

IgG and IgE antibody responses to cow dander and urine in farmers with cow-induced asthma

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Summary

Cow-asthmatic farmers' and negative control subjects' IgG and IgE antibody responses to bovine epithelial antigen (BEA) and urinary antigen (BUA) were studied by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The anti-BEA IgE responses of 10 highly reactive sera were also studied by crossed radioimmuno-electrophoresis (CRIE). The relative amount of allergens common to both BEA and BUA was measured by IgE ELISA inhibition and found to be 3%. In immunoblotting the IgG reactivity of the asthmatic farmers to BEA and BUA declined along their anti-BEA IgE ELISA titres. Control subjects had IgG antibodies mainly to high molecular weight components (50–70 kD) but lacked detectable IgE responses. The IgE reactivity of the asthmatic farmers was directed to only a few components. A total of two main allergens were found in cow dander (20 and 22 kD) and one in cow urine (20 kD). The 20 kD component was shown to be the most important allergen in cow antigen extracts. In CRIE, seven reactive arcs were detected. Arcs 1, 2 and 5 were detected by all 10 sera and arc 3 by six and arc 7 by seven sera.

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Introduction

Cow epithelium is one of the most important inducers of occupational asthma in Finland [1]. We have recently shown that a protein component of 20 kD is a major allergen of both cow dander and urine [2]. Farmers with allergic rhinitis of bovine origin had IgE and IgG antibodies to this allergen. Control subjects did not usually show any IgE reactivity to this 20 kD protein, and their IgG responses were considerably weaker than those of allergic persons. According to previous studies of Prahl *et al.* [3,4] cow hair and dander contain four major allergens detectable by crossed radioimmuno-electrophoresis (CRIE), with molecular weights of 20, 22, 24 and 200 kD. However, by sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with immunoblotting, we found only one major allergen in cow dander and urine. We have now expanded our analyses to a larger group of allergic dairy farmers suffering from cow-derived asthma instead of rhinitis. It was assumed that more allergens might possibly be found with these more reactive sera. Some of the sera of cow-

asthmatic farmers were also studied by CRIE to determine the influence of different methods on the results. In addition the relative amount of common allergens in cow dander and urine was determined.

Materials and methods

Antigen preparation

Bovine epithelial antigen (BEA) was prepared essentially as described by Virtanen *et al.* [5]. The freeze-dried raw material was purchased from Allergon AB (Ängelholm, Sweden). The material was defatted in diethyl ether (1:10 w/v) at 4°C for 24 hr, dried at room temperature and extracted for 24 hr at room temperature in Coca's solution, pH 7.2 (1:10 w/v; 0.5% sodium chloride, 0.275% sodium hydrogen carbonate, 0.4% phenol). After centrifugation at 36 600 g for 30 min, the supernatant was dialysed (MW cutoff 3500) for 72 hr at 4°C in a vast excess of 0.05 M ammonium hydrogen carbonate (pH 7.8), lyophilized and stored at –20°C.

Bovine urinary antigen (BUA) was prepared by freeze-drying the urine and suspending the dry powder in ion-exchanged water (1:5 w/v) as described previously [5].

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The solution was then handled as BEA, beginning from the step of centrifugation.

The protein concentrations of the antigen preparations were measured according to the method of Lowry *et al.* [6] using bovine serum albumin as a standard.

Rabbit antiserum

Two rabbits were immunized with BEA every 2 weeks for several months, each time receiving 2.0 mg of protein/animal. The antigen was emulsified with Freund's incomplete adjuvant and injected subcutaneously. Blood was drawn several times, and the antisera were stored at -70°C . Before use, the antibody titres were measured by ELISA (data not shown) and the high-titred antisera were pooled so that the final pool contained equal amounts (ml) of the two rabbits' sera. The pooled sera was stored in aliquots at -70°C .

