

Murine passive cutaneous anaphylaxis test (PCA) for the 'all or none' determination of allergenicity of bovine whey proteins and peptides

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Summary

A passive cutaneous anaphylaxis test (PCA) for determination of the allergenicity of bovine whey proteins and peptides was developed in mice. Antisera against whey proteins raised in rabbits and in mice, using a procedure for high IgE titre mixed with Freund's incomplete adjuvant, were applied intradermally, and various whey proteins and whey protein hydrolysates were tested for positive PCA reaction. Unhydrolysed whey and peptides larger than 6500 Da were found to react positively, peptides between 6500 Da and 3400 Da reacted weakly, whereas peptides smaller than 3400 Da were unable to initiate a reaction. The studies indicate that guinea-pigs, widely used by tradition, may be successfully replaced by mice in determination of the allergenicity of various compounds. The sensitivity of the two species is similar, but the husbandry and handling of mice is more convenient, and they are less expensive.

Introduction

The study of the allergenicity of proteins and peptides has attracted considerable research activities in the past two decades, utilizing several *in-vivo* and *in-vitro* techniques. The usefulness of the *in-vitro* techniques, such as immuno-precipitation test and radio-allergo-sorbent test, is limited due to a poor correlation between results obtained by the *in-vitro* tests and provocation tests in patients [1,2]. Consequently, the *in-vivo* assay, passive cutaneous anaphylaxis test (PCA) using guinea-pigs, is still widely employed when assessing the allergenicity of various compounds [3,4,5].

However, several difficulties are associated with the use of guinea-pigs for PCA testing. Sedatives, anaesthetics and stress tend to give a reduced or lacking response [3]. Intravenous injections are rather complicated, and since only one antigen/allergen can be tested per animal, the use of guinea-pigs also becomes a rather expensive, space- and time-consuming method.

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The present report describes the use of mice in PCA testing for the *in-vivo* determination of the allergenicity of bovine whey proteins and peptides, and advocates a more general replacement of guinea-pigs by mice.

Materials and methods

Animals

The mice employed in the PCA test were as follows.

Group A: Inbred BALB/c Bom maintained at our laboratory for several generations on a diet without milk proteins (Ewos Brood stock feed—R-3, DK 8600 Silkeborg, Denmark).

Group B: Inbred BALB/c Bom maintained for several generations on pellets containing 15% skimmed bovine milk proteins (The Panum Institute, University of Copenhagen, Denmark).

Group C: BALB/c Bom maintained by random breeding in a closed colony at the Royal Veterinary and Agricultural University, Copenhagen. These mice were maintained on the milk containing diet.

Mice of both sexes aged 5–8 weeks were used and given water and feed *ad libitum*.

Measurement of protein content of antigen preparations

Protein content of antigen preparations was measured with the micro Kjeldahl method [6], or by measuring the absorbance at 280 nm using bovine serum albumin (Sigma) as standard. The protein contents of dilution series of bovine whey protein and hydrolysates were measured with the two methods, and complete agreement between the two methods was observed. Absorbance at 280 nm, which was the most sensitive of the two methods, was used to measure the protein content of diluted samples (i.e. fractions obtained by column chromatography), whereas the micro Kjeldahl method was used to measure the protein content of turbid samples with high protein content (i.e. whey protein concentrates).

Antibody preparations

Rabbit antiserum against bovine whey proteins was donated by Dr B. Gjesing (The Protein Laboratory, University of Copenhagen). The antiserum preparations were purified by precipitation with ammonium sulphate [7], after which the antibody preparations were desalted in a column (LKB, Bromma, Sweden) packed with 60 ml Biogel P-6 (100–200 Mesh, Biorad Laboratories) using a 0.1 M NH_4HCO_3 buffer. The fractions containing antibodies were pooled, adjusted to a concentration of 13 mg/ml and stored in aliquots of 3 ml at -20°C until use.

