

## **Cow's milk protein allergy – Results of skin-prick test with purified milk proteins**

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*Summary:* The diagnosis of milk-protein allergies is difficult. Therefore, the main cow's milk proteins ( $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin A + B,  $\alpha_s$ -casein,  $\beta$ -casein,  $\kappa$ -casein), purified to the highest available standards, were used for differential diagnosis of allergic individuals by applying skin-prick test. In the case of adults with uncertain medical history, milk proteins rarely caused skin reactions, while distinct skin reactions were observed in 11 of 13 children with strongly suspected milk-protein allergy. In the presented study  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin B were the main allergens, because skin reactions to these proteins were provoked with highest prevalence and intensity. Intensity and prevalence of reactions to  $\alpha$ - and  $\kappa$ -casein were significantly less. In two cases skin reactions to  $\alpha$ -lactalbumin were observed exclusively.

*Zusammenfassung:* Die Diagnose von Milchproteinallergien ist schwierig. Aus diesem Grunde wurden hochgereinigte Hauptproteine der Kuhmilch ( $\alpha$ -Lactalbumin,  $\beta$ -Lactoglobulin A + B,  $\alpha_s$ -Casein,  $\beta$ -Casein,  $\kappa$ -Casein) unter Anwendung des Hautpricktestes für die Differentialdiagnose bei Allergikern eingesetzt. Hierbei zeigte sich, daß Milchproteine bei Erwachsenen mit unklarer Anamnese nur selten Hautreaktionen auslösten, während bei 11 von 13 Kindern mit starkem Verdacht auf Milchproteinallergie deutliche Hautreaktionen beobachtet wurden. In der vorliegenden Untersuchung waren  $\alpha$ -Lactalbumin und  $\beta$ -Lactoglobulin die Hauptallergene, weil sie mit höchster Prävalenz und Intensität Hautreaktionen auslösten. Reaktionen auf  $\alpha$ - und  $\kappa$ -Casein erfolgten mit deutlich geringerer Häufigkeit und Intensität. In zwei Fällen wurde eine ausschließliche Reaktion auf  $\alpha$ -Lactalbumin beobachtet.

*Key words:* cow's milk protein allergy; purified milk proteins; skin-prick test; main allergen

*Schlüsselwörter:* Kuhmilchproteinallergie, gereinigte Milchproteine, Hauttest, Hauptallergen

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*Abbreviation index:* PNU = protein nitrogen unit

## Introduction

Differential diagnosis of food allergies is a serious problem. It is argued that egg and milk proteins are the most common dietary allergens (9). Eggs and milk are the most valuable dietary protein sources and it is important to secure diagnostic assay for them.

As there is a large variety of milk proteins which can be potential allergens (4), it might be more successful to treat patients with a hyposensibilization therapy if it is possible to identify a single protein as the sole allergen. For both diagnosis and therapy, respectively, highly purified proteins must be available. Therefore, we purified cow's milk proteins to the highest standards available at the present time. The development of the purification method of these proteins was accompanied with control by immunological techniques (5). These milk proteins were applied in clinical use for the differential diagnosis of milk protein allergies.

The data presented in this paper are restricted to results of a skin-prick test.

## Materials and Methods

Immunological skin reactions to whole cow's milk proteins, the fractions of casein and whey protein, and the five individual main proteins, respectively, were investigated by applying a skin-prick test to two groups of patients. Members of the first group were ambulant patients of the clinical department of dermatology of the University of Kiel, consisting of 12 females and eight males (age: 2 to 65 years). While only a few of these patients apparently suffered from milk-protein allergy, the remaining patients revealed an unclear medical history which did not exclude milk allergy. The other group were ambulant patients of a pediatric allergist in a village 30 km west of Kiel. This group consisted of seven girls and six boys (age 8 months to 8 years). In these cases, manifest milk-protein allergy was strongly suspected.

