

Comparative analysis of bovine extracts by immunoblotting and ELISA inhibition

Rabbit and human responses against cow epithelial and urine antigens

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Bovine epithelial and urine antigen extracts were compared using enzyme-linked immunosorbent (ELISA) inhibition assay and sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with immunoblotting. According to ELISA inhibition results, the two extracts share about 2% of their antigenic components. Urine-derived antigens seem to be antigenically closer to serum proteins than epithelial antigens, as determined by rabbit immune sera. The IgG responses of allergic farmers against epithelial antigens were directed mainly against a protein of 20 kD, while non-allergic farmers had only very weak reactions. Generally, the IgG response against urinary antigens seemed to be more heterogeneous than against epithelial antigens. Almost all cow-allergic farmers reacted with a band at 20 kD in IgE immunoblotting against urinary and epithelial antigen while all non-allergic farmers showed negative results.

Key words: allergens; cattle; immunoassay; immunoblot; respiratory hypersensitivity.

Accepted for publication 12 July 1989

Allergic reactions caused by inhalation of cow-derived dust are relatively common among dairy farmers. One constituent of the dust, bovine epithelium, has been shown to be an important occupational allergen (15, 18). Several studies have been published concerning antigens derived from cow hair and dander (2, 3, 11, 12, 13). In these studies, the major allergens have been considered to originate from the epithelium or hair. However, urinary proteins of rat, mouse, cat and dog have also been shown to be responsible for respiratory allergy symptoms (4, 6, 8, 16, 17, 20, 21). According to previous studies, urinary antigens appear to be more specific, showing less cross-reactions between animal

breeds than epithelial antigens (4, 21). In addition, urinary antigens are easier to prepare. We have recently shown that dairy farmers also have antibodies to urinary antigens (23). Further, a relation was seen between farmers' anti-urinary antigen antibodies and the atmospheric level of bovine-derived antigens in the working environment. According to earlier findings, cow hair and dander extract and urinary antigens share common allergens (11). However, little is known about the nature of urinary constituents in cow allergy. The aim of this study was to characterize and compare the antigenic structure and allergenic activity of the bovine epithelial and urinary extracts.

MATERIAL AND METHODS

Antigen preparation

Bovine epithelial antigen (BEA) was prepared as described by Virtanen et al. (24). The freeze-dried raw material of bovine epithelium was purchased from Allergon AB (Ängelholm, Sweden). The material was defatted in diethyl ether (1:10 w/v) at 4°C for 24 h, dried at room temperature, and extracted for 72 h 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). The solution was centrifuged at 36,600 *g* for 30 min, and the supernatant was dialysed (MW cutoff 3,500) for 72 h at 4°C in a vast excess of 0.05 M ammonium hydrogen carbonate (pH 7.8), sterilized by filtration through a Gelman Acrodisc (pore size 0.45 µm) and lyophilised. Storage was at -20°C.

Bovine urinary antigen used for rabbit immunization (BUA 1) was prepared from the urine of newly slaughtered cows. After filtering the urine through a Gelman Metrical GA-8 filter (pore size 0.2 µm), it was concentrated 16-fold by filtration through a Millipore filter (PCAC 04710, cutoff 1,000 daltons) and then stored at -20°C.

Bovine urinary antigen (BUA 2) for assays was made by freeze-drying the urine of newly slaughtered cows and suspending the dry powder in ion-exchanged water (1:5 w/v) as described previously (23). 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. (7) using bovine serum albumin as a standard. The BEA-extract contained 11.0 mg protein/ml. The protein content of BUA 1 was 5.80 mg/ml and of BUA 2 4.9 mg/ml.

Rabbit antisera

Rabbits were immunized with BEA or BUA 1 every 2 weeks for 6 weeks (BEA) or several

months (BUA), each time receiving 1.0 mg of protein/kg (BEA) and 1.5 mg of protein /animal (BUA). For the first injection, the antigen solution was emulsified with Freund's complete adjuvant and for later injections with Freund's incomplete adjuvant. Injections were given subcutaneously. Blood was drawn several times, and the antisera were stored at -70°C.