Murine antiserum against whey proteins was prepared using very low doses of proteins, as suggested by Jarret and colleagues [8], for production of sera with high IgE titre. Mice of group A ($n=20$) were injected intraperitoneally with 25 μl Freund's complete adjuvant to obtain a general stimulation of the immune system, whereas Jarrett *et al.* used an intraperitoneal injection of heat-killed pertussis bacteria to obtain this effect in rats. On the same day, the mice received 4.0 μg whey proteins subcutaneously. Ten days later the mice received a second injection of 0.4 μg subcutaneously. The mice were bled 4 days after the last injection.

Antigen preparations

Preparation of whey protein. The whey fraction of bovine milk proteins was purified from pasteurized skimmed milk (73°C/15 sec). The casein fraction was precipitated by adjusting the pH to 4.6, and the whey fraction was decanted followed by sedimentation of the residual caseins by centrifugation (23 000 $\times g$, 20 min, 5°C). The whey preparations contained 7 mg/ml [6] and were stored in aliquots of 3 ml at -20°C until assayed.

Preparation of enzymatically hydrolysed whey protein. Bovine whey was ultrafiltered and diafiltrated (DDS LAB-20 unit mounted with GR61PP-filter, cut off 20 000 Da, DDS-RO Division, Denmark) to obtain a preparation of lactose-free concentrated bovine whey protein (CWP) with a protein content of 49 mg/ml.

CWP (200 ml) was hydrolysed at 50°C and pH 7.5 with 0.2 ml *Bacillus licheniformis* proteinase (Alcalase 0.6 L. Novo Industri A/S, Denmark). The progressivity of hydrolysis was followed by the pH-stat method [9], and the reaction was terminated (100°C for 10 min), when a hydrolysis of 17.5% was reached. No residual activity could be measured by the electrophoretic method described previously [10]. Aliquots (10 ml) of the hydrolysate (termed CWP-hydrolysate) were stored at -20°C and tested for allergenicity by the PCA technique.

Preparation of peptides by partial enzymatic hydrolysis of whey protein

CWP (150 ml) was hydrolysed with 0.1 ml Alcalase 0.6 litre at 50°C and pH 7.5 for 5 min. The enzyme was irreversibly inactivated by adjusting the reaction mixture to pH 3.2 with 1.0 N HCl and incubating at room temperature for 24 hr. Inactivation by heating at 100°C for 10 min could not be used since this treatment at a low degree of hydrolysis resulted in precipitation of the unhydrolysed whey proteins and the largest product peptides. The hydrolysates were centrifuged at 23 000 g for 20 min, and the clarified supernatant (20 ml) was separated by gel filtration on a Biogel P-30 column (100–200 Mesh, Biorad Lab.).

The following molecular weight markers were used: bovine serum albumin (BSA, M_r 67000), β -lactoglobulin (β -LG, M_r 18300) and α -lactalbumin (α -LA, M_r 14200), trypsin inhibitor (M_r 6500, Serva), insulin chain B (M_r 3494, Serva) and insulin chain A (M_r 2532, Serva).

The eluted proteins or peptides were pooled in five molecular weight groups as indicated in Fig. 1. The composition of pools IV and V was further analysed by gel filtration in a Biogel P-6 column (Fig. 2). Aliquots of the five different pools of peptides were lyophilized, resuspended in deionized water and tested for allergenicity by the PCA technique.

Evans blue preparation. A solution of 10 mg/ml of Evans blue (Sigma) in isotonic saline was prepared and stored at -20°C in aliquots of 10 ml until use.

Samples of proteins ranging from 0 to 4800 μg were mixed with 3 ml samples of the Evans blue solution just prior to the intravenous administration.

Passive cutaneous anaphylaxis test

The mice were anaesthetized by intraperitoneal injection of propanidid (Sombrevin) (500 mg/kg), and the fur on a 3 $\times$ 4 cm square on the backside was removed using an electric razor. Samples of 100 μl , containing either antibody preparation mixed with saline (1:1) or antibody preparation mixed with Freund's incomplete adjuvant (1:1), were injected intradermally in the centre of the shaved area using 26-gauge needles.

