

In vitro and *in vivo* responses to the recombinant bovine dander allergen Bos d 2 and its fragments

P. RUOPPI*, T. VIRTANEN†, T. ZEILER†, M. RYTKÖNEN-NISSINEN†, J. RAUTIAINEN†, J. NUUTINEN* and A. TAIVAINEN‡

*Department of Otorhinolaryngology, Kuopio University Hospital, Kuopio, Finland, †Department of Clinical Microbiology, University of Kuopio, Finland, and ‡Department of Pulmonary Diseases, Kuopio University Hospital, Kuopio, Finland

Summary

Background About one in every four cases of occupational rhinitis recorded in Finland is animal-induced. Bovine allergens are the most important in this respect and the largest patient group consists of dairy farmers. Allergen immunotherapy, if proven effective, safe and feasible, would be ideal for their treatment. The development of recombinant allergens has offered new potential therapeutic prospects. Fragments of recombinant *Bos domesticus* (Bos d 2) allergen could be suitable for this purpose because they are recognized by T cells but their IgE-binding capacity is attenuated.

Objective The aim of this study was to verify whether the potential of the two fragments of recombinant Bos d 2 (corresponding to amino acids 1–131 and 81–172) to induce immediate allergic reaction in a shock organ (nose) was decreased compared to the complete recombinant allergen, which would be an advantageous property for a preparation intended for allergen immunotherapy.

Methods The study group consisted of 10 dairy farmers with cow-induced allergic rhinitis. We used the IgE titres against native Bos d 2 measured by indirect IgE ELISA to characterize the level of sensitization and compared the IgE titres in the rhinitis patients with 12 cow-sensitized asthmatic farmers and 12 healthy students. *In vitro* reactivity against recombinant Bos d 2 and its two fragments was studied by indirect IgE ELISA and *in vivo* reactivity by nasal provocation tests.

Results The IgE titres against native Bos d 2 of patients with rhinitis tended to be lower than the titres of asthmatics. The healthy students did not exhibit any detectable IgE reactivity to native Bos d 2. In the patients with rhinitis, there was no statistically significant difference between IgE responses against native and recombinant Bos d 2, whereas with both *in vitro* and *in vivo*, the reactivity to both fragments of recombinant Bos d 2 was lower than the reactivity to the complete recombinant allergen.

Conclusions Due to the decreased *in vivo* capacity to induce immediate allergic reactions, the fragments may be better tolerated in allergen immunotherapy than the complete allergen.

Keywords: allergic rhinitis, bovine, cow allergy, occupational, nasal provocation recombinant allergen

Clinical and Experimental Allergy, Vol. 31, pp. 915–919. Submitted 17 November 1999; revised 22 April 2000; accepted 2000.

Introduction

Animal allergy has been one of the most important and frequent causes of occupational rhinitis in Finland during

Correspondence: Dr Pirkko Ruoppi, Department of Otorhinolaryngology, Kuopio University Hospital, P.O. Box 1777, FIN-70211 Kuopio, Finland

recent years, being responsible for one in every four of the total 283 cases in 1998, the latest year for which official records are available [1]. At the present time, the treatment in most cases consists of anti-allergic medication and minimizing the exposure to allergens by means of good ventilation of the cow barn and the use of a personal breathing protector. It is not easy for farmers to change their occupation or even working conditions and therefore allergen immunotherapy could represent a potential treatment for cow allergy, if it could be shown to be effective, safe and convenient.

Allergen immunotherapy with conventional commercial preparations is known to have a risk of side-effects due to immediate allergic reactions. Therefore, a reduction of the IgE-binding capacity of the preparations would be advantageous. Bos domesticus allergen 2 (Bos d 2) has recently been cloned and produced as a recombinant protein [2, 3]. This preparation is produced in *E. coli* as a fusion protein with glutathione-S-transferase (GST) [2]. It has been shown previously *in vitro* that the two overlapping fragments of recombinant Bos d 2 (rBos d 2), corresponding to amino acids 1–131 and 81–172, have a good capacity to induce T cell proliferation [3]. In comparison with the complete recombinant allergen, they have a decreased capacity to bind IgE in the indirect enzyme-linked immunosorbent assay (ELISA) and ELISA inhibition tests. They also induce weaker skin prick test (SPT) reactions than the complete allergen. According to current knowledge, in allergen immunotherapy the immunomodulatory effects are specifically directed to T cells [4]. Due to their lower allergenicity, the fragments of rBos d 2 could be more tolerable and thus safer in immunotherapy than conventional native preparations. Since the IgE-binding capacity does not sufficiently reflect the biological activity of an allergen, it should be verified always in *in vivo* tests [5]. In this study, rBos d 2 preparations were for the first time tested in the shock organ, i.e. used in a nasal provocation test (NPT). NPT is a straightforward and reliable *in vivo* test which links SPT results to clinical symptoms. In Finland, NPT is routinely used in clinical practice for confirming the diagnosis of occupational allergic rhinitis. It is also usually carried out before any decision is made to initiate allergen immunotherapy for rhinitis.