Patient sera

Nineteen dairy farmers were selected for this study from the patients of the Otorhinolaryngological Clinic in Kuopio University Hospital. They were divided into three groups according to their allergic symptoms and their results in the radioallergosorbent test (RAST; Pharmacia Diagnostics), skin prick test (allergen by ALK) and nasal challenge with BEA. Two of the three tests had to be positive before a patient was classified as being allergic to materials of bovine origin. The results of the tests were considered positive as follows: 1) cow-RAST score (scale 0-4) of 2 or higher, 2) skin prick test result with cow-allergen of 2 or higher according to Finnish standards (scale 0-4 +, 3+ being the histamine control equivalent and 2+ being half of the histamine control), and 3) in a nasal challenge an increase of mucous secretion, oedema of the mucous membrane or colour change in the mucous membrane. Consequently, 10 farmers were included in the group of cow-allergic patients (Group 1). Four farmers had allergic rhinitis of non-bovine origin with no allergic symptoms of bovine origin or not fulfilling our requirements of allergy (Group 2). Group 3 consisted of five non-allergic patients who had never had any clinical symptoms of allergy and were negative in all tests. Two non-farming control subjects from our department who had never shown allergic reactions against animal-derived antigens were also included in the study.

ELISA-inhibition procedure

This procedure was carried out as described previously, with minor modifications (22). A

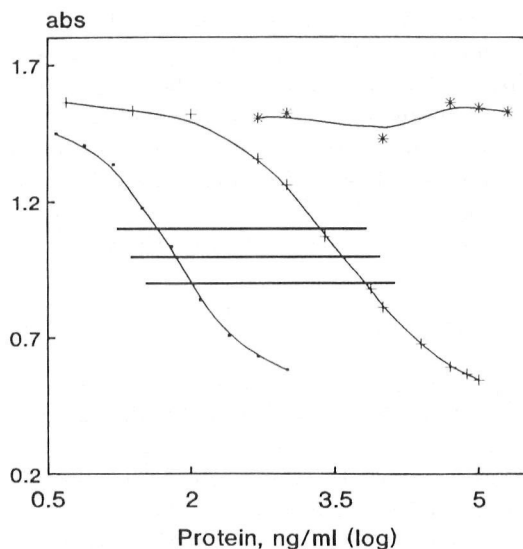


Fig. 1. ELISA-inhibition curves of BUA (+), BEA (·) and gelatin (*) standards against anti-BEA antiserum.

microelisa plate (Nunc-Immuno Plate I, Denmark) was coated overnight with BEA at a concentration of 1.0 µg/ml of protein in coating buffer (0.05 M carbonate buffer, pH 9.6) in volumes of 200 µl/well at 4°C. The next morning the plate was washed with 0.01 M PBS, pH 7.4, containing 0.5% gelatin (w/v) (Bio-Rad M 2845) and 0.5% (v/v) nonionic detergent, Nonidet P40 (BDH Chemicals Ltd., UK), later referred to as PBS-NP40-buffer. After incubation, the plate was washed and stored at -70°C. Before use, the plate was allowed to regain room temperature and was then washed with PBS-NP40-buffer. The BEA-standard dilutions, the BUA-dilutions and the gelatin standard dilutions in 100 µl vol. were added in duplicate. Rabbit anti-BEA antiserum at a dilution of 1:15, 000 (100 µl/well) was added immediately. The plate was incubated at 37°C for 1 h with an agitation rate of 300 rpm. After washing, the plate was incubated with PBS-NP40-buffer for 30 min at 37°C. After emptying the wells, horseradish peroxidase conjugated swine anti-rabbit antiserum (Dako-immunoglobulins a/s, P217) at a dilution of 1:1,000 was added, and the plate was incubated for 1 h at room temperature with an agitation

Table 1

The amounts of BEA and BUA at three OD levels and the corresponding percentages of common antigens (Fig. 1)

OD	BEA ng	BUA ng	%
1.100	46	2100	2.2
1.000	71	3700	1.9
0.900	100	6500	1.5

rate of 150 rpm. After washing, 200 µl of substrate solution (tetramethylbenzidine, 0.1 mg/ml; Merck, FRG) and urea hydrogen peroxide (BDH Chemicals, UK) corresponding to 0.05% of H₂O₂ in 0.1 M sodium acetate buffer (pH 6.0), were added. The reaction was stopped with 2 M H₂SO₄ (50 µl/well), and the absorbance was measured at 450 nm using a Titertek Multiskan spectrophotometer (Flow Laboratories, Finland).