Patient sera

This study included 49 currently working dairy farmers with clinically diagnosed asthma of bovine origin. All were patients of the Pulmonary Clinic of Kuopio University Hospital. Their asthma was confirmed by the skin prick test using a panel of 19 common environmental allergens including epithelial allergen preparation of cow (Allergologisk Laboratorium (ALK), Denmark), inhalation test using epithelial antigen from cow (ALK; De Villbiss-nebulizer) and occasionally by the RAST panel (Pharmacia). For a person to be classified as being asthmatic to materials of bovine origin the skin prick test and/or RAST and inhalation test had to be positive. The results of the tests were considered positive as follows: the skin prick test result with cow antigen 3+ or higher, 3+ being the histamine control equivalent RAST score of 2 or higher (scale 0–4) according to the Finnish standards and the inhalation test a decrease of 20% in PEF or FEV1 values. The negative controls were 16 patients from the same clinic who had various pulmonary symptoms, but none indicating cow-allergy. This control group included five dairy farmers who were all negative in the inhalation, skin prick and RAST tests with cow allergen. Also included were 36 dental students from Kuopio University. Sera from 10 of these students were randomly selected for the immunoblotting studies.

IgE measurements

A microELISA plate (NUNC-Immuno Plate I, Denmark) was coated for 2 hr at 37°C with BEA at a concentration

of 5 $\mu\text{g}/\text{ml}$ of protein in a coating buffer (0.05 M carbonate buffer, pH 9.6) in volumes of 100 μl /well. Next, the plate was washed and incubated for 2 hr at 37°C with a diluent (phosphate-buffered saline [PBS] containing 0.5% [v/v] Nonidet P40 [BDH Chemicals Ltd., U.K.], 0.1% [w/v] casein [BDH Chemicals Ltd., U.K.] and 0.02% [w/v] thiomersal). After incubation, the plate was stored at -70°C . Before use, the plate was allowed to reach room temperature and was then washed. Next, serum samples at a dilution of 1:5 in 100 μl volumes were added in duplicate to the plate. The plate was then incubated at 37°C for 2 hr. After washing and incubation with diluent for 0.5 hr at 37°C , goat anti-human IgE (Cappel, U.S.A.; 0201-0061) at a dilution of 1:500 was added; and the plate was incubated for 2 hr at 37°C . After washing, biotinylated rabbit anti-goat IgG (Cappel, U.S.A.; 8606-3712) was added at a dilution of 1:2000. After incubation for 1 hr at 37°C and washing, Vectastain ABC Standard Kit (Vector Laboratories, U.S.A.; PK-4000) reagents (avidin DH and horseradish peroxidase H) were added at a dilution of 1:500 and left at room temperature for 0.5 hr. After a further wash, the substrate solution (tetramethylbenzidine, 0.1 mg/ml; Merck, Germany) and urea hydrogen peroxide (BDH Chemicals Ltd, U.K.), corresponding to 0.05% H_2O_2 in 0.1 M sodium acetate buffer (pH 6.0), were added. The reaction was stopped (2 M H_2SO_4), and the absorbance was measured at 450 nm using a Titertek Multiskan spectrophotometer (Flow Laboratories, Finland).

Variation of the results between plates was corrected as follows: a coefficient for the plate was calculated by dividing the mean absorbance of the positive control wells of all the plates by the respective mean of an individual plate (four wells/plate). The results for the plate were then multiplied by this coefficient, and the corrected absorbances were considered to be the antibody titres.

IgE ELISA inhibition for the analysis of common allergens

For the IgE ELISA inhibition, a microELISA plate was coated with BEA as described above for the measurement of IgE antibodies, except that the coating concentration of BEA was 10 $\mu\text{g}/\text{ml}$. For the comparison of the extracts, several dilutions of BEA and BUA in 50 μl volumes were added in duplicate to the plate. Human anti-BEA antiserum (a pool consisting of sera containing a high level of cow-reactive IgE antibodies, as determined by IgE ELISA) was added immediately at a dilution of 1:5, 50 μl /well. The plate was then incubated at 37°C for 2 hr. After washing and incubation with diluent, the procedure was completed as described above for the measurement of IgE antibodies, starting with the step of adding goat anti-human IgE (Cappel).