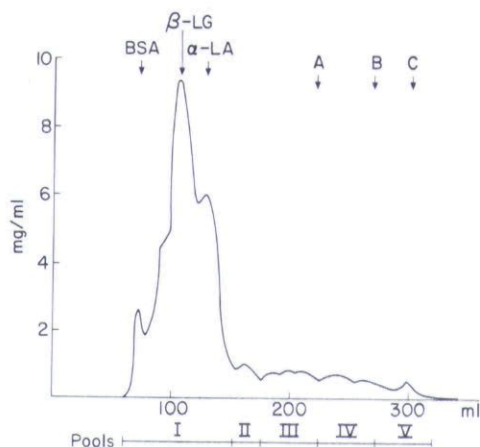


Fig. 1. Gel filtration of hydrolysed whey protein on Biogel P-30. Samples of hydrolysed whey protein (5.0 ml) were separated on Biogel P-30. The eluted proteins and peptides were pooled as indicated. Molecular weight markers were bovine serum albumin (BSA), β -lactoglobulin (β -LG), α -lactalbumin (α -LA), trypsin inhibitor (A), insulin chain B (B) and insulin chain A (C).

Following a period of 1, 3 or 24 hr after administration of antibody, samples of 250 μ l of the antigen–Evans blue solutions were injected intravenously in the tail vein. The blue skin reactions of the surface were recorded 30 min after the injection, at which time the mice were killed by dislocation of the neck and the skin removed to observe the colour reaction on the inside (corium) of the skin.

Results

The PCA reaction

Freund's incomplete adjuvant (FIA) was mixed with the antibody preparation (1:1) to obtain a (100 μ l) localized intradermal deposit of antibody and to minimize migration of antibody. The use of FIA resulted in a well-defined local colour reaction, whereas

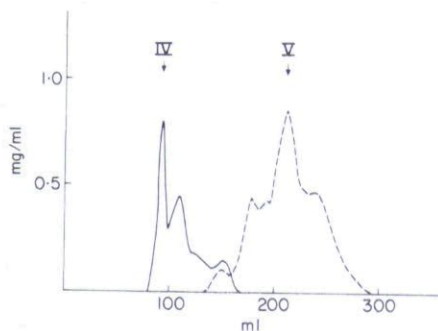


Fig. 2. Rechromatography of pool IV and pool V on Biogel P-6. Aliquots (5.0 ml) of pool IV and pool V were separated on Biogel P-6. The arrows indicate the major peak of pool IV (> 6000 Da) and pool V (2300 Da), respectively.

Table 1. Sensitivity of the PCA test using rabbit or murine antibodies

Antibody preparation	Type of mice		
	Group A	Group B	Group C
Rabbit	2.0 μg	10 μg	40 μg
Mouse	0.1 μg	2.0 μg	10 μg

The values represent the lowest concentration of whey protein per animal that could induce a positive PCA reaction.

antiserum mixed with saline (1:1) gave a more diffuse, less intensive blue colouration of the entire backside once the dose of antigen was above the detection limit. Three latent periods, i.e. the time between intradermal administration of antiserum and intravenous administration of antigen-Evans blue solution, of 1, 3 and 24 hr were compared, and a period of 3 hr was found to be optimal when using FIA.

A dose of 650 μg in 100 μl of rabbit anti bovine whey antibodies mixed with FIA was necessary to obtain a strong sensitization of the mice. Smaller amounts (65 μg and 6.5 μg) were found to be insufficient to mediate a PCA reaction, even with an intravenous administration of 160 μg whey proteins in 250 μl 1% Evans blue solution.

The PCA reaction period, i.e. the time between administration of antigen-Evans blue solution and appearance of blue zones of reaction on the back of the mice, was studied for 24 hr using rabbit antibody-FIA and 160 μg whey protein in 250 μl Evans blue solution. A reaction period of 15–30 min was found to be optimal. Long reaction periods (24 hr) gave diffuse coloration of the entire back of the animals and resulted in a tendency to produce coloration in the control animals as well. Similar results concerning the reaction period were obtained using whey protein peptides with molecular weights between 11 000 Da and 6500 Da (pool III, Table 2).