To determine the amount of inhibition, curves of absorbance against the log concentration of the standard antigen (BEA), BUA and gelatin were plotted. By drawing a straight line across the straight parts of BEA and BUA curves, the amount of inhibition caused by BUA was calculated.

Polyacrylamide gel electrophoresis

Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis was carried out in a 12.5% acrylamide gel (18 × 20 × 0.3 cm) using the discontinuous buffer system of Laemmli (5). Freeze-dried antigens were dissolved in a sample buffer containing 0.125 M Tris, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% v/v glycerol and 0.002% w/v bromophenolblue (pH 6.8). After an incubation period of 7 h incubation at room temperature, the samples were boiled for 2 min. Samples and Pharmacia molecular weight standards (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and alfa-lactalbumin) were loaded onto the gels, and electrophoresis was carried out at 110 V until the marker dye had migrated to the bottom of

the gel. After electrophoresis, gels were stained by the silver staining method of Morrissey (9), photographed and stored in heat-sealed plastic bags.

Electrophoretic transfer

Proteins in unstained gels were transferred to nitrocellulose membrane (0.45 μ m pore size, Millipore) using the method described by Towbin et al. (19). The transfer buffer was 25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.3. The gel and a prewetted nitrocellulose membrane were placed between two sheets of wet Whatman 3M paper, two Scotch Brite scouring pads and then between two hole-punched plastic plates. Care was taken to re-

move air bubbles between the gel and the nitrocellulose. This sandwich was held together tightly with two rubber bands and placed between electrode plates in a transfer tank. The nitrocellulose membrane was placed anodically and proteins were transferred at 36 V for 2.5 hours. After transfer, the nitrocellulose was cut vertically into strips and the effectiveness of the transfer was checked by staining one sample strip and the standard strip with 0.15% amido black in water, methanol and acetic acid (65:25:10) for 10 min. The strips were then destained with the same solvent.

Detection of IgG and IgE-binding bands

The unstained membrane strips were incubated for 2 h at 37°C in PBS-buffer (20 mM PBS, 115 mM NaCl, 0.1% Tween 20; Batteiger et al. (1), containing 0.1% human serum albumin and 0.1% NaN₃). After the incubation the strips were packed in heat-sealable plastic bags, and 2 ml of diluted primary antibody was added before sealing. Rabbit antiserum was used at a dilution of 1:10 and patient sera at a 1:5 dilution for detection of IgG antibodies, and at 1:2 dilution for the detection of IgE antibodies. The diluent buffer was the same as mentioned above. After an incubation period of 6–12 h with a rolling movement at room temperature, the strips were washed 3 $\times$ 15 min with PBS-buffer and packed again in plastic bags. Conjugates were diluted to the same buffer as the primary antibody. ¹²⁵I-anti-rabbit IgG (Amersham), ¹²⁵I-anti-human IgG (NEN, Research Products) and ¹²⁵I-anti-human IgE (Phadebas Rast, Pharmacia Diagnostics) were used. For the detection of IgG binding bands, 250,000 cpm was used on two strips and for the detection of IgE binding bands approximately 200,000 cpm. After an overnight incubation in the same conditions as before, the strips were washed 2 $\times$ 15 min with PBS-buffer, 2 $\times$ 10 min with ion-exchanged water and air dried. The ¹²⁵I-labelled bands were detected by autoradiography at -70°C using Amersham Hyperfilm MP-film and a Kodak X-Omatic cassette with intensifying screen.