For the comparison of the amount of allergens common to both BEA and BUA extracts, standard curves of absorbance against the log concentrations of the extracts were plotted. At the level of 50% inhibition of maximal absorbance achieved in wells containing no inhibitor, the amounts of BEA and BUA were quantitatively determined.

SDS-PAGE

Sodium-dodecyl-sulphate-polycrylamide gel electrophoresis (SDS-PAGE) was carried out in a 12.5% acrylamide gel using the discontinuous buffer system of Laemmli [7]. Briefly, freeze-dried antigens were dissolved in a sample buffer containing SDS and 2-mercaptoethanol and boiled for 90 sec. A sample containing 0.9 mg of protein was loaded onto each gel. After electrophoresis at 200 V, the separated proteins were transferred to nitrocellulose membrane (Millipore 0.45 μ m) using the method of Towbin *et al.* [8]. The proteins were transferred at 60 V for 1 hr 45 min.

Detection of IgG- and IgE-binding bands

After electrophoretic transfer, the nitrocellulose membrane was incubated for 1 hr at +37°C in Tween-PBS [9], pH 7.3 (0.020 M PBS, 0.115 M NaCl and 0.05 M Tween 20) containing 0.1% w/v casein (BDH) and 0.05% w/v sodium azide. All subsequent steps were carried out at room temperature. The membrane was cut into strips and incubated with patient serum at a dilution of 1:100 for the detection of IgG antibodies and at a dilution of 1:5 for the detection of IgE antibodies. All dilutions were made with the Tween-PBS buffer containing casein and sodium azide. After 6 hr incubation, the strips were washed four times for 10 min with Tween-PBS. For the detection of human IgG antibodies, the strips were first incubated overnight with rabbit anti-human IgG antiserum (Dako A423) diluted 1/200, washed and treated with ^{125} I-labelled anti-rabbit IgG (Amersham IM 143) for 6 hr. Human IgE reactivity was detected with ^{125} I-labelled anti-human IgE (Phadebas Rast, Pharmacia Diagnostics). All conjugates were diluted to contain radioactivity approximately 100 000 c.p.m./ml. Autoradiography was carried out at -70°C using Amersham Hyperfilm MP-film and a Kodak X-Omatic cassette with an intensifying screen.

Crossed immunoelectrophoresis (CIE)

CIE was performed in 1% (w/v) agarose in 0.025 M barbital buffer, pH 8.6, containing 0.03% NaN₃, accord-

ing to the method of Hoiby and Axelsen [10]. Antigen (30 μ l with 20 μ g protein) was loaded onto the first dimension gel and separated at +4°C for 90 min using constant voltage of 8 V/cm. A second dimension gel containing 20% anti-BEA antiserum was used, and the second electrophoresis was carried out at +4°C for 16–18 hr using 2 V/cm. After electrophoresis the gels were pressed four times for 5 min (filter paper—paper towel—glass plate—3 kg) and washed overnight in 0.9% NaCl and then for 2 hr with distilled water, and dried at +37°C.

Crossed radioimmuno-electrophoresis (CRIE)

The CIE gel was incubated with patient serum diluted 1/10 with 70 mM PBS for 3 hr according to the method of Weeke *et al.* [11]. After washing four times for 10 min with 0.15 M NaCl, the gel was further incubated overnight with ^{125} I-labelled antihuman IgE (Pharmacia). The conjugate was diluted with 0.05 M PBS, pH 7.0 containing 15 mM NaN₃ and 7.2 mM dinatrium-EDTA. After incubation, the gel was washed three times for 10 min with 0.15 M NaCl and once with distilled water, and air-dried. Radioactivity was detected as described in immunoblotting. After autoradiography the gels were stained for 10 min with Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and destained in the same solution.

