstrated by the lack of IgE-reactivity to the spotted components when serum from a non-allergic person (C) was used (Fig 1, *C*).

Frequencies of IgE reactivity to micro-arrayed milk allergens

A summary of the frequencies of IgE reactivities to micro-arrayed milk allergens is shown in Fig 2. In total 78 sera (Table I) were tested for IgE reactivity to micro-arrayed components. Seventy one of those sera were positive in the cow's milk ImmunoCAP, one serum was negative and six sera were not tested by ImmunoCAP measurements. The black bars in Figure 2 show the percentages of IgE reactivities for all tested patients. Results from each of the three age groups are also displayed.

Among the whole milk extracts those from cow (n=66; 84.6%), sheep (n=63; 80.8%), and goat (n=53; 67.9%) were most frequently recognized. Mare's milk and human milk were less frequently recognized (30.8% and 17.9%, respectively) (Fig 2, *A*). The casein fractions of cow's, goat's, and sheep's milk reacted with IgE from approximately 50% of the tested sera (Fig 2, *A*).

Among the natural cow's milk proteins (Fig 2, *B*) ALA reacted with 62.8% of the sera and thus was the most frequently detected natural cow's milk allergen followed by BLG and AC which were recognized by approximately 50% of the tested sera. BC (43.6%) and KC (29.5%) were also frequently recognized, whereas Lf, BSA, and SSA showed low IgE binding frequencies (5.1%, 3.8%, 2.6%, Fig 2, *B*). Interestingly, natural human ALA reacted almost with 30% of the sera. In summary, 65 of the sera (83.3%) showed IgE reactivity with at least one of the natural cow's milk allergens.

Comparing the IgE binding frequencies of natural and recombinant cow's milk allergens some interesting differences were noted (Fig 2, *B* and *C*, black bars). While the recombinant caseins showed comparable IgE binding frequencies as the natural counterparts, rALA and also rBLG reacted with less than half of the sera which had reacted with their natural counterparts. Recombinant BSA fragments reacted with a similar low percentage of sera as purified natural BSA (Fig 2, *B* and *C*). Overall, 49 (62.8%) sera showed IgE reactivity to at least one of the recombinant allergens.

A comparison of the IgE recognition frequencies in the different age groups showed that the frequencies of recognition were highest in group B (age 6-14 years; Fig 2, white bars) followed by group A (age 3 months-5 years; grey bars) and group C (age 16-70 years; Fig 2, hatched bars). These results reflected the levels of cow's milk-specific IgE determined by ImmunoCAP being highest in group B followed by A and C (Table I).

Natural cow's milk allergen preparations are contaminated with unrelated milk allergens

In order to analyze whether the discrepancies in the IgE recognition frequencies between the recombinant forms of ALA and BLG and their natural counterparts are due to contaminations in the natural allergen preparations, we tested the latter with specific antibody probes. For this purpose, rabbit antibodies had been raised against each of the purified recombinant allergens which showed specific reactivity to the protein backbones but did not recognize carbohydrates or other post-translational modifications. Each of the antisera reacted with the corresponding natural allergens (Table II). However, most of the casein-specific antibodies recognized natural AC, BC, and KC preparations which suggests that the natural caseins are not pure (Table II). Cross-reactivity between AC, BC, and KC is unlikely because no relevant sequence homologies were found when their amino acid sequences were aligned (data not shown).

Natural ALA, BLG, Lf, and BSA preparations did not react with antisera raised against other milk allergens and hence appeared to be pure. Yet, the biggest discrepancies of IgE reactivity between natural and recombinant components were observed in case of ALA and BLG, both representing glycoproteins containing 6 and 1 glycosylation sites, respectively (data not shown).

Mono- and oligo-sensitization to cow's milk allergen components in cow's milk allergic patients

Since impurities were detected in natural casein preparations and since IgE reactivities to carbohydrates present in natural allergen preparations should be excluded, mono- or oligo-sensitization to cow's milk allergen components was assessed with purified recombinant allergens. It was found that 16 (32.7%) out of the 49 patients who had reacted with recombinant allergens reacted only with one allergen. The other 33 sera (67.3%) reacted with at least 2 allergens (2 allergens: n=13; 3 allergens: n=9; 4 allergens: n=1; 5 allergens: n=5; 6 allergens: n=3; 7 allergens: n=2). There was no obvious difference regarding mono- or oligo-sensitization in the three age groups.

In the mono-sensitized group (n=16) six patients exhibited >10 kUa/l cow's milk-specific IgE in the ImmunoCAP test. Far more, i.e., 27 patients exhibited >10 kUa/l

cow's milk-specific IgE in the oligo-sensitized group (n=33) indicating that higher cow's milk-specific IgE levels are due to oligo-sensitization.

Comparison between IgE reactivity and allergenic activity of cow's milk allergens

In order to determine the biological relevance of IgE reactivity to cow's milk allergens, rat basophil leukaemia (RBL) cells which had been transfected with the α-chain of the human Fcε receptor were loaded with sera and challenged with the allergens (Fig 2 *A-C*; right panels). Basophil degranulation was observed for 47.4% (n=37) of tested patients sera (n=78) with cow's milk extract, 37.2% (n=29) of the sera showed degranulation with at least one natural and 34.6% (n=27) of the sera with at least one recombinant allergen. Generally, fewer sera gave positive results in the basophil degranulation assay than in the IgE reactivity test. Eighty five percent of sera showed IgE reactivity to cow's milk extract in the microarray but only 47.4% induced basophil degranulation (Fig 2, *A*). The differences between IgE reactivity and basophil degranulation were less pronounced for the natural caseins (e.g., AC: IgE reactivity: 48.7%; basophil degranulation: 25.6%; BC: IgE reactivity: 43.6%; basophil degranulation: 34.6%; KC: IgE reactivity: 29.5%; basophil degranulation: 25.6%), whereas striking differences were noted for natural ALA and BLG (ALA: IgE reactivity: 62.8%; basophil degranulation: 11.5%; BLGA: IgE reactivity: 48.7%; basophil degranulation: 28.2%; BLGB: IgE reactivity: 50%; basophil degranulation: 19.2%) indicating that ALA and BLG have a lower allergenic activity than the caseins. When the recombinant proteins were compared regarding their frequency of IgE reactivity and allergenic activity, we found for rALA comparable results (rALA: IgE reactivity: 24.4%; basophil degranulation: 21.8%) and for rBLG a small difference (rBLG: IgE reactivity: 21.8%, basophil degranulation: 12.8%) (Fig 2, *C*). In contrast, recombinant caseins induced much less frequently basophil degranulation than IgE reactivities were observed (Fig 2, C).

Thus the association of IgE reactivity with basophil degranulation was poor. However, it was noted that sera from group B with the highest levels of cow's milk-specific IgE (Table I) which also contained more IgE positive sera than the other two groups induced more frequently basophil degranulation than sera from groups A and C (Fig 2, *A-C*).

Identification of patients without and with severe symptoms based on microarray and basophil degranulation results

In the next step we analyzed whether there are any associations between clinical symptoms, IgE reactivity and basophil degranulation data (Fig 3). For this purpose, patients were grouped in individuals (i) without clinical reaction, (ii) with solely oral allergy syndrome, (iii) with only gastrointestinal symptoms, (iv) with gastrointestinal symptoms and other symptoms, (v) with only skin symptoms, (vi) with only respiratory symptoms, (vii) with skin and respiratory symptoms, and (viii) with severe systemic reactions.

The most striking result was that persons containing IgE antibodies against cow's milk extract and allergens without clinical symptoms showed no relevant basophil degranulation with any of the tested components (Fig 3). By contrast, sera from most cow's milk allergic patients who suffered from severe systemic reactions and from those who suffered from gastrointestinal symptoms induced basophil degranulation with many of the tested components. It appeared that patients with severe systemic reactions also showed stronger IgE reactivity to more components than the other groups (Fig 3). However, IgE reactivity testing did not allow distinguishing persons without symptoms from patients with severe and gastrointestinal symptoms.

IgE reactivity and basophil activation tests with purified cow's milk allergens may identify patients who grew out cow's milk allergy

In the course of our study nine of our patients had outgrown cow's milk allergy and for eleven patients it was known that they were still suffering from cow's milk allergy. Figure 4 shows the IgE reactivity profiles and basophil degranulation results from these patients. Patients with persistent cow's milk allergy showed more frequent and more intense IgE reactivity to natural as well as recombinant cow's milk allergens than patients who had outgrown their milk allergy. Likewise, patients with persistent cow's milk allergy showed more often and more pronounced basophil degranulation than patients who had outgrown milk allergy.

DISCUSSION

In this study we analyzed 78 sera from cow's milk-sensitized persons from different age groups and with different clinical manifestations of cow's milk allergy for IgE reactivity to natural and recombinant milk allergen preparations. The allergens were micro-arrayed on a chip to allow testing of small serum volumes. It was therefore possible to study the IgE reactivity profiles to several allergen components in infants and children. Based on IgE reactivity profiles established with natural cow's milk allergens, ALA > BLG > AC > BC > KC represented the most frequently recognized cow's milk allergens. Recombinant caseins appeared to exhibit similar IgE binding properties as the natural caseins and offered the additional advantage of purity. "Purified natural caseins" were contaminated with unrelated milk allergens and therefore seemed unsuitable for component-resolved diagnosis of cow's milk allergy. A considerable discrepancy in IgE reactivity was noted for ALA and BLG, where the recombinant allergens reacted only with approximately half of the sera that reacted with the natural allergen. However, only approximately half of the sera which showed IgE reactivity to purified natural ALA and BLG showed allergenic activity in the basophil degranulation assays, indicating that the natural allergens contain IgE-reactive structures, such as carbohydrates or other post-translational modifications, with low allergenic activity.[12] By contrast, approximately the same percentage of sera which had reacted with the recombinant ALA and BLG proteins also gave positive results in the basophil degranulation assay, indicating that these allergen preparations perhaps resemble more closely clinically relevant structures.