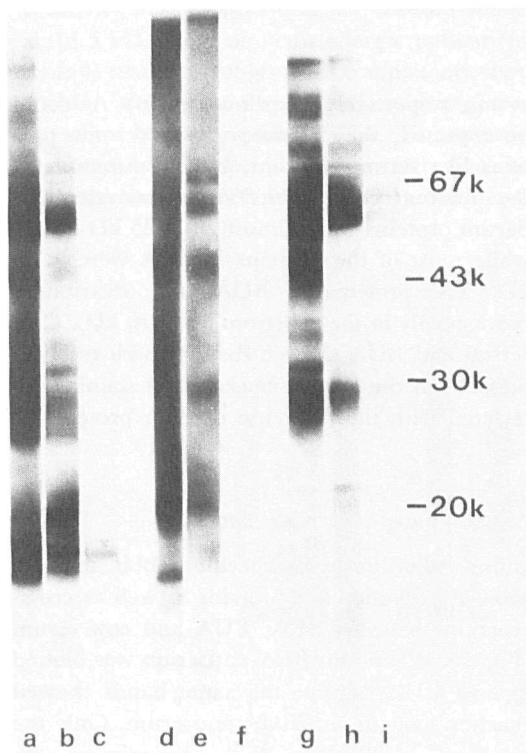


Fig. 2. Immunoblotting results with rabbit sera (antigen/antiserum). a) BUA/anti-BUA, b) BUA/anti-BEA, c) BUA/normal rabbit serum, d) BEA/anti-BEA, e) BEA/anti-BUA, f) BEA/normal rabbit serum, g) cow serum/anti-BUA, h) cow serum/anti-BEA, i) cow serum/normal rabbit serum.

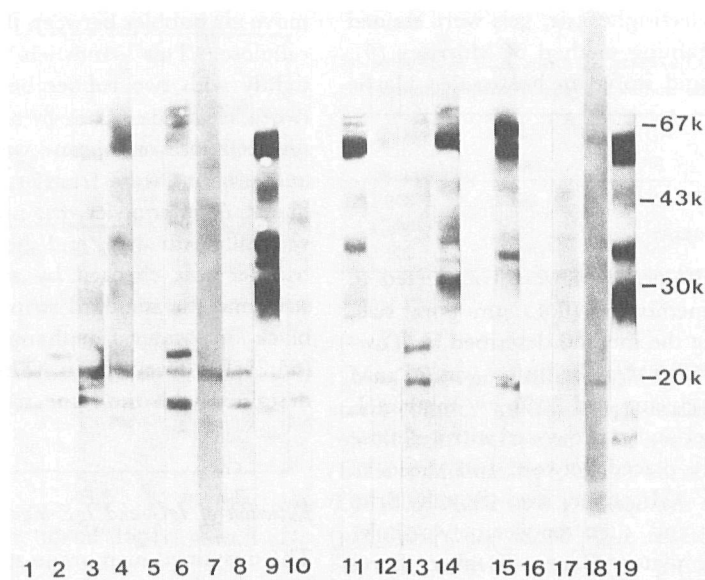


Fig. 3. Human IgG-antibody immunoblotting results against BUA-antigen. Patients 1–10 are cow-allergic (Group 1), patients 11–14 have allergic rhinitis of non-bovine origin (Group 2) and patients 15–19 are non-allergic farmers (Group 3).

RESULTS

ELISA-inhibition

The amount of antigens common to BEA and BUA was measured using the ELISA-inhibition method. BEA and BUA standard curves were plotted and three horizontal lines were drawn across the straight parts of both curves (Fig. 1). The quantities of BEA and BUA corresponding to each line were calculated and the percentages of common antigenic components were determined (Table 1). According to these calculations, 1.9% of the antigenic components of BEA and BUA are common to both antigens. The gelatin standard did not show any reactivity in the analysis.