Results

Protein concentrations of BEA and BUA

The protein concentrations of BEA and BUA were 9.20 and 2.23 mg protein/ml, respectively.

Determination of the amount of allergens common to both urine and epithelium

The amount of allergens common to both BEA and BUA was determined by IgE ELISA inhibition. At the level of 50% inhibition of the maximal absorbance achieved in wells containing no inhibitor, the amount of common allergens was 3% (Fig. 1).

IgE measurements

The levels of IgE antibodies to BEA in the different study groups are shown in Table 1. The level of anti-BEA IgE antibodies was statistically significantly higher in the group of farmers with cow-induced asthma than in the other two groups (2-tailed *t*-test; *P*-values both <0.001).

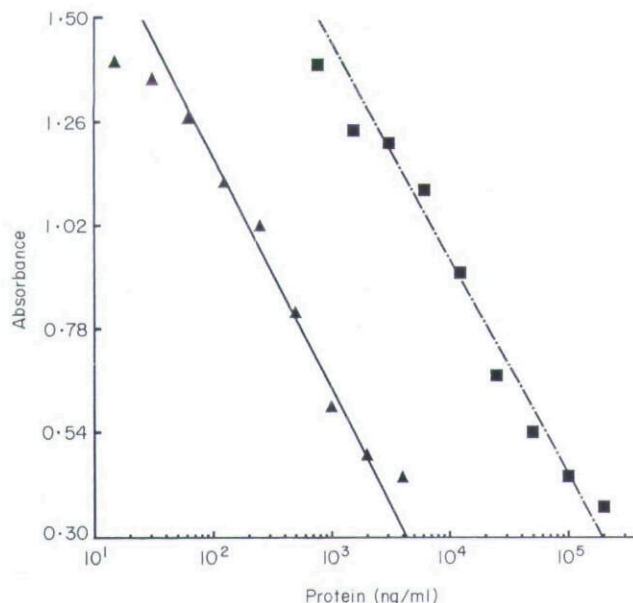


Fig. 1. The IgE-ELISA inhibition curves of BEA (triangle) and BUA (square). The cross-reactivity was determined at the level of 50% inhibition (optical density 0.73).

IgG immunoblotting results

All the immunoblotting strips were arranged within each group according to the subjects' anti-BEA IgE ELISA titre in a declining order. As determined visually, the strength of the anti-BEA IgG response seemed to decrease with the declining anti-BEA IgE titre in the group of asthmatic farmers (Fig. 2a). This phenomenon was also demonstrated in the reaction to the band at 20 kD. Responses to BUA were more heterogeneous because of the greater number of separate components. The anti-BUA IgG immunoblotting results for the first 18 farmers showing the highest level of anti-BEA IgE antibodies are shown in Fig. 3a. As is evident, several high molecular

weight bands between 50 and 80 kD were strong antigens. Occasionally, strong IgG responses were also seen to bands at 17 and 24 kD.

The non-asthmatic controls' anti-BEA and anti-BUA IgG immunoblotting results are shown in Fig. 4. Their IgG reactivity to BEA and BUA as well as that of the students was mainly directed against one or two bands around 60 kD. IgG responses to the lower molecular weight antigens of BEA were considerably weak or absent. Strong anti-BUA IgG responses were detected also to lower molecular weight components, especially to those at 17 and 24 kD.

IgE-immunoblotting results

In IgE immunoblotting, 30 of the 51 asthmatic farmers' sera reacted to BEA and 26 to BUA. The anti-BEA IgE results of all the cow-asthmatic farmers are presented in Fig. 2b, and a portion of the anti-BUA IgE results (patients 1–18) in Fig. 3b. The number of separate allergenic components detected in BEA was seven (16, 20, 22, 24, 30, 35 and 55 kD) and in BUA four (20, 22, 24 and 67 kD). The most important allergenic component in both of these extracts was at 20 kD. It was recognized by 97% of the IgE-immunoblot positive farmers' sera in BEA and by 88% in BUA. A component of 22 kD was also an important allergen in BEA and in BUA (67% and 58% reactivity, respectively) as well as a 24 kD component in BUA (63% reactivity). All of the subjects in the two control groups showed negative results in the anti-BEA and anti-BUA IgE immunoblotting analyses.