A finding of potential relevance for future allergen-specific therapeutic and prophylactic strategies is that the described milk allergen array allowed establishing the patients' sensitization profiles to individual components. Using the microarray we identified a considerable percentage of patients who appeared to be mono-sensitized towards one major allergen and could discriminate these patients from oligo-sensitized patients.

The combination of IgE reactivity testing to micro-arrayed allergens and the testing for allergenic activity using RBL cells expressing the human FcεRI turned out to provide additional useful diagnostic information. In fact, several studies described that basophil activation tests provide additional information for the diagnosis of food allergy.[13-15] While IgE serology to cow's milk and cow's milk allergens was positive for many individuals with cow's milk sensitization but also for individuals without clinical

symptoms, the RBL assay may identify the subgroup of individuals without clinical symptoms. Also patients with only skin symptoms gave weak or no reaction in the basophil degranulation test, whereas all patients with severe systemic and the majority of patients with gastrointestinal symptoms induced basophil degranulation. Accordingly, a recent study showed that basophil activation tests are capable of distinguishing sensitized but clinically tolerant from clinically allergic patients with peanut or egg allergy.[16]

Moreover, it appeared that the basophil degranulation assay could distinguish between cow's milk allergic patients who outgrow their allergy from those who did not because those who grew out milk allergy displayed no basophil activation capacity. In this context, a recent study showed that children who are likely to outgrow their cow's milk allergy show reduced basophil activation with heat denatured milk.[17]

The gold standard for the diagnosis of clinically relevant cow's milk sensitization is certainly the controlled food challenge test, but it must be kept in mind that this test cannot always be easily used, particularly not in small infants and in patients who mounted life-threatening reactions upon exposure to cow's milk.

The component-resolved microarray test combined with an assessment of the allergenic activity of the individual components using a basophil activation assay may therefore provide interesting additional diagnostic information. Component-resolved diagnosis of cow's milk allergy may also represent a first step towards the development of new immunotherapeutic strategies for cow's milk allergy similar to those which have been developed based on recombinant allergens for respiratory allergies.[18]

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TABLE I. Characterization of milk-sensitized patients[a]

Patient group	Sex m/f	Country	Milk-related symptoms	SPT CM	Challenge	CMA outgrown	Other allergies	Total IgE (kU/l)	Specific IgE to CM (kUA/l)
A: A1-A53 3mo-5y (n=53)	29/22 nk (n=2)	S (n=15) A (n=8) G (n=30)	no reaction (n=2) GI (n=1) GI+others (n=5) skin (n=15) resp (n=1) skin+resp (n=5) sys (n=4) nk (n=20)	pos (n=20) neg (n=7) nd/nk (n=26)	pos[a] (n=17) pos[b] (n=6) neg[a] (n=1) neg[b] (n=2) nd/nk (n=27)	yes (n=9) no (n=6) nk (n=38)	GP (n=1) M,C (n=1) HE (n=7) HE+various others (n=6) no (n=11) nk (n=27)	8.36 - 3295 mv: 161 (n=49)	<0.35 - >100 mv: 10.7 (n=49)
B: B54-B69 6y-14y (n=16)	10/5 nk (n=1)	G (n=14) I (n=2)	no reaction (n=1) GI+others (n=3) skin (n=1) skin+resp (n=4) sys (n=2) nk (n=5)	pos (n=9) nd (n=7)	pos (n=4) pos[b] (n=3) np (n=2) nd/nk (n=7)	no (n=2) nk (n=14)	HE (n=6) HE+various others (n=1) Candida (n=1) nk (n=8)	14.1 - 14525 mv: 866 (n=15)	3.77 - >100 mv: 18.9 (n=16)
C: C70-C78 16y-70y (n=9)	3/6	G (n=3) A (n=4) F (n=1) S (n=1)	no reaction (n=2) OAS (n=1) resp (n=1) skin (n=2) sys (n=1) nk (n=2)	pos (n=4) neg (n=2) nd (n=3)	pos (n=2) pos[a] (n=1) neg[b] (n=2) nd/nk (n=4)	no (n=3) nk (n=4) na (n=2)	HE (n=1) M,C (n=1) PO,AH,M (n=1) Meat,C (n=1) C,WF,T,K,P (n=1) GP,dog (n=1) nk (n=3)	148 - 3736 mv: 433 (n=7)	1.3 - >100 mv: 5.34 (n=7)

[a]Abbreviations used in the table: y, year; mo, month; f, female; m, male; A, Austria; F, France; G, Germany; I, Italy; S, Spain; CM, cow's milk; OAS, oral allergy syndrome; GI, gastrointestinal symptoms; skin, skin symptoms; resp, respiratory symptoms; sys, systemic reactions; nk, not known; SPT, skin-prick test; pos, positive; neg, negative; nd, not done; na, not applicable because patients were asymptomatic; np, not possible; GP, grass pollen; M, mite; C, cat; HE, hen's egg; PO, pollen; AH, animal hair; WF, wheat flour; T, tomato; K, kiwi; P, pork; kU/l, kilo units per liter; kUa/l, kilo units antigen per liter; [a]open challenge as described by Garcia-Ara C. et al.;[19] [b]double-blind placebo-controlled food challenge; mv, median value.

TABLE II. Reactivity of natural cow's milk proteins with specific antibody probes[a]

Natural proteins	Rabbit antisera						
	raS1C	raS2C	rBC	rKC	rALA	rBLG	rLF
AC	**1.3**	**1.3**	**0.3**	**0.4**	0.0	0.0	0.0
BC	**0.4**	**1.2**	**1.1**	**0.8**	0.0	0.0	0.0
KC	**1.1**	**1.2**	0.1	**1.4**	0.1	**0.4**	0.0
ALA	0.0	0.0	0.0	0.0	**1.5**	0.0	0.0
BLGA	0.0	0.0	0.0	0.0	0.0	**1.5**	0.0
BLGB	0.0	0.0	0.0	0.0	0.0	**1.8**	0.0
Lf	0.0	0.0	0.0	0.0	0.0	0.0	**0.5**
BSA	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Antibody reactivities are presented as OD values, which are calculated as the OD values obtained with the immune sera minus the OD values obtained with the corresponding pre-immune sera. Elevated values are given in bold and contaminations are highlighted in grey.

FIGURE LEGENDS

FIG 1. Microarray. **(A)** Application scheme of micro-arrayed milk components: 0: IgE; 1: GM; 2: CM; 3: SM; 4: HM; 5: MM; 6: Lf; 7: BLGA; 8: BLGB; 9: rBLG; 10: parv; 11: BSA; 12: SSA; 13: HSA; 14: rBSAF1; 15: rBSAF2; 16: rBSAF3; 17: hALA; 18: ALA; 19: rALA; 20: GC; 21: CC; 22: SC; 23: KC; 24: rKC; 25: BC; 26: rBC; 27: AC; 28: raS1C; 29: raS2C. **(B)** Visualization of protein dots on a microarray slide at a wavelength of 635 nm. **(C)** Detection of IgE-reactive protein spots at a wavelength of 670 nm after incubation with sera from milk allergic patients (A18, B63, B68) and a non-allergic individual (C) and visualization with a fluorescence-labelled anti-IgE antibody.

FIG 2. Frequencies of IgE recognition and basophil degranulation. The percentages of sera exhibiting IgE reactivity and inducing mediator release are displayed for all investigated patients (n=78; black bars) and for the three age groups (Group 1: 3 mo-5 years; n=53; grey bars; group 2: 6-14 years; n=16; white bars; group 3: 16-70 years; n=9; hatched bars). **(A)** Milk samples: CM, GM, SM, HM, MM, CC, GC, SC. **(B)** Purified natural cow's milk allergens: AC, BC, KC, ALA, BLGA, BLGB, Lf, BSA, SSA, HSA, hALA and **(C)** recombinant allergens/allergen fragments: raS1C, raS2C, rBC, rKC, rALA, rBLG, rBSAF1, rBSAF2, rBSAF3, parv.

FIG 3. Associations of milk-related symptoms with IgE reactivities and basophil degranulation. Milk-allergic patients were grouped according to milk-related symptoms as follows: No reaction; OAS (oral allergy syndrome); GI only (gastrointestinal reactions); GI + others (gastrointestinal symptoms and/or skin and/or respiratory symptoms); Skin (skin symptoms); Resp (respiratory symptoms); Skin+resp (skin symptoms and respiratory symptoms); Severe systemic reactions. Displayed items are: Age, RAST class to CM. The intensities of IgE reactivities and mediator release to cow's milk (CM), purified natural cow's milk allergens (AC, BC, KC, ALA, BLGA, BLGB, BSA, Lf), and recombinant cow's milk allergens (raS1C, raS2C, rBC, rKC, rALA, rBLG) are indicated in different colors.

FIG 4. IgE reactivities and inductions of basophil activation in patients with persistent and transient cow's milk allergy. IgE reactivities and basophil degranulation in response to cow's milk (CM), purified natural cow's milk allergens (AC, BC, KC, ALA,

BLGA, BLGB, BSA, Lf), and recombinant cow's milk allergens (raS1C, raS2C, rBC, rKC, rALA, rBLG) are shown for patients with persistent (not outgrown) or transient (outgrown) cow's milk allergy. The intensities of IgE reactivity and basophil activation are displayed in different colors.