SDS-PAGE

The silver stained protein profiles of BEA, BUA and cow serum in 12.5% SDS-PAGE gel revealed 13–17, 25–30 and over 30 resolving

bands, respectively (data not shown). As could be expected, they seemed to share some proteins like serum albumin, but the amounts of the different components varied considerably. Serum proteins were almost all > 25 kD in size while most of the proteins in BEA were < 30 kD. The proteins of BUA were distributed more evenly in the gel (from 80 to 10 kD). Cow serum and BUA showed distinct, well resolved bands, but the intense background staining interfered with the detection of BEA proteins.

Immunoblotting with rabbit antisera

Immunoblotting with specific rabbit antisera showed immunogenic proteins as well as cross-reactions between BEA, BUA and cow serum (Fig. 2). When anti-BEA antiserum was blotted against BUA, almost the same bands showed reaction as with anti-BUA antiserum. Only the bands above 70 kD in BUA were missing with the anti-BEA antiserum, and the band at 20 kD gave a stronger reaction than with anti-BUA antiserum. When BEA antigen was blotted with these two antisera, a band at 16 kD proved to

be BEA specific. According to the results (Fig. 2), anti-BUA antiserum detected most of the serum proteins, while anti-BEA antiserum detected only a few serum proteins, and gave the strongest reactions with two bands at 55 and 27 kD. We used normal rabbit serum as a negative control, and it reacted weakly with some bands. When normal rabbit serum was blotted against BUA, reactions were seen at 24, 20, 17 and 16.5 kD. With epithelial antigen, a reaction was seen only at 20 kD, and when cow serum was used bands were detected at 71, 67, 55 and 27 kD.

Immunoblotting with patient sera

Patient sera showed heterogeneous IgG responses towards BUA as can be seen in Fig. 3. However, all allergic patients (Group 1) showed reactivity to components at 20, 17 and weaker at 16.5 kD. Only one cow-allergic patient (patient 9) reacted strongly with components above 25 kD. When non-allergic patient sera (Group 3) were blotted against BUA, no common bands could be detected. Only two sera reacted with the band at 20 kD and only one serum

detected bands at 17 and 16.5 kD. The results of the patients with allergen rhinitis of non-bovine origin were not significantly different from the results of the cow-allergic group, but the reactions towards components at 20, 17 and 16.5 were on average weaker. The IgG response against BEA seems to be more homogeneous than the anti-BUA IgG response (Fig. 4). All patients in Groups 1 and 2 reacted with a band at 20 kD, while the results of the non-allergic group were almost negative or responded better to heavier bands. Only one cow-allergic patient had IgG antibodies against antigen components above 25 kD and only one showed a strong response to a BEA specific component at 16 kD. Antibodies were also found commonly in Group 1 to a band at 22 kD. The two non-farming controls from our department showed no bands in BEA or BUA IgG immunoblotting (data not shown).

The distribution of IgE antibodies towards BEA and BUA were also analysed from the sera of cow-allergic patients (Fig. 5). Two patients failed to show any bands towards BUA, but the rest showed a reaction to the band at 20 kD and four to the band at 22 kD. In addition to these allergens, a band weighing approximately 24

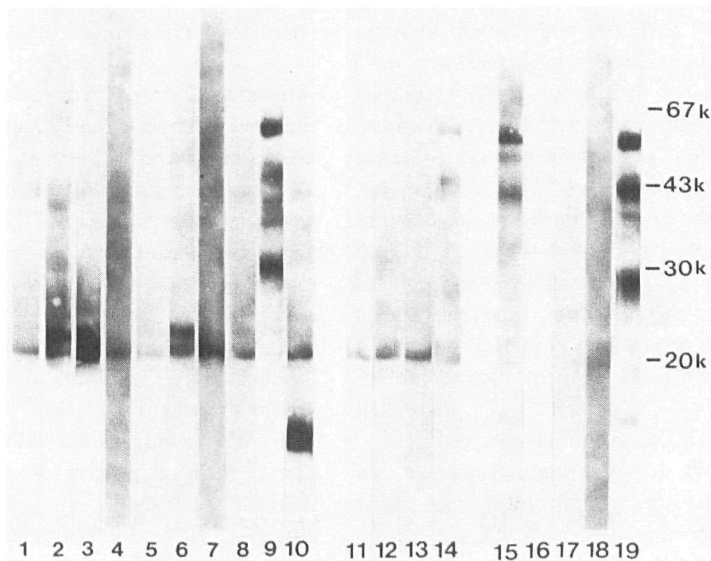


Fig. 4. Human IgG-antibody immunoblotting results against BEA-antigen. Patients 1–10 are cow-allergic (Group 1), patients 11–15 have allergen rhinitis of non-bovine origin (Group 2) and patients 16–19 are non-allergic farmers (Group 3).