Materials and methods

Volunteering dairy farmers who were healthy apart from being previously diagnosed with cow dust-induced allergic rhinitis were recruited to the study. According to routine clinical practice, the diagnosis of cow allergy was based on a positive history of occupational symptoms as well as positive SPT and positive NPT performed with commercial

cow dander solution from ALK (Allergologisk Laboratorium A/S, Hørsholm, Denmark). SPT was performed and interpreted according to the recommendations of European Academy of Allergology and Clinical Immunology [6]. Histamine hydrochloride (10 mg/mL) and allergen diluent were used as positive and negative controls respectively. A weal diameter of at least 3 mm and half of the histamine control was considered positive. In NPT, the patients were at first challenged with the negative control, which was the diluent of the allergen solutions, to avoid misinterpretation of possible non-mast cell related nasal reactions. A volume of 0.05 mL of the test fluid was sprayed towards the inferior and middle turbinate of one nasal cavity at a time with a 1-mL syringe filled with air and equipped with a thin needle while the patient was holding the breath. If no placebo reaction occurred, the nasal challenge test was performed on the other nasal cavity with allergen solution. In cow challenge, Aquagen SQ (ALK) in a concentration of 100 000 SQ-units/mL was mostly used but if, on the grounds of history or SPT result, a very high degree of sensitization was suspected, the NPT was started with a hundred- or ten-fold dilution. Nasal reactions were observed during the following 30 min and recorded in rhinoscopy by an otorhinolaryngologist in the study group. The interpretation of the NPT was based on rhinoscopy findings, which were hypersecretion and mucosal oedema, and on subjective symptoms (sneezing, nasal itching and obstruction). The test was considered positive if hypersecretion and at least one of the other two signs and symptoms were recorded [7].

For inclusion, the otorhinolaryngologist in the study group initially examined the patients. Infection rhinitis, ongoing medications (except contraceptive pills), pregnancy and lactation were exclusion criteria. For the person to be finally included, the NPT result with allergen diluent (negative control) and GST fusion protein had to be negative, but positive with native bovine allergen (nBos d 2). From 15 candidates, 10 farmers fulfilled the criteria. Five were excluded, due to a negative NPT with nBos d 2 in 4, while 1 was positive with GST. There were 5 women and 5 men, aged from 27 to 51, mean $38 \pm$ SD 8.4 years.

The group of patients with cow-induced asthma consisted of 12 patients, 5 women and 7 men. The age ranged from 23 to 61 years, mean $44 \pm$ SD 13.8 years. They all suffered from occupational symptoms and work related airway obstruction was verified by recording of peak expiratory flow (PEF). The bovine origin of the asthma was confirmed at the Department of Pulmonary Diseases of Kuopio University Hospital according to the criteria of American Thoracic Society [8]. For a person to be classified as being asthmatic to cow, the SPT (ALK) and/or radioallergosorbent test (RAST, Pharmacia &

Upjohn, Stockholm, Sweden) and inhalation test, using cow epithelial antigen (ALK) had to be positive with a RAST score of 2 or higher (scale 0–4) being considered positive. The criterion for positive inhalation test (automatic dosimeter nebulization, Spira Elektro 2, Hämeenlinna, Finland [9,10] was a decrease of 15% or more in forced expiratory volume in 1 second (FEV₁) values.

The third group comprised 12 healthy medical students, aged 21–28, mean $23 \pm \text{SD } 2.2$ years. They had no allergic symptoms verified by a questionnaire. The crucial questions were if they suffered now or had earlier suffered from atopic disorders (rhinitis, dermatitis or asthma). An atopic constitution, even if only suspected, was an exclusion criterion.

nBos d 2 was purified from commercial raw material with affinity chromatography and gel filtration as described previously [11]. The productions of rBos d 2 and its two overlapping fragments rBos d 2 (1–131) and rBos d 2 (81–172) as GST fusion proteins has been described elsewhere [3]. In nasal provocation tests, 10-fold dilutions of nBos d 2 (0.1–100 µg/mL) or rBos d 2, its two fragments (1–131 and 81–172), or GST (0.2–200 µg/mL) were used. To guarantee the stability of aqueous test solutions, they were kept frozen until the NPT was performed.