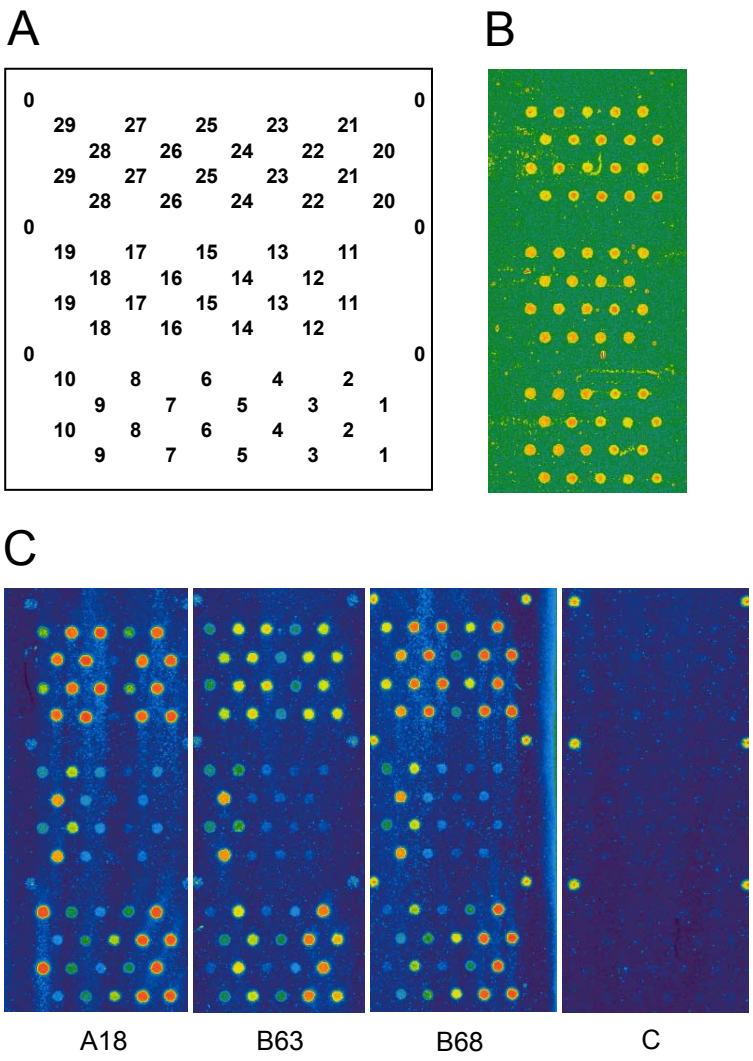

A
B
C
0 0
29 27 25 23 21
28 26 24 22 20
29 27 25 23 21
28 26 24 22 20
0 0
19 17 15 13 11
18 16 14 12
19 17 15 13 11
18 16 14 12
0 0
10 8 6 4 2
9 7 5 3 1
10 8 6 4 2
9 7 5 3 1
A18
B63
B68
C

FIG 1.

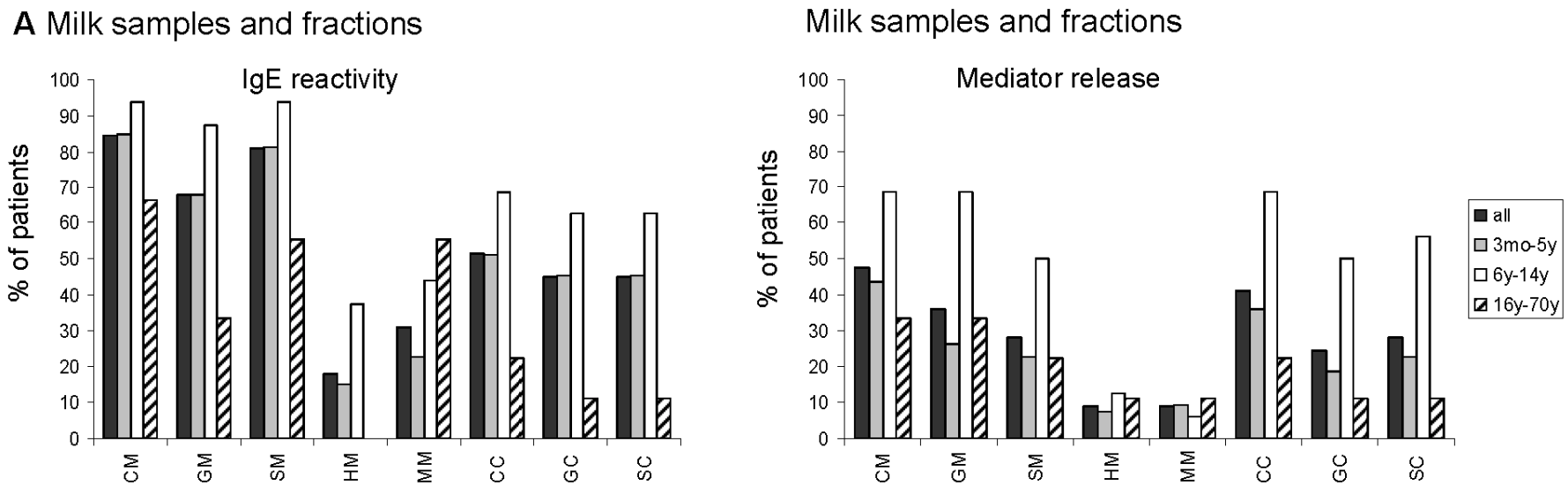

A Milk samples and fractions
IgE reactivity
% of patients
100
90
80
70
60
50
40
30
20
10
0
CM
GM
SM
HM
MM
CC
GC
SC
Milk samples and fractions
Mediator release
% of patients
all
3mo-5y
6y-14y
16y-70y

FIG 2A.

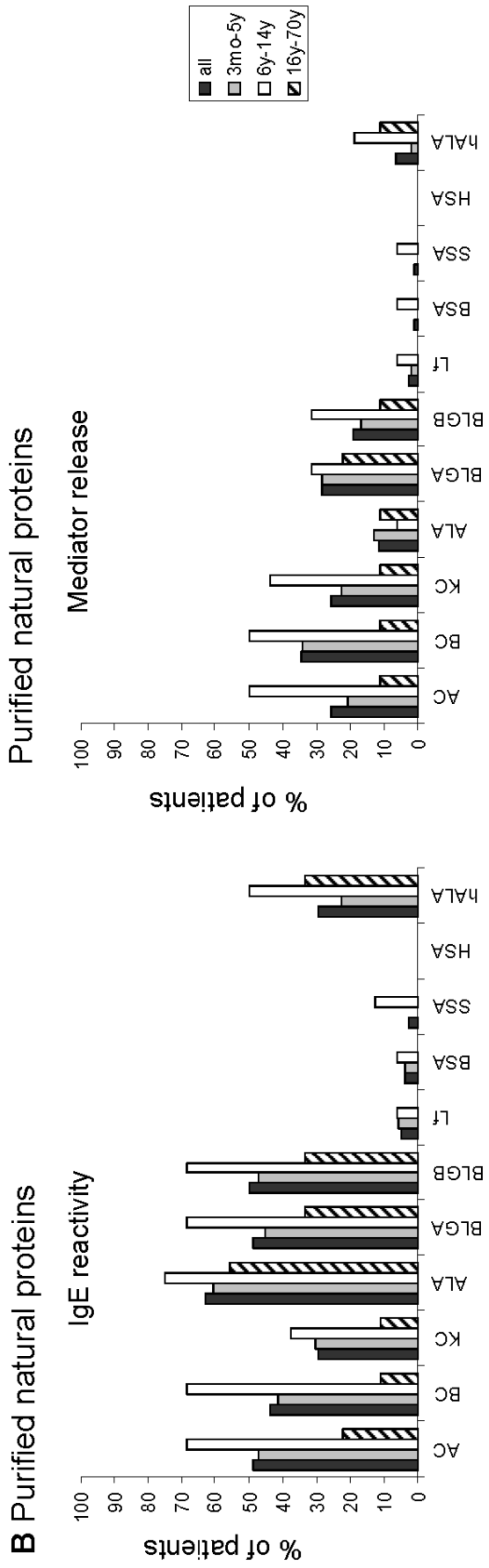
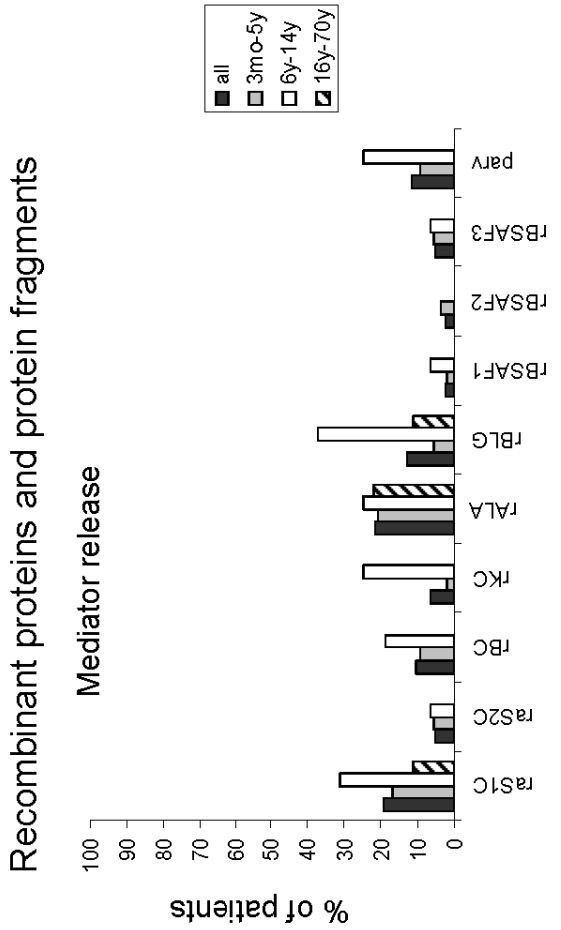

Purified natural proteins
Mediator release
% of patients
100
90
80
70
60
50
40
30
20
10
0
all
3mo-5y
6y-14y
16y-70y
AC
BC
KC
ALA
BLGA
BLGB
LF
BSA
SSA
HSA
hALA
B Purified natural proteins
IgE reactivity
% of patients
AC
BC
KC
ALA
BLGA
BLGB
LF
BSA
SSA
HSA
hALA

FIG 2B.