CIE and CRIE

In CIE, using anti-BEA antiserum, seven arcs were separated from BEA and five arcs from BUA (Fig. 5a, c). To determine which BEA arcs had allergenic activity, 10 strongly positive cow-asthmatic farmers' sera were randomly selected for CRIE analysis. To roughly estimate the amount of common allergens between BEA and BUA, the serum of one farmer (patient 14) was used in the anti-BUA CRIE analysis. All 10 sera detected arcs 1, 2 and 5 of BEA. Arc 4 was non-reactive, and arc 3 was detected by seven sera, arc 6 by four sera, arc 7 by 7 sera and arc 8 by two sera. Arc 8 was detected only in autoradiography. The cow-asthmatic farmer's serum (patient 14) that was also tested against BUA detected six arcs in the BEA gel (Fig. 5b) and two arcs in the BUA gel (Fig. 5d). As shown in Fig. 5b,d, BUA contains significantly less allergenic material than BEA. To compare the anti-BEA CRIE results to those of anti-BEA IgE immunoblots, the results of the 10 sera are presented schematically in Table 2. It seems that the most intense

Table 1. Level of IgE antibodies to BEA in ELISA

	Optical density $\pm$ s.d.	n
A	0.78 $\pm$ 0.30	49
B	0.34 $\pm$ 0.16	16
C	0.35 $\pm$ 0.19	38

A: patients with bovine asthma.

B: non-asthmatic farmers.

C: students.

Optical density at 450 nm.

Table 2. Results of BEA analyses made by CRIE and SDS-PAGE and immunoblotting

Patient‡	CRIE arc*								IgE immunoblot (kD)							
	1	2	3	4	5	6	7	8	55	35	30	24	22	20	16	
1	x	X	X		X		x		x		x	X	x	X		
2	x	X	x		x					x	x	x		X	x	
4	x	X	x		X		X	x					X	X		
5	x	x	x		x		x		x			x	x	x	X	
6	x	X			x									X	X	
13	x	X			X	x	X	x					X	X		
14	x	X	x		X	x	X						X	X	X	
23	X	X	x		x	x	X						X	X	x	
26	x	x			x	x	x						x	x	x	
32	x	X			x									X		

* CRIE arcs are numbered randomly. The most reactive arcs are indicated with a larger mark.

† Reactive bands are presented according to their molecular size. The most reactive bands are indicated with a larger mark.

‡ Patients are numbered according to their anti-BEA IgE titre so that the patient with the highest titre is number 1.