At the first visit, the farmers with rhinitis were tested for final inclusion by a unilateral challenge, one at a time, to the control solutions and to nBos d 2. The controls were the diluent of the allergen solutions (0.15 M NaCl/0.01 M bicarbonate buffer, pH 9.2) and a fusion part of the allergens (GST). On the following three visits (at least one week between visits), the 10 farmers with rhinitis were challenged to the complete rBos d 2 and its two fragments (1–131 and 81–172), in concentrations from 0.2 to 200 µg/mL or until a nasal reaction occurred. The confusing effect of possible nasomaxillary reflexes was avoided by inducing only one positive response during each visit. Technically the NPT was performed and interpreted in the same way as described above in this part and it was single-blind with the patients being blinded. Blood samples were taken for indirect IgE ELISA tests from patients with rhinitis and asthma as well as from healthy controls. A blood sample from one patient was not obtained. The levels of specific IgE antibodies against nBos d 2, rBos d 2, fragments and GST were measured with ELISA, the technique having been described in detail previously [11,12]. Beginning from the dilution 1 : 10, serum samples were tested at several dilutions in duplicate. Reagent blank absorbance values were subtracted from the results. The final results were then estimated as titres from the dilution curve, with the absorbance of 0.3 as a cut-off.

The overall difference between the three patient groups was tested by Kruskal Wallis test. The pairwise comparisons

between the groups were made by Mann–Whitney *U*-test with Bonferroni correction. Wilcoxon Signed Rank test and McNemar test were used in other statistical analyses of the results. The Ethics Committee of the University of Kuopio and Kuopio University Hospital approved the study design. All patients provided informed consent before entry into the study.

Results

There was an overall difference of the means of IgE titres against nBos d 2 (indirect ELISA) between the three patient groups (Kruskal Wallis; $P = 0.0002$). In dairy farmers with cow-induced allergic rhinitis, titres were lower than the corresponding values in the group of cow-asthmatic farmers but the difference was not statistically significant (Mann–Whitney *U*-test with Bonferroni correction; $P = 0.33$) (Fig. 1). In the control group of healthy students, there were no measurable antibody titres against nBos d 2 and the difference was significant with respect to farmers with asthma ($P < 0.001$) but not significant with respect to farmers with rhinitis ($P = 0.09$).

In the farmers with rhinitis, there was virtually no statistically significant difference between the nBos d 2- and rBos d 2-specific IgE-titres (Table 1). With respect to the IgE-binding capacity, the two fragments of rBos d 2 had a tendency to a lower IgE binding than the corresponding values with complete rBos d 2. The differences were not, however, statistically significant (Wilcoxon Signed Rank test, $P = 0.08$). In two patients, there was also some reactivity against the GST fusion part.

In NPT, there was one dairy farmer with cow-induced rhinitis who did not react at all to the recombinant allergens, and three farmers who suffered rhinitis only from complete rBos d 2 but not from either of the fragments (Table 2). In all, positive NPT reactions to different cow allergens were recorded as follows: to nBos d 2 in 100%, to rBos d 2 in 90%, to fragment 81–172 of rBos d 2 in 50%, and to fragment 1–131 of rBos d 2 in 30% of challenged farmers. The *in vivo* reactivity of fragment 1–131 of rBos d 2 was significantly lower than the reactivity of nBos d 2 (McNemar test, $P < 0.01$) and complete rBos d 2 ($P = 0.02$). There was also a tendency to attenuated *in vivo* reactivity of fragment 81–172 of rBos d 2 with respect to nBos d 2 ($P = 0.03$) and complete rBos d 2 ($P = 0.06$). In NPT, neither immediate side-effects were experienced nor late reactions reported.

Discussion

We characterized the overall sensitization level of farmers with cow-allergy manifested in rhinitis by determining IgE antibodies against nBos d 2 and comparing the results with