Recombinant proteins and protein fragments
Mediator release
% of patients
all
3mo-5y
6y-14y
16y-70y
rnS1C
rnS2C
rBC
rKC
rALA
rBLG
rBSAF1
rBSAF2
rBSAF3
parv

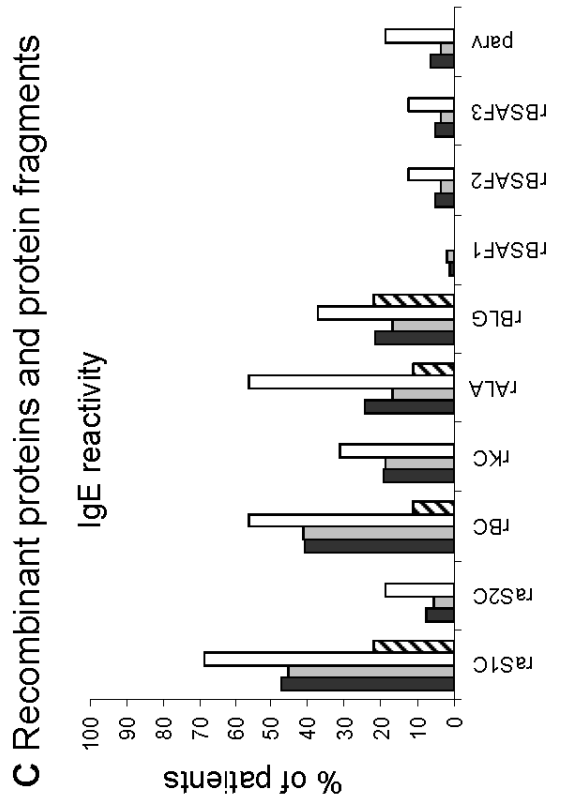

C Recombinant proteins and protein fragments
IgE reactivity
% of patients
rnS1C
rnS2C
rBC
rKC
rALA
rBLG
rBSAF1
rBSAF2
rBSAF3
parv

FIG 2C.

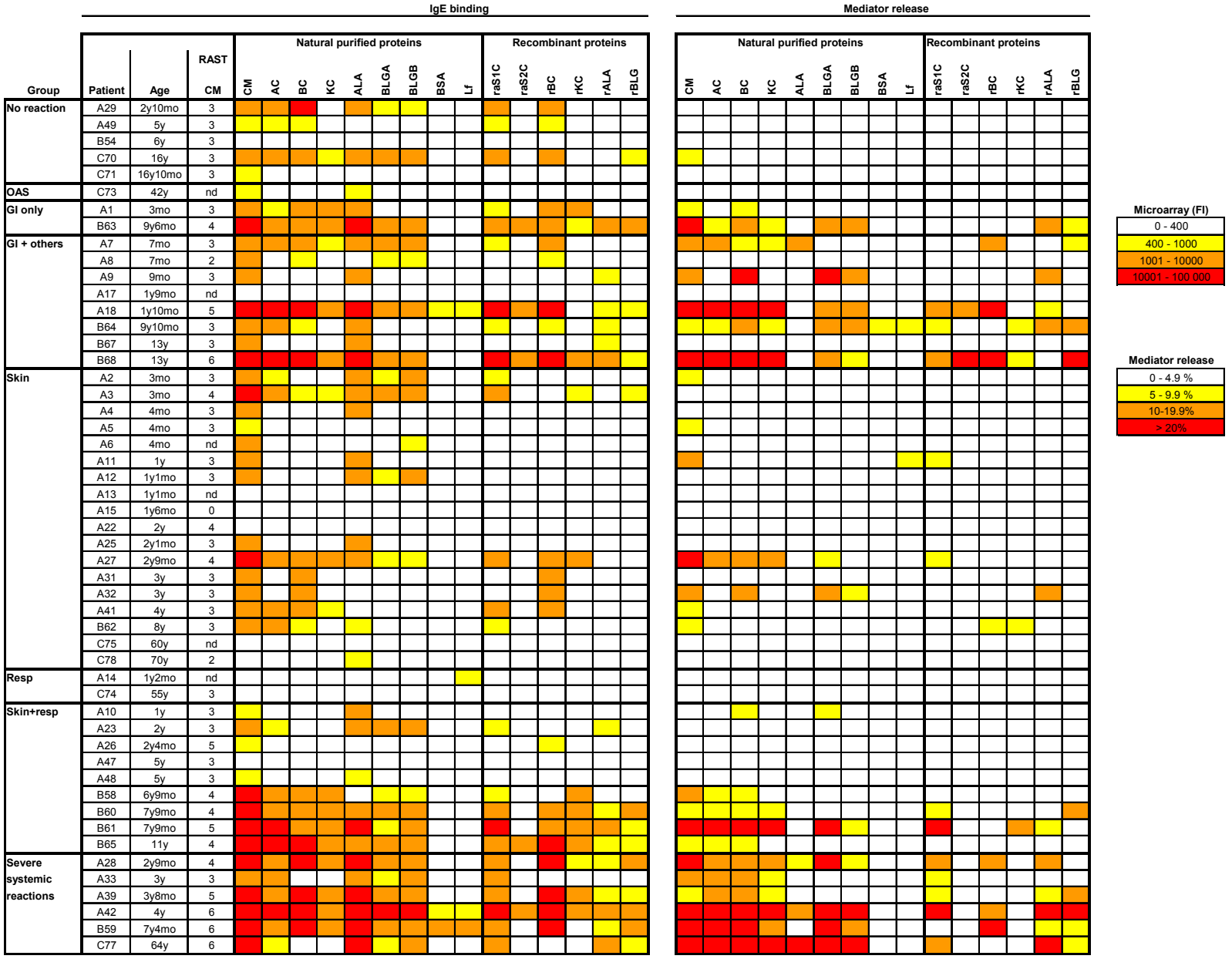

IgE binding
Mediator release
Natural purified proteins
Recombinant proteins
Group
Patient
Age
RAST
CM
AC
BC
KC
ALA
BLGA
BLGB
BSA
Lf
raS1C
raS2C
rBC
rKC
rALA
rBLG
No reaction
A29 2y10mo 3
A49 5y 3
B54 6y 3
C70 16y 3
C71 16y10mo 3
OAS
C73 42y nd
GI only
A1 3mo 3
B63 9y6mo 4
GI + others
A7 7mo 3
A8 7mo 2
A9 9mo 3
A17 1y9mo nd
A18 1y10mo 5
B64 9y10mo 3
B67 13y 3
B68 13y 6
Skin
A2 3mo 3
A3 3mo 4
A4 4mo 3
A5 4mo 3
A6 4mo nd
A11 1y 3
A12 1y1mo 3
A13 1y1mo nd
A15 1y6mo 0
A22 2y 4
A25 2y1mo 3
A27 2y9mo 4
A31 3y 3
A32 3y 3
A41 4y 3
B62 8y 3
C75 60y nd
C78 70y 2
Resp
A14 1y2mo nd
C74 55y 3
Skin+resp
A10 1y 3
A23 2y 3
A26 2y4mo 5
A47 5y 3
A48 5y 3
B58 6y9mo 4
B60 7y9mo 4
B61 7y9mo 5
B65 11y 4
Severe systemic reactions
A28 2y9mo 4
A33 3y 3
A39 3y8mo 5
A42 4y 6
B59 7y4mo 6
C77 64y 6
Microarray (FI)
0 - 400
400 - 1000
1001 - 10000
10001 - 100 000
Mediator release
0 - 4.9 %
5 - 9.9 %
10-19.9%
> 20%

FIG 3.