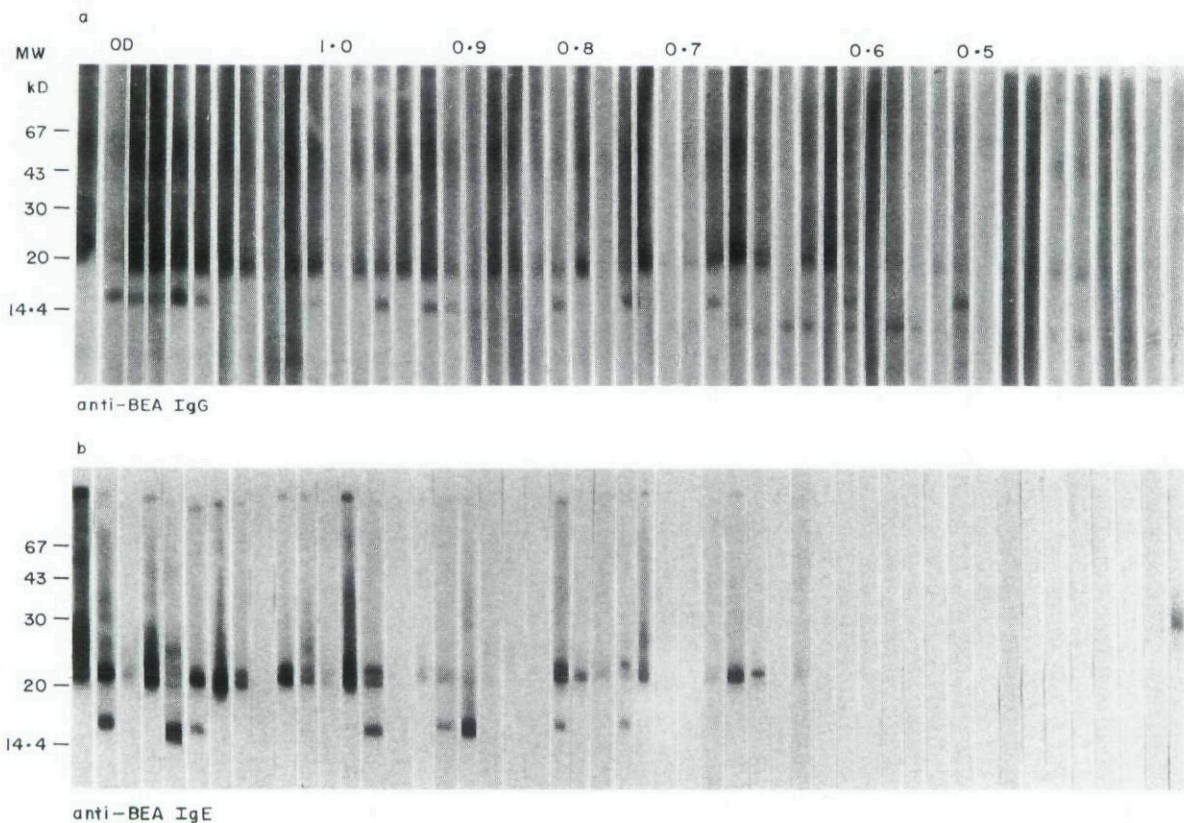


Fig. 2. The anti-BEA IgG (a) and IgE (b) immunoblotting results of the cow-asthmatic farmers. The strips are arranged from left to right according to the patients' anti-BEA IgE ELISA titres.

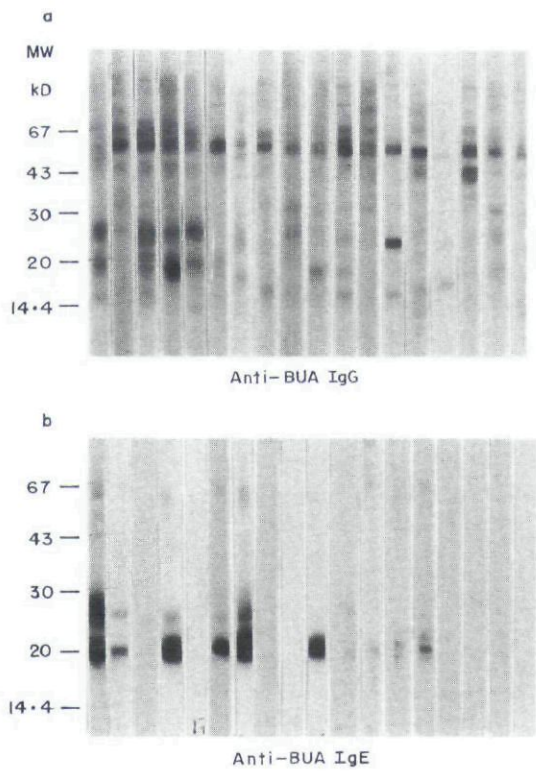


Fig. 3. The anti-BUA IgG (a) and IgE (b) immunoblotting results of cow-asthmatic farmers (1-18).