Whole cow's milk protein, the fractions of whey protein and casein, and the purified individual main proteins ( $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin A and B,  $\alpha_s$ -casein,  $\beta$ -casein,  $\kappa$ -casein), respectively, were prepared from fresh raw milk as described elsewhere (5) for the skin-prick test. While only  $\alpha_s$ -casein and  $\beta$ -casein were immunologically pure substances, the other purified proteins contained traces of different major milk proteins which could be detected by immunoblotting (5). Whereas  $\alpha$ -lactalbumin did not contain any  $\beta$ -lactoglobulin,  $\beta$ -lactoglobulin contained varying traces of  $\alpha$ -lactalbumin.

Solutions of the different milk proteins for the skin-prick tests were prepared freshly on the day of use. The concentrations corresponded to 5000 or 50 000 PNU per ml 0.9 % NaCl (PNU = protein nitrogen unit, 1 PNU =  $10^{-5}$  mg protein nitrogen). 5000 PNU per ml were applied in the testing of Group-1 patients in the clinical department of dermatology of the University of Kiel, while 50 000 PNU per ml were used for the Group-2 patients of the pediatric allergist.

Skin-prick tests were carried out on the palmar surface of the forearm. For this purpose, one drop of the respective test solution was applied to the test area. The skin below this drop then was superficially pricked with a prick lancet. The test area then was inspected after 20 min for recording the individual skin reactions to the different test proteins and the positive and negative control solutions. The negative control solution consisted of 0.9 % NaCl in H<sub>2</sub>O; the positive control solution contained 0.1 % histamin.

The evaluation of skin reactions of the children's group was done according to the diameters of the wheals and erythemas. The results were classified as described by

Ring (8) using a scale from no reaction <-> over doubtful positive reaction <1>, weak reaction <2>, distinct reaction <3> to strong reaction <4>. One patient showed an extremely strong reaction which was noted as <5>. For final evaluation only weak, distinct, strong, and extremely strong reactions (2 to 5) were judged positive.

Statistic evaluation of data was done with the aim to find out which milk protein caused, with highest prevalence, positive skin reactions. For this purpose, we carried out ranking of the purified milk proteins by reaction intensities and ranking of the purified milk proteins only according to positive or non-positive reactions applying Kruskal-Wallis one way analysis by ranks (10). Tukey multi-range analysis (10) was applied to the same data sets to detect homogeneous groups.

## Results

One group of selected patients, mainly adults, was tested at the dermatological department of the University of Kiel. In this case, the protein concentration for skin prick test corresponded to 5000 PNU per ml. The results are summarized in Table 1. In comparison, 10 volunteers of the staff of the clinical department who did not suffer from any allergic disorder were tested by applying the same solutions as in the patient group. In this healthy control group no skin reaction to the prick test (except for positive control solution) was observed in any case.

As can be seen in Table 1, skin reactions which were judged positive rarely occurred in adults. The most prominent reaction was observed in the case of a 2-year-old child (patient no. 7), who revealed positive reactions to whole milk (homogenized, pasteurized), whey proteins and  $\alpha$ -lactalbumin (distinct reaction), but not to  $\beta$ -lactoglobulins and caseins. In this patient  $\alpha$ -lactalbumin was identified as the individual allergen.

Only in four of the 18 adults were skin reactions observed in the skin-prick test. While one patient (no. 4) reacted weakly to raw milk, another (no. 13) reacted with medium intensity but only to homogenized pasteurized milk. In case of Patient 9 the reaction to whole casein was

Table 1. Results of skin-prick tests in patients of the clinical department of dermatology. Only those results of the 21 selected patients are shown that were at least doubtfully positive. Protein concentration of test solutions corresponded to 5000 PNU. The skin reactions were evaluated according to a scale (see methods) ranging from <-> (no reaction) to <4> (strong reaction). Samples tested: RM = raw milk; HM = whole milk, homogenized and pasteurized; WP = whole whey protein;  $\alpha$ -L =  $\alpha$ -lactalbumin;  $\beta$ -LG =  $\beta$ -lactoglobulin A/B; CN = casein; GMP = glykomacropeptide.