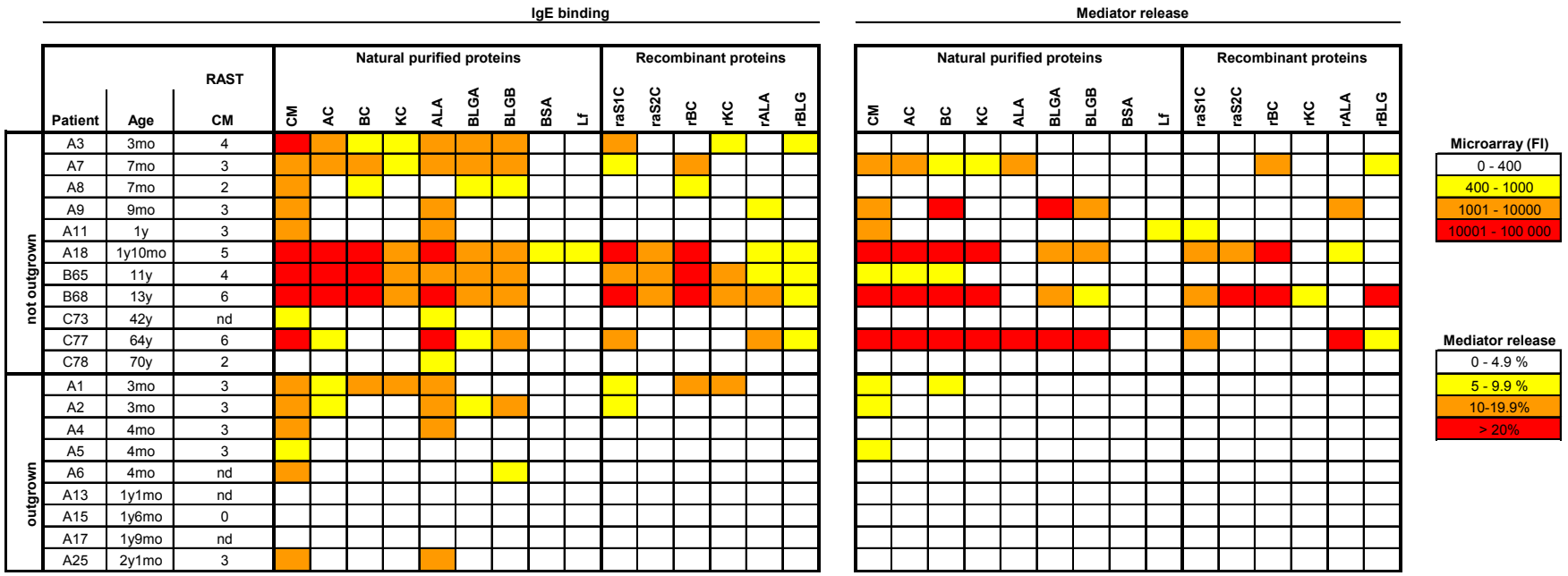

IgE binding
Mediator release
Natural purified proteins
Recombinant proteins
Patient
Age
RAST
CM
CM
AC
BC
KC
ALA
BLGA
BLGB
BSA
Lf
raS1C
raS2C
rBC
rKC
rALA
rBLG
not outgrown
A3 3mo 4
A7 7mo 3
A8 7mo 2
A9 9mo 3
A11 1y 3
A18 1y10mo 5
B65 11y 4
B68 13y 6
C73 42y nd
C77 64y 6
C78 70y 2
outgrown
A1 3mo 3
A2 3mo 3
A4 4mo 3
A5 4mo 3
A6 4mo nd
A13 1y1mo nd
A15 1y6mo 0
A17 1y9mo nd
A25 2y1mo 3
Microarray (FI)
0 - 400
400 - 1000
1001 - 10000
10001 - 100 000
Mediator release
0 - 4.9 %
5 - 9.9 %
10-19.9%
> 20%

FIG 4.

5. Patients suffering from non-IgE-mediated cow's milk intolerance cannot be diagnosed based on IgG-subclass or IgA responses to milk allergens

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Submitted, 2009

Patients suffering from non-IgE-mediated cow's milk intolerance cannot be diagnosed based on IgG-subclass or IgA responses to milk allergens

Running title: Cow's milk intolerance cannot be diagnosed based on IgG-subclass and IgA responses to milk allergens

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Summary

Background Cow's milk represents one of the most common causes of food allergy in the first years of life. In two thirds of patients adverse symptoms following milk ingestion are caused by IgE-mediated allergic reactions whereas for one third the mechanisms are unknown.

Objective The aim of this study was to investigate whether persons suffering from non-IgE-mediated cow's milk intolerance can be distinguished from persons without cow's milk intolerance based on their humoral immune responses to cow's milk antigens.

Methods We determined IgG_{1-4} subclass and IgA antibody levels to purified recombinant αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin in four groups of clinically defined individuals by ELISA. Group I consisted of patients with IgE-mediated cow's milk allergy (CMA, n=9), group II were patients with non-IgE-mediated cow's milk intolerance (CMI, n=14), group III were patients with gastrointestinal symptoms not associated with cow's milk ingestion (GI, n=7), and group IV were control persons without gastrointestinal problems (C, n=26). Cow's milk specific IgE was determined by ImmunoCAP.

Results Only patients with CMA, but none of the individuals belonging to the other groups, had IgE antibodies to cow's milk. CMA patients mounted the highest IgG_1 and IgG_4 antibody levels to αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin. No statistically significant differences regarding levels of IgG_4, IgA, and complement-binding IgG subclasses (IgG_1, IgG_2, IgG_3) to purified cow's milk allergens were found between CMI patients and persons without cow's milk intolerance (GI and C group).

Conclusion Cow's milk intolerant patients cannot be distinguished from persons without cow's milk intolerance on the basis of IgG subclass or IgA reactivity to cow's milk allergens. Antibody-mediated forms of hypersensitivity against cow's milk allergens, besides those mediated by IgE, are unlikely to contribute to cow's milk intolerance.

Key words cow's milk intolerance, non-IgE-mediated, cow's milk allergy, IgG

Introduction

Cow's milk is a very common cause of food allergy in the first years of life, affecting about 2.5% of infants [1]. IgE-mediated allergy is responsible for approximately 60% of cow's milk-induced adverse reactions. However, for patients without cow's milk-specific IgE, the mechanisms are not yet understood [2].

Non-IgE-mediated cow's milk intolerance (CMI) affects infants but is more common in adults. These patients do not have circulating cow's milk protein-specific IgE, they also show negative results in skin prick tests and RAST [3-7]. The clinical symptoms in CMI patients are normally delayed, starting from one hour to several days after the consumption of cow's milk and affect mainly the gastrointestinal system such as nausea, bloating, intestinal discomfort, and diarrhea. Immunological as well as non-immunological mechanisms may be responsible for non-IgE-mediated reactions to cow's milk proteins [8]. Among the immunological mechanisms basically humoral and cellular mechanisms may be considered. Symptoms may be caused by cow's milk-specific T cell responses of the Th1 or perhaps Th17 phenotype whereas antibody-mediated mechanisms may involve Type II or Type III hypersensitivity mechanisms such as ADCC or complement activation [8, 9].

A matter of debate is also the selection of the most reliable method for diagnosis of non-IgE-mediated cow's milk intolerance: either *in vitro* cellular or antibody-based test systems [3, 10-15]. Already studies that tried to investigate IgG and IgA levels to cow's milk allergic as compared to healthy individuals gave controversial results. Some studies reported increased levels of milk-specific IgG_1 and IgG_4 in children with atopic dermatitis compared to healthy individuals [16], high IgG_1, IgG_4, and IgA antibodies to α-lactalbumin in atopic children until one year of age and then decreasing antibody levels [17]. Other studies showed similar levels of IgA and IgG antibodies to cow's milk proteins in healthy individuals and in patients with CMA [18, 19] or low cow's milk-specific IgG levels in CMA patients whether or not tolerance was achieved [20]. In studies that also included patients with non-IgE-mediated milk hypersensitivities higher IgG_4 antibodies to β-lactoglobulin were described for atopic children [21] and higher IgG_1, IgG_4, IgA to α-casein (α-cas), β-casein (β-cas), κ-casein (κ-cas), α-lactalbumin (α-la), β-lactoglobulin (β-lg) were found in IgE-mediated cow's milk allergic patients as compared to patients with non-IgE-mediated disorders and controls [7]. However, the question whether patients suffering from non-IgE-

mediated cow's milk allergy can be distinguished from persons without cow's milk related symptoms based on IgG reactivity to cow's milk allergens and whether antibody-complement-mediated immune mechanisms may play a role has not yet been answered.

In this study we expressed and purified the recombinant cow's milk allergens αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin. The recombinant antigens were used to compare milk allergen-specific IgG_{1-4} and IgA antibody levels in patients suffering from IgE-mediated cow's milk allergy, in patients suffering from non-IgE-mediated cow's milk intolerance, in patients with gastrointestinal symptoms, and in healthy individuals without cow's milk-related symptoms. Our results demonstrate that patients suffering from non-IgE-mediated cow's milk allergy cannot be distinguished from persons without cow's milk-related symptoms based on IgG subclass or IgA reactivity to cow's milk allergens, which contradicts that humoral mechanisms might be involved in non-IgE-mediated cow's milk intolerance.

Material and methods

Study groups

Patients with cow's milk allergy (CMA, n=9) were diagnosed according to positive case history and/or positive provocation test and determination of specific IgE to cow's milk using the ImmunoCAP System (Phadia, Uppsala, Sweden). Patients with cow's milk intolerance (CMI, n=14) had a positive history of reproducible symptoms after milk consumption but without any specific IgE to cow's milk (cut off level 0.35 kUA/l). Lactose-intolerance was excluded by a negative lactose-intolerance test (lactose H2 breath test) in 7 CMI patients (CMI 2, 3, 4, 10, 11, 12, 13). Patients with gastrointestinal problems (GI, n=7) had gastrointestinal symptoms not related to cow's milk consumption. Persons without any gastrointestinal problems were recruited as controls (C, n=26). They comprised non-allergic as well as patients with IgE-mediated allergy to allergen sources other than milk (Table 1).

Total IgE levels (kU/l) as well as cow's milk specific IgE levels were measured using the ImmunoCAP System (Phadia) for all patients, specific IgE levels to respiratory allergens (sx1: timothy grass, rye, birch, mugwort, mite *Dermatophagoides pteronyssinus*, cat dander, dog dander, mould *Cladosporium herbarium*) and to food allergens (fx5: hen's egg, cow's milk, codfish, wheat, peanut, soy) were determined by ImmunoCAP (Phadia) for cow's milk intolerant patients and patients with gastrointestinal symptoms.

The study was approved by the Ethics-Committee of the Medical University of Vienna and the General Hospital of Vienna.

Biological materials, expression, and purification of recombinant cow's milk allergens

Pasteurized cow's milk containing 3.5% fat was bought at a local market (NÖM, Austria, batch: 333 402:51).

cDNAs coding for αS1-casein (rαS1-cas), αS2-casein (rαS2-cas), β-casein (rβ-cas), κ-casein (rκ-cas), α-lactalbumin (rα-la) and β-lactoglobulin (rβ-lg) were either isolated by IgE immunoscreening from a cDNA expression library prepared from bovine mammary glands or by reverse transcription-PCR from the same tissue [22]. Recombinant allergens were expressed in *Escherichia coli* strain BL 21 Codon Plus (DE3)-RIPL (Stratagene, La Jolla, CA) as hexahistidine-tagged proteins and purified

using Ni-NTA resin affinity columns according to the manufacturer's instructions (QIAGEN, Hilden, Germany) [22].