kD was detected in the serum of one patient (patient 7). The same two patients were negative against BEA as in the anti-BUA IgE analysis. The most important allergens of BEA seem to be protein components at 20 and 22 kD, but reactivity to other components is also seen, e.g. against bands at 24 and 45 kD. We also screened the IgE reactivity of six patients from Groups 2 and 3. In Group 2, two of the three patients tested showed a very weak reaction against the band at 20 kD, and the three patients tested from Group 3 were negative as well as were the two non-farming controls from our department (data not shown).

DISCUSSION

Allergens have been demonstrated in different cow-derived extracts like hair, dander, saliva, urine, whey, amniotic fluid, beef and even from

cow-hide products (11). However, cow allergy among dairy farmers is most likely due to sensitization to antigens in an aerosol form, and therefore cow dander and urine seem to be the most probable sources of allergens in cowsheds. Earlier studies of cow allergen extracts have mainly focused on hair and dander. We think, however, that cow urine might also be a significant sensitizer (23). It is for this reason that we wanted to compare dander and urine extracts in detail.

The silver-stained protein profiles of BEA, BUA and cow serum were remarkably different in SDS-PAGE gel. We separated 25–30 bands from BUA and about 15 bands from BEA. Exact counting was impossible due to some weak bands in BUA and the intense background of BEA. When immunoelectrophoretic methods have been used in the studies of other authors to analyse cow hair and dander extracts, 18 components have been separated (2).

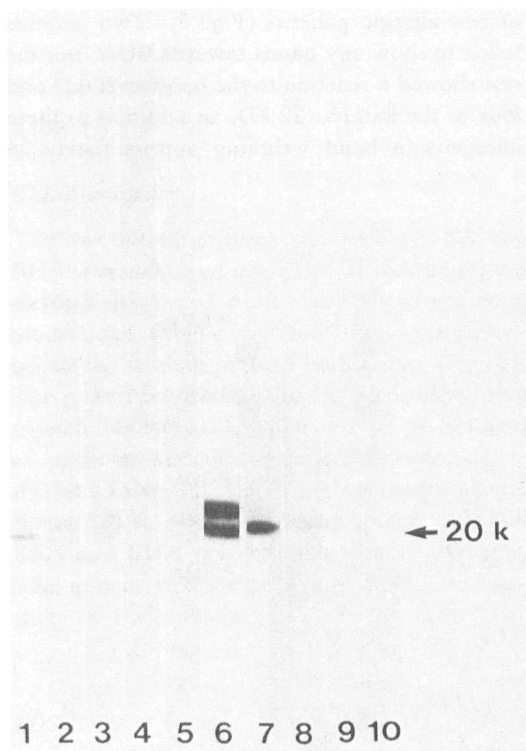


Fig. 5. Human IgE-antibody immunoblotting results against BUA-antigen. All patients (1–10) belong to the cow-allergic group.