Patient (No.) Age/Sex	Tested samples						
	RM	HM	WP	$\alpha$ -L	$\beta$ -LG	CN	GMP
(4) 55y/f	2	-	-	-	-	-	-
(7) 2y/m	1	2	2-3	3	-	-	-
(9) 48y/f	-	-	1	-	-	2-3	-
(13) 3y/m	-	3	-	-	-	-	-
(16) 47y/f	1	1	-	-	-	1	-
(20) 46y/f	-	1	2	2	1	1	2

Table 2. Results of skin prick tests in patients of a pediatric allergist who were preselected for strongly suspected milk protein allergy. Protein concentration of test solutions corresponded to 50 000 PNU. The skin reactions were evaluated according to a scale (see methods) ranging from <-> (no reaction) to <5> (extremely strong reaction). Samples tested: RM = raw milk; HM = whole milk, homogenized and pasteurized; WP = whole whey protein;  $\alpha$ -L =  $\alpha$ -lactalbumin;  $\beta$ -LGA =  $\beta$ -lactoglobulin A;  $\beta$ -LGB =  $\beta$ -lactoglobulin B; CN = casein;  $\alpha_s$ -C =  $\alpha_s$ -casein;  $\beta$ -C =  $\beta$ -casein;  $\kappa$ -C =  $\kappa$ -casein.

Patient (No.)Age/Sex	Tested samples									
	RM	HM	WP	$\alpha$ -L	$\beta$ -LGB	$\beta$ -LGA	CN	$\alpha_s$ -C	$\beta$ -C	$\kappa$ -C
(22) 3y/f	-	-	1	1	1	1-2	1	-	-	-
(23) 9mo/m	3	2	3	3	4	3	4	1	3	1
(24) 12mo/m	2	2	3-4	3-4	2	1	1	1-2	1	1
(25) 4y/m	1	1	2	1	2	2	1	-	1	-
(26) 8mo/f	2	1	2	1	2	1-2	2	1	1	1
(27) 5y/m	-	-	-	-	-	-	-	-	-	-
(28) 4y/f	-	-	-	-	-	-	-	-	-	-
(29) 2y/f	2	2	1	1-2	1-2	-	1-2	-	2	1-2
(30) 2y/m	-	2	3-4	4	4	4	2	2	2	2
(31) 4y/f	2	1	2	3	3	1	1	-	2	1-2
(32) 8y/f	2	2	3	5	-	-	-	1	-	-
(33) 2y/f	2	1	4	3-4	3	3	-	1-2	-	-
(34) 18mo/m	-	2	2	3	3	1-2	1	-	1-2	1-2

positive. One patient (no. 20) showed polyvalent sensibility with weak reactions to almost all samples tested, including glycomacropeptide, the pepsinolytic soluble fragment of  $\kappa$ -casein, but with the exception of raw milk.

The second group of test subjects, the children, were tested by a pediatric allergist. The protein concentrations applied to patients of this group corresponded to 50 000 PNU per ml. The results are summarized in Table 2.

The data presented in Table 2 clearly demonstrate that children who apparently suffer from milk protein allergy, in most cases exhibited more distinct reactions compared with the other group of mainly adult patients. At present, we cannot exclude that this is also due to the higher PNU values of the test solutions applied.

Only two of the 13 tested children did not show any positive skin reactions to the prick test with milk proteins. In most cases of positive skin reactions the reaction to  $\alpha$ -lactalbumin was positive. In one case (patient 32) again  $\alpha$ -lactalbumin was identified as the individual allergen, because the reaction to  $\alpha$ -lactalbumin was extremely strong and quite distinct compared to whole whey protein, while no positive reaction was observed by using  $\beta$ -lactoglobulins A and B and the caseins. In most cases skin reaction on skin-prick test was more distinct for whey proteins than for caseins and, in tendency, most distinct was  $\alpha$ -lactalbumin. Positive skin reactions to  $\alpha$ -lactalbumin were most frequent, since in two cases (patients 7 and 32) skin reactions to purified milk proteins were positive only in case of  $\alpha$ -lactalbumin.

## Discussion

The presented data were obtained from two selected groups of patients who did not belong to the same statistical population, since one group was children with strongly suspected milk protein allergy, while in the other group, which mainly consisted of adults, milk-protein allergy could not be excluded by medical history. Due to this background, statistical evaluation is limited and results may be generalized only from the group of milk-allergic children to other groups of milk-allergic children selected according to the same criteria.