ELISA for measurement of IgG_{1-4} and IgA antibodies specific for purified recombinant cow's milk allergens

Serum levels of IgG_{1-4} and IgA to six purified recombinant cow's milk allergens were measured by means of ELISA [26]. In brief, sera were diluted 1:50 for detection of allergen-specific IgG_{1-4} and IgA. Murine mAbs with specificity for IgG_1, IgG_2, IgG_3, IgG_4, and $IgA_{1/2}$ (BD Biosciences, San Jose, CA) were used as recommended by the manufacturer to detect allergen-specific human immunoglobulins. Murine mAbs were traced with a 1:1000 dilution of a horseradish peroxidase-linked sheep anti-mouse IgG antibody (GE Healthcare, Little Chalfont Buckinghamshire, UK).

In order to compare the OD values from different ELISA plates, the results were normalized for each immunoglobulin isotype by including a reference serum on each ELISA plate. Therefore the first ELISA plate served as a reference plate and the OD values from the other ELISA plates were normalized by the ratio between the OD value of the reference serum from the reference plate and the OD value of the reference serum from the measured plate (mean of duplicate determinations). The cut-off level for a positive IgA and IgG measurement was set at 2-fold the OD value obtained for the highest buffer control.

IgA and IgG immunoblot analysis

For immunoblot analysis approximately 40 µg/cm of total cow's milk proteins were separated by SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) as described [23, 24]. The nitrocellulose strips were blocked with PBST (PBS, 0.5% v/v Tween 20) and then incubated with sera from individuals of the four study groups diluted 1:1000 in PBST over night at 4°C. Bound IgA and IgG antibodies were detected with an anti-human IgA (clone MH14-1, Sanquin, Amsterdam, Holland) and an anti-human IgG antibody (clone MH16-1, Sanquin) respectively, which had been ^{125}I-labelled using the ChloramineT method [25] and were diluted 1:1000 in PBST. Antibody-binding was visualized by autoradiography using Kodak XOMAT films with intensifying screens (Kodak, Heidelberg, Germany).

Statistics

Statistical comparisons were done by analysis of variance. For this, OD values were first logarithmized to get homogeneity of variance. In case of statistical significance ($p<0.05$), a post-hoc test (Tukey Hsd) was performed. For all calculations the statistical Programm SPSS (2008, version 16.0 SPSS Inc, Chicago, USA) was used.

Results

Characterization of the study groups

Group I consisted of 9 patients with IgE-mediated cow's milk allergy, 5 children (age 2 years-16 years; 2 females and 3 males) and 4 adults (age 39 years-69 years; 1 female and 3 males). These patients suffered from gastrointestinal symptoms, respiratory symptoms, atopic dermatitis, and/ or in the worst case systemic reactions after consumption of cow's milk. The diagnosis of IgE-mediated cow's milk allergy was confirmed by the demonstration of cow's milk allergen-specific IgE in their serum.

Group II consisting of 14 adults (age 24 years-58 years; 8 females and 6 males) with a clearly documented intolerance to cow's milk was designated cow's milk intolerant group (CMI). These patients reported reproducible symptoms after ingestion of cow's milk. Eleven of these patients exhibited gastrointestinal symptoms such as diarrhea, abdominal pain, and flatulence after ingestion of milk. Certain patients showed angioedema and throat swelling, respiratory symptoms (e.g., cough, rhinitis), atopic dermatitis-like symptoms or rhinoconjunctivitis after milk consumption. Five of the 14 patients suffered also from IgE-mediated allergy to allergen sources other than cow's milk, but none of the 14 patients showed any IgE reactivity to cow's milk allergens. A lactose intolerance test was performed with negative outcome in 7 of the 14 CMI patients. One of the patients suffered from celiac disease, another one from irritable bowel syndrome and for two patients celiac disease or irritable bowel syndrome was suspected.

A third group of patients with gastrointestinal problems such as diarrhea and flatulence not related to cow's milk consisted of 7 patients (age 19 years-56 years; 4 females and 3 adults). These patients, designated GI, had no problems when consuming cow's milk, had a negative lactose intolerance test and the presence of a chronic inflammatory bowl disease such as ulcerative colitis, Crohn's disease or celiac disease was excluded.

Group IV, a control group, represented individuals without any gastrointestinal symptoms and consisted of 18 persons without any IgE-mediated allergies and 8 persons with IgE-mediated allergy to allergen sources other than cow's milk (n=26; age 18 years-53 years; 16 females and 10 males). Each of these persons consumed regularly cow's milk without any problems.

Only cow's milk allergic patients have milk allergen-specific IgE

Sera from CMA, CMI, GI patients, and controls were analyzed by ImmunoCAP regarding their IgE reactivity to cow's milk (Fig. 1). Only CMA patients but none of the individuals belonging to the other groups (CMI, GI, C) showed IgE reactivity to cow's milk. The IgE antibody levels against cow's milk in the sera from the CMA patients ranged from 0.53 to 209.6 kUA/l (Fig. 1).

Cow's milk allergic patients exhibit elevated IgG_1 and IgG_4 levels to cow's milk allergens

Sera from the four groups were tested for IgG_1 and IgG_4 reactivity to purified recombinant cow's milk allergens including rαS1-casein, rαS2-casein, rβ-casein, rκ-casein, rα-lactalbumin, and rβ-lactoglobulin by ELISA (Fig. 2). The cow's milk allergic patients showed always the highest IgG_1 and IgG_4 antibody responses to each of the recombinant allergens (Fig. 2 and Table 2).

After statistical evaluation the following results were considered as statistically significant: The IgG_1 antibody levels of the CMA patient group were significantly higher against rαS1-cas (GI: $p<0.01$; C: $p<0.01$) and rβ-cas (GI: $p<0.01$; C: $p=0.03$) when compared with the GI and C group and against rα-la when compared with the GI group ($p=0.04$). The IgG_4 levels were significantly higher against rαS1-cas in the CMA group than in the C group ($p=0.01$) and against rβ-cas (CMI: $p=0.02$; GI: $p=0.02$; C: $p<0.05$) compared to the CMI, GI, and C group.

Cow's milk intolerant patients do not show significantly elevated IgG_1 or IgG_4 levels to cow's milk allergens compared to persons without cow's milk-related symptoms

The median IgG_1 and IgG_4 antibody levels of the CMI patients were generally very low or close to the cut off level (Fig. 2, Table 2). Statistical evaluation showed that patients with cow's milk intolerance did not have significantly higher IgG_4 levels against the cow's milk allergens compared to persons without any cow's milk-related symptoms (i.e., GI and C group). Only the IgG_1 and IgG_4 levels against rαS1-cas were slightly elevated compared to GI and C groups, but without reaching any significance.

Cow's milk intolerant patients lack significantly elevated levels of cow's milk allergen-specific complement-binding IgG subclasses or IgA

Besides cow's milk allergen-specific IgG_1 and the non-complement-binding IgG_4 subclass we have also analyzed cow's milk allergen-specific IgG subclasses which can activate complement (i.e., IgG_2, IgG_3) and cow's milk allergen-specific IgA antibody levels (Table 2). Cow's milk allergen-specific IgG_2 and IgG_3 antibodies were very low in each of the tested groups. Only rκ-casein-specific IgG_2 levels were slightly elevated in the CMI group but there was no statistically significant difference when compared with the GI and the control group (Table 2). Likewise, cow's milk allergen-specific IgA levels were low in each of the groups for all tested allergens (Table 2).

We also investigated the presence of IgG and IgA antibodies specific for cow's milk proteins other than allergens using nitrocellulose-blotted cow's milk extract (data not shown). IgG and IgA reactivity was found mainly against bands migrating at a molecular weight of approximately 30 kDa most likely representing the casein fraction. No relevant differences regarding IgG and IgA anti-milk reactivity were found between the CMI group and the GI and control group (data not shown).

Hierarchy of antigenicity of individual cow's milk allergens

If one compares the levels of IgG responses to the individual cow's milk allergens it appears notable that in cow's milk allergic patients the IgG_1 and IgG_4 levels to rαS1-cas were by far the highest. RαS1-cas thus seemed to be far more "antigenic" than the other caseins, whereas rα-lactalbumin and rβ-lactoglobulin seemed to be the least antigenic milk allergens. Interestingly, the IgG_4 levels to β-casein and κ-casein were lower than those to α-lactalbumin and β-lactoglobulin in the CMA group. Furthermore, we noted that κ-casein was relatively well recognized by IgG_1 from the CMA, CMI, GI, and control group and by IgG_2 antibodies from the CMI and GI group.

Discussion

Adverse reactions to food and in particular to cow's milk represent an important health problem. Food intolerance can be caused by immune-mediated as well as by non-immune-mediated mechanisms [8]. Among the immune-mediated mechanisms for cow's milk intolerance, IgE-mediated reactions, also commonly termed 'cow's milk allergy' represent well defined entities because they are based on the IgE recognition of cow's milk allergens, IgE-mediated effector cell activation and the unambiguous diagnosis can be performed by detection of allergen-specific IgE antibodies, anamnesis and provocation testing [27]. However, for one third of patients suffering from cow's milk intolerance other mechanisms are operative [28]. For non-immunological mechanisms it is possible to test for lactose intolerance by breath test and/or genetic testing [29, 30]. Regarding immunological mechanisms it is assumed that milk-induced gut damage is caused by humoral and/or cellular mechanisms [31].
Our study demonstrates that patients suffering from non-IgE-mediated cow's milk intolerance cannot be discriminated from persons without milk intolerance on the basis of IgG or IgA reactivity to cow's milk allergens. Previously performed studies have provided controversial results and there is currently considerable uncertainty regarding the diagnostic value of serological tests measuring IgG antibodies against food components for the diagnosis of non-IgE-mediated cow's milk allergy [3, 7, 10-21]. We have expressed and purified the major allergens of cow's milk and used the purified proteins to test for IgG_{1-4} subclass and IgA reactivity in four defined groups of patients, comprising patients suffering from IgE-mediated cow's milk allergy (CMA), patients suffering from non-IgE-mediated cow's milk intolerance (CMI), patients with gastrointestinal problems not mediated by cow's milk (GI) and persons without gastrointestinal problems (C). Our results clearly demonstrate that there is no significant difference regarding IgG and IgA antibody recognition of cow's milk allergens between the CMI and C group. In fact we have tested for non-complement-activating IgG_4 antibodies as well as for complement-activating IgG subclasses (IgG_1, IgG_2, IgG_3) and therefore can exclude with high probability that IgG-mediated complement activation and IgG-mediated cellular cytotoxicity may play a significant role for milk intolerance in the CMI group.
When testing for antibody reactivity in cow's milk allergic patients (CMA) to purified recombinant cow's milk allergens we found that certain allergens were more