Sensitivity of the PCA method

The sensitivity of the PCA method was studied using the rabbit antibody preparation (650 μg /animal) or the murine antibody preparation (diluted 1:8). Furthermore, the sensitivity using the two antibody preparations was studied in the three different groups of mice. A dilution series ranging from 0.04 $\mu\text{g}/\text{ml}$ to 400 $\mu\text{g}/\text{ml}$ of whey protein in Evans blue solution was prepared. At each dilution step three mice of each group passively sensitized with rabbit or mice antiserum were challenged intravenously with 250 μl antigen-Evans blue solution. The sensitivity results are presented in Table 1. At low concentrations of antigen (i.e. close to the detection limit) the reaction could be seen on the inside of the skin only. At higher concentrations of antigen the PCA reactions were also visible on the surface of the skin as dark blue circles.

The highest sensitivity was obtained when using the murine antibody preparation (mixed with FIA) on the inbred BALB/c Bom fed the diet without milk proteins. This choice of antibody preparation and type of mice was used throughout the following experiments.

Table 2. Molecular weight range of peptides in pools I-V

Pool	Molecular weight
I	> 14 000
II	14 000-11 000
III	11 000-6500
IV	6500-3400
V	< 3400

No PCA reaction was seen when group A mice received undiluted murine antibody preparation that had been subjected to 56°C for 1 hr prior to intradermal injection.

PCA-test for allergenicity of hydrolysed bovine whey proteins and peptides

Peptides from enzymatically hydrolysed whey proteins were partially separated by gel filtration (Fig. 1). Pool I contained the unhydrolysed whey proteins, whereas pools II-V contained peptides of decreasing molecular size (Table 2). The values of Table 2 should merely be considered as the molecular weight range of the majority of the peptides in the different pools, since there was a certain extent of overlapping (Fig. 2).

Sixty-six mice of group A were passively sensitized by intradermal injection with 100 μ l undiluted murine antibody:adjuvant (1:1), whereas nine control mice were given 100 μ l saline:adjuvant intradermally. CWP, CWP-hydrolysate and peptide pools I-V were diluted in Evans blue solution to give samples with a protein content of 100 μ g/ml, 400 μ g/ml and 1600 μ g/ml, respectively. Three hours after the antibody injection the mice were challenged with 250 μ l antigen-Evans blue solution. At each antigen dilution three mice were challenged intravenously. The control mice were given 250 μ l CWP-Evans blue solution. After 30 min the reaction on the surface of the skin was recorded. If no reaction was observed the mouse was killed and the skin inverted in order to inspect the inside of the skin. The results are shown in Table 3. Reduced allergenicity was observed of CWP hydrolysate and pool IV, and no allergenicity could be recorded of peptide pool V.

Discussion

A number of *in-vitro* methods have been developed to examine the potential allergenicity of various compounds. The PCA method, however, has several advantages and is not replacable by one single *in-vitro* assay, because these assays are designed to monitor isolated events, e.g. antigen-IgE binding, not taking other physiological events in the complex allergic reaction into account.

This tends to result in false negative results from *in-vitro* assays, but false positives are also seen. O'Donnell and Mitchell [11] found that the body fluid of *Ascaris lumbricoides* contained a number of allergens using a radio-allergo-sorbent test and serum from infected rats, but when they used a PCA assay it was found that only one of these 'allergens' induced the degranulation of mast cells, which is a key element in the allergic reaction.

In the present study, the CWP hydrolysate was able to induce a PCA reaction in mice, indicating that the hydrolysate contained peptides or proteins of sufficient size to

induce a histamine release of the mast cells. Electrophoretic studies employing SDS-PAGE revealed that all whey protein components were hydrolysed by Alcalase, but also that large peptides (molecular weight > 14 000 Da) persisted throughout the period of hydrolysis. Less than 5% of the peptides of the CWP hydrolysate turned out to be larger than 6000 Da when analysed by gel filtration on Biogel P-6 (results not shown), explaining the weak PCA reaction induced by this preparation.