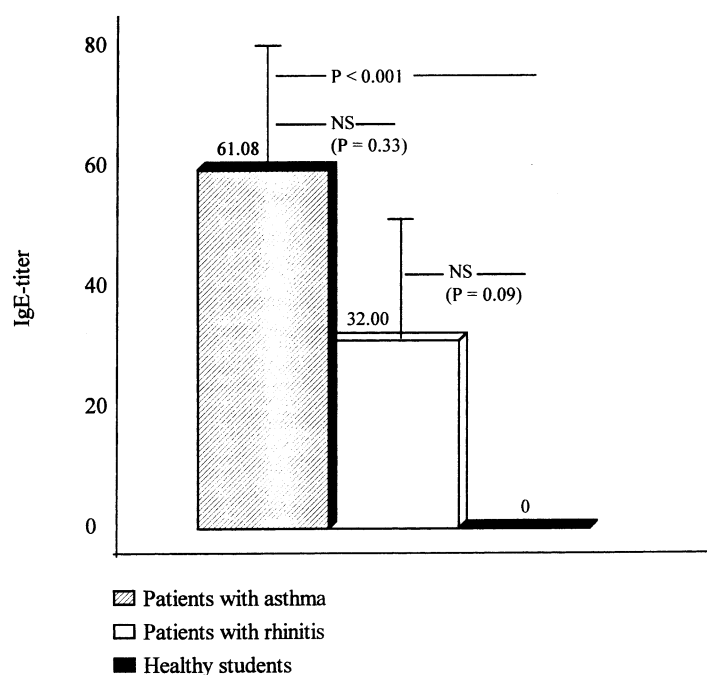


Fig. 1 IgE-titres determined by indirect ELISA (mean $\pm$ SE) against nBos d 2 in patients with cow-induced asthma or allergic rhinitis and healthy controls.

negative (healthy medical students) and positive (cow-allergic asthmatics) control groups. None of the 12 healthy students had notable levels of IgE antibodies against nBos d 2. Furthermore, the dairy farmers with rhinitis had clearly lower IgE levels against the native bovine allergen than the cow-allergic asthmatics. These results are in agreement with the clinical experience [13]. Quantitative antibody formation correlates directly with the area of the shock organ. In the statistical analysis, all titres lower than

10 were interpreted as 0, which may in some cases modify the individual results but would be unlikely to distort the overall tendencies.

When the results on the group of dairy farmers with cow-allergic rhinitis were examined, we observed no statistically significant difference between *in vitro* immune reactivity to complete nBos d 2 and rBos d 2. In a previous study, the IgE binding capacity of rBos d 2 was lower than

Table 1 IgE-titres (indirect ELISA) of 9 rhinitis patients to different cow antigen preparations and GST

Patient number	IgE titres				GST
	nBos d 2	rBos d 2	rBos d 2 (1–131)	rBos d 2 (81–172)	
1	0	107	0	0	0
2	60	0	0	0	0
3	0	18	0	0	0
4	0	0	0	0	0
5	31	53	0	15	0
6	0	0	0	0	17
7	0	0	19	19	29
8	39	91	0	0	0
10	0	19	0	0	0

0 = < 10 in ELISA.

The serum sample of patient 9 was not available.

Table 2 Allergen dilutions, which caused a positive reaction in a nasal provocation test

Patient number	Allergen dilutions			
	nBos d 2	rBos d 2	rBos d 2 (1–131)	rBos d 2 (81–172)
1	1	–	–	–
2	1 : 10	1 : 10	1 : 100	–
3	1	1	1	1 : 10
4	1 : 10	1	–	–
5	1	1	–	1
6	1 : 1000	1 : 1000	–	1 : 100
7	1 : 10	1	–	1
8	1	1	–	–
9	1 : 10	1 : 100	1	1
10	1	1	–	–

1 = undiluted allergen solution.

– = no reaction to any concentration.

that of nBos d 2 [3]. Similar data has been published for other recombinant allergens [14,15]. This difference between studies may be related to the difference in the levels of sensitization between patient groups; in the former study, the sera of asthmatic patients were used whereas in this study, the sera were from patients with allergic rhinitis. As shown also in this study, asthmatic patients had higher levels of native Bos d 2-specific IgE in their serum than the patients with rhinitis. *In vitro*, we observed a tendency for lower IgE binding capacity of both fragments of rBos d 2, when compared with nBos d 2 or the complete rBos d 2.

Two patients had low levels of GST-specific IgE. One explanation for the positive *in vitro* finding may be that insect-derived GST can be allergenic and that these two patients had cross-reacting antibodies in their sera [16,17]. On the other hand, it is possible to produce allergens without the fusion portion [18] and so the problem can be avoided in the future. These patients were not excluded from the study because the NPT result with GST was negative.

The *in vivo* capacity to induce an immediate allergic reaction was evaluated. Complete rBos d 2 induced rhinitis in 90% of NPTs in the patients, whereas the NPT result with nBos d 2 was positive in all patients. Therefore the complete rBos d 2 does not seem to offer any real advantage over nBos d 2 in allergen immunotherapy. The most interesting finding in this study was that in NPT, the lower reactivity of the fragments compared to the complete rBos d 2 was expressed very clearly. Since the fragments have been shown to be potent stimulators of specific T cell clones, they seem to be suitable for allergen immunotherapy. However, further studies are needed for establishing their feasibility for this purpose.

Acknowledgement

Financial support from the Kuopio University Hospital (Project no. 5035) is gratefully acknowledged.

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