antigenic than others and αS1-casein appeared as the allergen with the highest antigenicity which is in good agreement with results from other studies [7]. In fact the majority of earlier studies investigated IgG responses in cow's milk allergic patients and found elevated levels of and IgG_1 and IgG_4 antibodies [18, 21, 32]. However, these studies have not really addressed the question whether IgG and IgA testing is a reliable parameter for the identification of patients suffering from non-IgE-mediated cow's milk intolerance (CMI) and whether IgG and IgA testing can distinguish CMI patients from persons without cow's milk intolerance. In addition to testing with purified recombinant allergens we have also probed nitrocellulose-blotted cow's milk extracts for antibody reactivity in order to investigate whether proteins other than the known cow's milk allergens may play a role in non-IgE-mediated cow's milk allergy but obtained negative results. We therefore can exclude the possibility that non-IgE-mediated cow's milk allergy is due to IgG or IgA recognition of other cow's milk proteins.

Since our study strongly suggests that humoral immune mechanisms other than those mediated by IgE are not of pathophysiological relevance for cow's milk intolerance, future studies may focus on studying the role of cellular immune mechanisms for non-IgE-mediated cow's milk intolerance. It is possible that CMI patients contain milk antigen-specific Th1 or perhaps Th17 which either *per se* or due to lack of appropriate control by regulatory T cells and/or Th3 can induce gut inflammation. In fact it has been demonstrated that certain CMI patients exhibit positive epicutaneous reactions to cow's milk antigens which would speak for the presence of Type IV hypersensitivity reactions at least in a subgroup of patients [2].

In conclusion, our study contradicts theories stating a role of humoral mechanisms in non-IgE-mediated cow's milk allergy and demonstrates that diagnostic tests based on the measurement of cow's milk antigen-specific IgG and IgA cannot be used to diagnose patients suffering from non-IgE-mediated cow's milk allergy.

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allergy and recurrent abdominal pains. Pediatr Allergy Immunol 2002;13: 129-36.

Figure legends

Fig. 1. Cow's milk specific IgE levels (kUA/l) in the four study groups as determined by ImmunoCAP. IgE levels (y-axis) are displayed for CMA (cow's milk allergic patients, n=9), CMI (cow's milk-intolerant individuals, n=14), GI (patients with gastrointestinal problems, n=7), and C (asymptomatic controls, n=26) as box plots. Fifty % of the values are within the boxes and non-outliers are between the bars. Upper and lower box plot margins represent the interquartile range, the bar inside the boxes indicates the median. The lower limit of detection of the assay is 0.35 kUA/l.

Fig. 2. Cow's milk allergen-specific IgG_1 and IgG_4 reactivity in the study groups. IgG_1 and IgG_4 antibody levels to rαS1-cas (a), rαS2-cas (b), rβ-cas (c), rκ-cas (d), rα-la (e), and rβ-lg (f) were measured by ELISA for the CMA, CMI, GI, and C group and OD values corresponding to the antibody levels (y-axes) are displayed as box plots. Outliers are not shown. The cut off for a positive reaction is indicated by the horizontal line.

Table 1. Demographic and clinical features of participants[a]

Patient	Age	Sex	Milk-related symptoms	GI disorders	Other allergies	Other allergic symptoms	Total IgE (kU/l)	Specific IgE to		
								CM (kUA/l)	fx5 (kUA/l)	sx1 (kUA/l)
Group I: Patients with IgE-mediated cow's milk allergy (CMA, n=9)										
CMA 1	13y	m	AS, E, U, V	nk	pollen, hen's egg	AS, GI, RC, U	3634	147.4	nd	nd
CMA 2	11y	f	AE, AS, U	nk	candida	AS	114	23.60	nd	nd
CMA 3	16y	m	Sys, AS, U	nk	fish, nuts, pollen, mite, Alternaria	AS, Rh	355	11.6	nd	nd
CMA 4	2y	m	U	nk	pollen, dog, cat, horse, hen's egg	AS, RC	45.6	2.85	nd	nd
CMA 5	39y	m	U (cheese)	nk	goat's milk, sheep's milk	nk	27.2	1.51	nd	nd
CMA 6	6y	f	AD, U, V	nk	hen's egg, fish	AE, U	1037	99.4	nd	nd
CMA 7	42y	m	GI	nk	cat, dog	nk	35.3	0.53	nd	nd
CMA 8	64y	f	Sys, GI, U	nk	nk	nk	1302	209.6	nd	nd
CMA 9	69y	m	GI	IBS	nk	nk	9.79	0.98	nd	nd
Group II: Patients with cow's milk intolerance (CMI, n=14)										
CMI 1	53y	m	CO	nk	mite, cats	CO, DY	64.9	<0.35	<0.35	10.3
CMI 2	57y	f	LS, TS	ni	rye, oat, wheat	RC	47.9	<0.35	<0.35	7.47
CMI 3	50y	f	GI, RS	sus CD	birch, shrimp, cockroach	nk	81.1	<0.35	<0.35	1.24
CMI 4	39y	m	GI	sus IBS	cat, horse	nk	26	<0.35	<0.35	2.61
CMI 5	25y	f	GI	nk	nk	nk	153	<0.35	<0.35	<0.35
CMI 6	29y	f	GI	nk	nk	nk	10.2	<0.35	<0.35	<0.35
CMI 7	29y	m	GI	nk	nk	nk	41	<0.35	<0.35	<0.35
CMI 8	48y	m	RI	nk	nk	nk	14.9	<0.35	<0.35	<0.35
CMI 9	58y	f	AE, GI, I	nk	nk	nk	8.33	<0.35	0.85	0.95
CMI 10	42y	m	AD, GI	IBS	nk	nk	16.7	<0.35	<0.35	<0.35
CMI 11	40y	m	GI, RC	ni	nk	nk	4.87	<0.35	<0.35	<0.35
CMI 12	55y	f	GI	HP-gastritis	nk	nk	84.6	<0.35	<0.35	<0.35
CMI 13	30y	f	GI	nk	nk	nk	8.21	<0.35	<0.35	<0.35
CMI 14	24y	f	AD, GI	CD	nk	nk	13.4	<0.35	<0.35	<0.35
Group III: Patients with patients with gastrointestinal symptoms not related to cow's milk (GI, n=7)										
GI 1	56y	f	no	ni	nk	nk	9.28	<0.35	<0.35	<0.35
GI 2	47y	f	no	food allergy	wheat, peanut, soy	nk	572	<0.35	pos (W, P, S)	nd
GI 3	36y	m	no	sus IBS	nk	nk	42.6	<0.35	<0.35	2.33
GI 4	33y	f	no	ni	nk	nk	39.7	<0.35	<0.35	7.67
GI 5	46y	f	no	ni	nk	nk	6.89	<0.35	<0.35	<0.35
GI 6	30y	m	no	IBS	nk	nk	131	<0.35	<0.35	23.2
GI 7	19y	m	no	inf. colitis	nk	nk	49.6	<0.35	<0.35	<0.35

Patient	Age	Sex	Milk-related symptoms	GI disorders	Other allergies	Other allergic symptoms	Total IgE (kU/l)	Specific IgE to		
								CM (kUA/l)	fx5 (kUA/l)	sx1 (kUA/l)
Group IV: Individuals without gastrointestinal problems (C, n=26)										
Non-allergic persons										
C 1	50y	f	no	no	no	no	62.3	<0.35	nd	nd
C 2	51y	f	no	no	no	no	6.16	<0.35	nd	nd
C 3	24y	f	no	no	no	no	70.2	<0.35	nd	nd
C 4	25y	f	no	no	no	no	91.9	<0.35	nd	nd
C 5	53y	m	no	no	no	no	12.3	<0.35	nd	nd
C 6	46y	f	no	no	no	no	8.31	<0.35	nd	nd
C 7	21y	m	no	no	no	no	8.89	<0.35	nd	nd
C 8	44y	f	no	no	no	no	55.1	<0.35	nd	nd
C 9	42y	m	no	no	no	no	27	<0.35	nd	nd
C 10	39y	m	no	no	no	no	11.5	<0.35	nd	nd
C 11	27y	m	no	no	no	no	12.08	<0.35	nd	nd
C 12	38y	f	no	no	no	no	12.2	<0.35	nd	nd
C 13	28y	f	no	no	no	no	7.85	<0.35	nd	nd
C 14	30y	f	no	no	no	no	22.3	<0.35	nd	nd
C 15	28y	f	no	no	no	no	3.69	<0.35	nd	nd
C 16	25y	f	no	no	no	no	5.2	<0.35	nd	nd
C 17	37y	f	no	no	no	no	39.1	<0.35	nd	nd
C 18	21y	m	no	no	no	no	41.4	<0.35	nd	nd
Persons with other allergies										
C 19	18y	m	no	no	mite	RC	20.30	<0.35	nd	nd
C 20	29y	f	no	no	bee	I, LR	16.8	<0.35	nd	nd
C 21	25y	f	no	no	cat, dog, pollen, Aspergillus	RC	203	<0.35	nd	nd
C 22	31y	f	no	no	bee	LR	37.4	<0.35	nd	nd
C 23	25y	m	no	no	mite, wasp, bee	LR, RC, U	139	<0.35	nd	nd
C 24	25y	m	no	no	mite	RC	393	<0.35	nd	nd
C 25	43y	m	no	no	pollen, bee, wasp, cat	AS	220	<0.35	nd	nd
C 26	30y	f	no	no	dog	nk	506	<0.35	nd	nd

[a]Abbreviations: y, years; f, female; m, male; AD, atopic dermatitis; AE, angioedema; AS, asthma; CO, cough; DY, dyspnoea; E, eczema; GI, gastrointestinal symptoms; I, itching; LR, local reaction; LS, labial swelling; RC, rhinoconjunctivitis; Rh, rhinitis; RS, respiratory symptoms; Sys, systemic reaction; TS, throat swelling; U, urticaria; V, vomiting; CD, celiac disease; HP-gastritis, *Helicobacter pylori*-gastritis; IBS, irritable bowel syndrome; inf. colitis, infectious colitis; ni, not identified; nk, not known; sus, suspected; nd, CM, cow's milk; kU/l, total IgE in kilo units/liter; kUA/l, allergen-specific IgE in kilo units antigen/liter; fx5, food allergens: hen's egg, cow's milk, codfish, W, wheat, P, peanut, S, soy; sx1, respiratory allergens: timothy grass, rye, birch, mugwort, mite *Dermatophagoides pteronyssinus*, cat dander, dog dander, mould *Cladosporium herbarium*, not done.