This result, and the results on pool III, are in agreement with Otani [12], who found that only peptides larger than 10 000 Da, produced by proteolytic degradation of β -lactoglobulin, induced histamine release in passively sensitized guinea-pigs. Pool IV, containing peptides in the range between 6500 Da and 3400 Da, induced weak PCA reactions in mice. This result is in disagreement with Otani [12], but confirms the observations of Lui *et al.* [4], who showed positive PCA reactions in guinea-pigs upon challenge with a 7200 Da peptide produced by hydrolysis of bovine serum albumin. As shown in Fig. 2, the majority of peptides in pool IV were larger than 6000 Da. Pool V (< 3400 Da) could not induce any PCA reactions in mice, indicating that the peptides had less than two epitopes per molecule [3].

In the present experiments the PCA reaction was used to compare titres of antigens, and consequently large amounts of antibodies were administered to ensure maximal sensitivity [3]. When using such large amounts of antibody preparations it turned out to be advantageous to administer the antibody intradermally in an emulsion with Freund's incomplete adjuvant, which resulted in smaller, more intense colour reactions than pure antiserum due to a reduced migration of antigen from the site of application. Administration of these large amounts of antibodies without FIA resulted in very large, diffuse reaction zones. Since it was difficult to administer all the large antibody preparations cutaneously, a part of the antibodies may have leaked out subcutaneously and activated mast cells in a large area of the skin.

A multitude of inbred mouse strains (which minimizes genetic variation) are available. In the present study, inbred BALB/c mice were chosen due to their relatively

Table 3. Allergenicity of hydrolyzed whey proteins and the resultant peptides separated by gel filtration

Intradermal injection	Intravenous injection antigen	PCA reaction (dose/animal)		
		25 μ g	100 μ g	400 μ g
Ab	CWP	*	*	*
Ab	CWP hydrolysate	None	†	†
Ab	Pool I	*	*	*
Ab	Pool II	*	*	*
Ab	Pool III	*	*	*
Ab	Pool IV	None	†	*
Ab	Pool V	None	None	None
Ab	Saline	None	None	None
Saline	CWP	None	None	None

Skin reaction: * deep blue coloured zone larger than 10 mm; † blue coloured zone smaller than 3 mm easily visualized on the inside of the skin.

high susceptibility to passive sensitization [13, 14]. Inbred BALB/c mice fed milk-free pellets (Table 1, group A) showed a higher responsiveness than inbred BALB/c mice fed pellets containing milk proteins (group B). It is well known that mice are immunized by the oral route [15, 16], and circulating antibodies against whey proteins in these mice form complexes with the intravenously injected whey proteins, thereby reducing the concentration of whey proteins available for induction of a PCA-reaction. The outbred mice (Table 1, group C) showed a lower responsiveness compared with group B, probably indicating that the inherited high responsiveness of inbred BALB/c mice [14] was lost. In all three groups of mice a higher sensitivity was observed using the murine antibody preparation as compared with the rabbit antibodies. The murine antibody preparation was prepared according to Jarrett *et al.* [8] for high IgE production. The lack of a PCA reaction following heat treatment (56°C) of the antibody preparation administered verifies that the PCA reactions relied on the presence of IgE antibodies. By contrast, the rabbits were immunized with whey proteins in order to produce a high titre of precipitating antibodies following a standard scheme. The differences in the immunization procedure and the species difference explain the observed differences in sensitivity using the two different antibody preparations. At best, the PCA reaction could be induced in group A mice sensitized with murine antibodies by intravenous challenge with 0.1 µg whey protein. This sensitivity was similar to the sensitivity reported in guinea-pigs [3].

The general agreement between the present results obtained on mice and the quoted results obtained on guinea-pigs indicates that mice should be considered to be of general interest for PCA tests applied to the assessment of allergenicity of proteins and peptides.

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