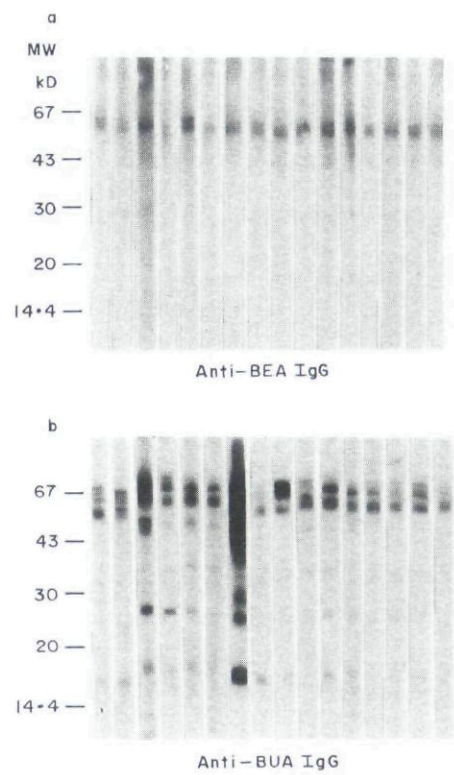


Fig. 4. The anti-BEA IgG and anti-BUA IgG immunoblotting results of the non-allergic controls. The strips are arranged from left to right according to their anti-BEA IgE ELISA results.

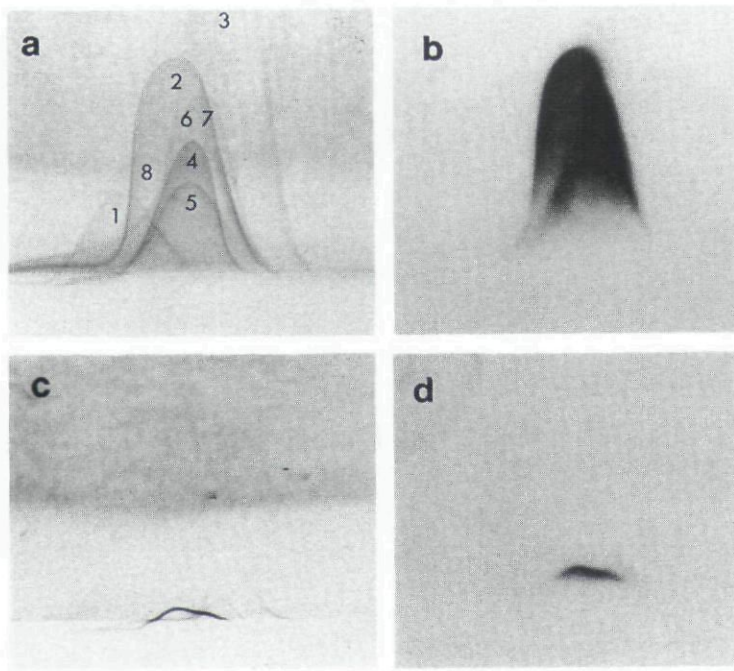


Fig. 5. CIE gels and their corresponding CRIE autoradiographs. (a) BEA/anti-BEA gel. The arcs are randomly numbered. (b) Corresponding CRIE (patient 14 in Table 2). (c) BUA/anti-BEA gel. (d) Corresponding CRIE (patient 14).

reactions are most frequently directed to the 20 kD component in immunoblotting and to arc 2 in CRIE.