Table 2. Median OD-levels of IgG_{1-4} and IgA to purified recombinant cow's milk allergens in the four study groups as determined by ELISA.

		CMA n=9	CMI n=14	GI n=7	C n=26
rαS1-cas	**IgG_1**	2.13	0.17	0.11	0.11
	IgG_2	0.12	0.06	0.04	0.06
	IgG_3	0.06	0.05	0.05	0.06
	IgG_4	1.28	0.15	0.06	0.06
	IgA	0.08	0.08	0.07	0.07
rαS2-cas	**IgG_1**	0.33	0.16	0.13	0.17
	IgG_2	0.10	0.06	0.05	0.07
	IgG_3	0.07	0.06	0.06	0.06
	IgG_4	0.25	0.05	0.05	0.05
	IgA	0.08	0.11	0.08	0.08
rβ-cas	**IgG_1**	0.31	0.17	0.10	0.14
	IgG_2	0.09	0.07	0.06	0.08
	IgG_3	0.04	0.04	0.04	0.05
	IgG_4	0.08	0.04	0.04	0.05
	IgA	0.09	0.07	0.06	0.07
rκ-cas	**IgG_1**	0.29	0.20	0.28	0.20
	IgG_2	0.06	0.12	0.11	0.09
	IgG_3	0.05	0.05	0.05	0.05
	IgG_4	0.09	0.08	0.05	0.06
	IgA	0.07	0.09	0.06	0.10
rα-la	**IgG_1**	0.25	0.15	0.12	0.14
	IgG_2	0.08	0.05	0.05	0.06
	IgG_3	0.06	0.06	0.05	0.07
	IgG_4	0.28	0.06	0.10	0.06
	IgA	0.13	0.10	0.12	0.12
rβ-lg	**IgG_1**	0.25	0.11	0.10	0.13
	IgG_2	0.05	0.04	0.04	0.05
	IgG_3	0.06	0.06	0.06	0.07
	IgG_4	0.12	0.12	0.09	0.10
	IgA	0.10	0.08	0.08	0.08

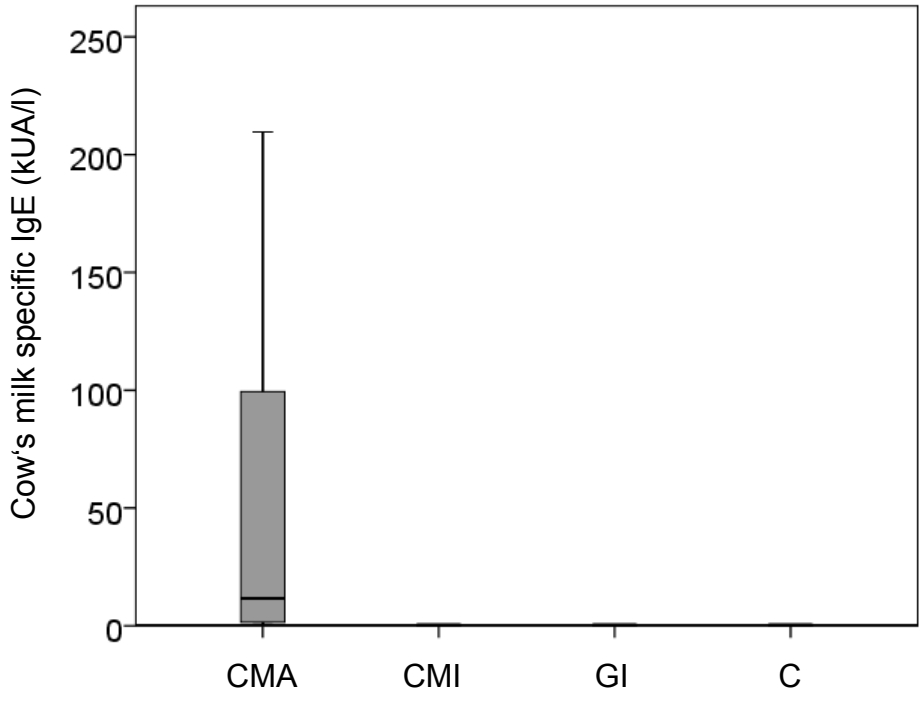
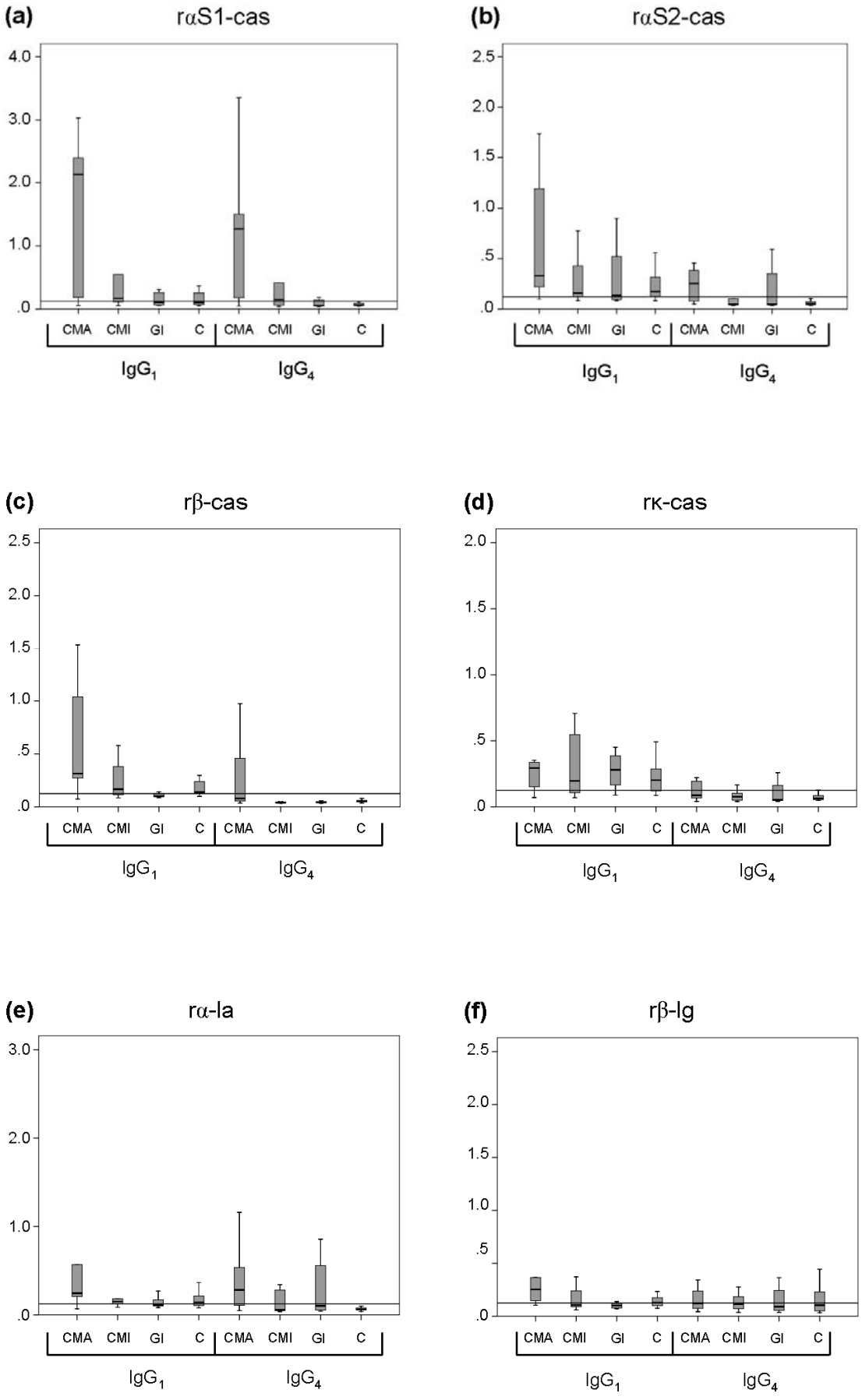


Fig. 1.


(a)
rαS1-cas
4.0
3.0
2.0
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CMA CMI GI C CMA CMI GI C
IgG1
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(b)
rαS2-cas
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IgG4

Fig. 2.

Curriculum vitae

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Education

since July 2005 PhD thesis at the Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna under supervision of Prof. Susanne Spitzauer and Prof. Rudolf Valenta. Topic: Immune responses to cow's milk allergens: Molecular allergen characterization and epitope mapping

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Publications

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