Furthermore, the protein concentration of the test solutions applied in the children's group was 10 times as high as the concentration applied in the other group. This difference originated from the fact that there was no experience with purified milk proteins. The different concentrations applied were suggested by experienced doctors and were found to be used also in applications with commercial test kits. It should be noted that standardisation is a must for getting comparable results. Standardized test substances for the investigation of effects of different concentrations and protein qualities on the results of skin tests are still missing (6, 10). No published information is available about useful concentrations of milk proteins to be applied in the skin-prick test.

Based on the presented results, we assume that the higher concentration of 50 000 PNU per ml is more likely to be useful than the lower one.

Another limitation is the fact that skin-test reactivity is different in infants and adults (6). Furthermore, positive skin reactions are often observed, also in the absence of clinical symptoms on oral challenge with milk (3). Therefore, the following conclusions are mainly extracted from the results of the more homogeneous group of children, who were strongly preselected for milk protein allergy.

With no consideration of the statistical limitations, we analyzed the data related to the purified milk proteins in order to discover which milk protein had the highest prevalence in causing positive skin-prick results. The result of the Kruskal-Wallis one-way analysis by ranks (10) of the data is shown in Table 3.

Table 3. Rank and multiple range analyses of skin-prick test results presented in Table 2.

Milk protein	Average rank (Kruskal-Wallis)		Homogeneous groups (95 % Tukey HSD intervals)	
	a	b	a	b
$\alpha$ -lactalbumin	52	51	*	*
$\beta$ -lactoglobulin B	50	51	**	*
$\beta$ -lactoglobulin A	41	39	**	**
$\beta$ -casein	36	39	**	**
$\kappa$ -casein	30	30	*	*
$\alpha_s$ -casein	27	30	*	*

a = Analysis for the intensities of skin reactions; b = analysis for positive skin reactions (intensity  $\geq 2$ ). Asterisks in the same columns denote homogeneous groups.

Ranking is mainly independent on the criterion used. While significance level of ranking by intensity of skin reactions was 0.015, ranking by positive skin reaction (intensity  $\geq 2$ ) resulted in a significance level of 0.005. In addition, the multiple-range analysis indicated that reactions to  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin do not belong to the same homogeneous group with reactions to  $\alpha$ -casein and  $\kappa$ -casein, while differences between the reactions to the other proteins are not significant.

The results of these analyses suggest a strong tendency toward  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin being the main allergens among the tested cow's milk proteins.

This impression may also be derived directly from the data. In any case, where the skin reaction to casein was judged positive the reactions to whey proteins were positive, too, and showed at least the same intensity. Additionally, in most cases in which a positive skin reaction to whey protein was observed there was also a positive reaction to purified  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin. Two individuals, one of each group, exclusively exhibited a positive skin reaction to  $\alpha$ -lactalbumin. Thus, whey proteins seem to be the main allergens of cow's milk proteins, with a slight tendency to favor  $\alpha$ -lactalbumin in our investigation.

In most studies it is suspected that  $\beta$ -lactoglobulin and also casein are the most potent or most common allergens present in cow's milk (1, 2, 3, 7, 12, 14). Vanto et al. (13), who applied various methods, including the skin-prick test, found  $\alpha$ -lactalbumin to be the main allergen among cow's milk proteins, which corresponds to our findings. The fact that, in our investigation, the casein fractions exhibited the lowest potential in causing positive skin reactions, may be in contrast to results of Wüthrich (15), who demonstrated in 15 cheese- or milk-allergic individuals by radio-allergo-sorbent-test (Rast) a highest prevalence of immunoglobulin E antibodies to casein, followed by those to  $\alpha$ -lactalbumin. Nearly no antibodies were found to  $\beta$ -lactoglobulin by Wüthrich. But skin test results and Rast results may diverge depending on the titres of specific IgG antibodies. A paper concerning these specific immunoglobulin levels of milk allergic individuals is in preparation.

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