Discussion

Previously, we have measured the amount of common antigenic components in BEA and BUA using the ELISA inhibition method [2]. The amount of common antigenic structures was found to be 1.9%. In this study, we measured common allergens by a modification of the ELISA inhibition technique using human sera containing a high level of IgE antibodies to BEA. The amount of allergenic components common to both BEA and BUA was shown to be 3%. An obvious explanation for the higher percentage figure with the IgE ELISA inhibition technique is that natural IgE antibodies against bovine materials are directed to fewer epitopes than the IgG antibodies obtained by active immunization of rabbits and used in the previous ELISA inhibition. However, Prahl *et al.* [4] detected only 0.5% common allergens in cow urine and dander. This result was based on a CRIE method, and consequently the difference in results may be explained by methodological differences.

In the analysis of IgG reactivity to BEA, the sera of control subjects reacted in general to only a few high molecular weight bands (Fig. 4a). On that basis, they could be differentiated from the group of asthmatic farmers, who frequently responded also to a 20 kD component as well as to other low molecular weight components (Fig. 2a). In the BUA immunoblots, the non-allergic controls' (Fig. 4b) and students' IgG reactions were similar to those of the asthmatic farmers (Fig. 3a). The results of the five farmers in the group of non-allergic control subjects did not differ from the results of the other controls. Only one of these five controls had an exceptionally high IgG response to BUA (Fig. 4b). In our earlier report [2], negative controls had totally negative results in IgG immunoblotting. The differences between the previous and current methods may possibly explain this phenomenon.

In IgE immunoblotting, many of the cow-asthmatic farmers' sera showed negative results. This negativity correlated well with the farmers' anti-BEA IgE ELISA titres, which had to be approximately 0.7 (optical density) before a positive immunoblot was seen (Fig. 2b). If we adapt the definition of Aukrust [12] for major allergens (an allergen that shows in CRIE some specific IgE binding in more than 50% and strong IgE binding in at least 25% of the allergic subjects is a major allergen) to our IgE immunoblotting results, two major allergens could be detected in BEA (20 and 22 kD) and one in BUA (20 kD). In addition, the BEA bands at 16 and 24 kD and BUA bands at 22 and 24 kD were considerable allergens. IgE

reactivity to other bands of BEA and BUA occurred only occasionally, and the responses were usually hardly detectable in the autoradiographs. So, our results are quite similar to those of Prahl *et al.* [3], who found four major allergens in cow hair and dander (20, 22, 24 and 200 kD). In their study, the highest IgE binding was directed to the 20 and 24 kD components.

When the anti-BEA IgE responses of 10 cow-asthmatic farmers were studied with CRIE, three of the arcs (1, 2 and 5) were reactive with all sera. Arc 3 was detected by six sera and arc 7 by seven sera. Thus, those five antigens could theoretically be considered to be major allergens. Comparisons with the previous results of Prahl *et al.* [3] are difficult to make because of the inherent variability of methods like CRIE between different laboratories. However, arcs 2 and 3 of BEA appear to be located into the same positions as those of the two major allergens (MW 20 and 24 kD) of Prahl *et al.* [3]. Also, the shapes of the arcs were similar. Even though CRIE and immunoblot results are not readily comparable, comparison of our CRIE and immunoblot results (Table 2) corroborates the view that the 20 kD allergen is arc 2 in CRIE, since both of them were detected by all 10 sera and the reactions were usually the most intense.

The 20 kD protein seems to be the most important allergen in cow-derived materials, confirming our earlier findings. Although cow urine contains many antigenic components, its allergenic activity is low in comparison to cow dander (as determined by IgE ELISA inhibition and CRIE). By immunoblotting, we detected also another major allergen in cow dander (MW 22 kD). However, by CRIE five major allergens were found. This difference in results is possibly caused by the differences in treatment of the protein antigens during the processes. Consequently, this study shows that more than one method has to be employed to obtain comprehensive information of allergenic components in animal-derived materials. However, we prefer immunoblotting, since in this method, animal immune serum is not required in the primary separation process of antigenic components. In addition, the comparison of individual antibody responses is more easily done by immunoblotting, by which several sera can be analysed simultaneously.

Acknowledgment

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