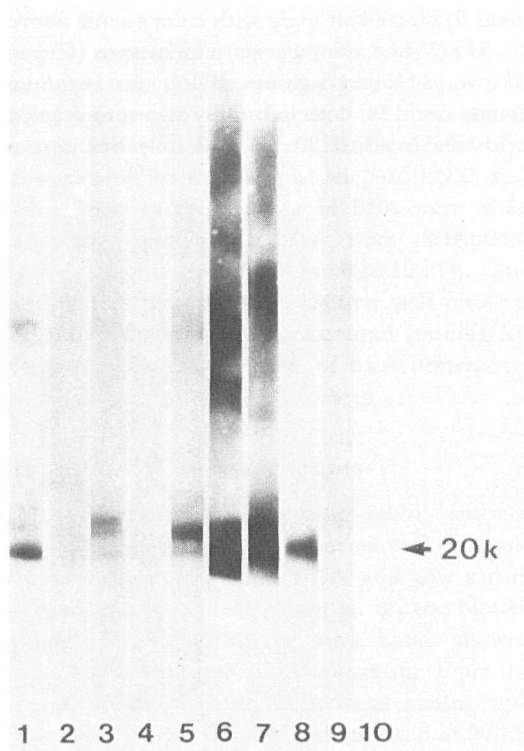


Fig. 6. Human IgE-antibody immunoblotting results against BEA-antigen. All patients (1–10) belong to the cow-allergic group.

When the amount of cross-reaction between BEA and BUA was studied using the quantitative ELISA inhibition method, only 1.9% of the antigenic components were common to both of these extracts. Prahl has compared the amounts of common allergens in hair and dander extract to urine using counterimmunoelectrophoresis, rocket immunoelectrophoresis and crossed-line immunoelectrophoresis (11). His results were 1, 1 and 0.5%, respectively. Our result is in good agreement with the result of Prahl in spite of the fact that we measured common antigens instead of allergens.

We also used SDS-PAGE combined with immunoblotting to demonstrate the cross-reactive components. According to the results, cow serum is antigenically closer to urine than dander because anti-BUA antiserum detected several serum proteins which anti-BEA antiserum did not. It seems, however, that all these antigen extracts share many common proteins but their relative amounts vary considerably. Some nonspecific reactions were seen with normal rabbit sera but they were weak and most likely do not interfere with the conclusions drawn from these results. According to earlier studies, cross-reactions between animal species are common and we assume that this is the basis for the observed phenomena (4, 21).

According to the results of immunoblotting, human humoral responses to bovine antigens are heterogeneous. It seems that farmers who have been exposed to cow-derived materials have anti-BUA IgG antibodies mainly to the protein components > 25 kD in size, while those especially cow-allergic patients also seem to respond to components of < 25 kD. This can be seen with both antigens, but mainly with BUA because it contains more "heavy" proteins than BEA. The IgG reactivity to bovine antigens was also found among Groups 2 and 3. This is not surprising because it was the sera of the farmers which was studied. Obviously, the reactivity found reflects the cow dust exposure encountered in dairy farm work. For instance, according to Virtanen et al. (23), there is a relationship between the

level of anti-BUA IgG antibodies in farmers' sera and the BEA concentrations in breathing zones. Further, in the study of Platt-Mills et al. (10), the quantity of IgG antibodies to rat urinary allergens was correlated with the degree of exposure to animals. Therefore, it is possible that the measurement of human IgG reactions to the heavier part of BUA which appears to be missing to a large extent from BEA might offer a better means for estimating the exposure to cow antigens.

The allergens of bovine epithelia have been analysed earlier by Prahl and his co-workers (2, 11, 12, 13, 14). They found four major allergens by crossed radioimmunoelectrophoresis. The MW of these allergens were 24, 20, 22 and 200 kD (12). The latter of these allergens was IgG molecule. When we studied the IgE response to BEA, we found that the strongest reactivity was directed to an allergen at 20 kD. Some reactivity was also seen to bands at 22, 24 and 45 kD. But, according to the results, only the component at 20 kD can be considered a major allergen. The discrepancy between our result and that of Prahl et al, may be due to the type of patients chosen for the study. We examined allergic rhinitis patients whose allergenicity is probably at a lower level than, e.g. that of asthmatics. We think that the 20 kD protein is possibly the allergen No. 1 of Prahl et al. (12). Our cow-allergic patients failed to show a response to allergens at 200 kD and the reason might be the SDS- and mercaptoethanol treatment of our antigens that dissociates IgG to polypeptides of 22 and 55 kD. Anti-BUA IgE-antibodies were directed to two bands at 20 and 22 kD. They are probably the same proteins as in the BEA preparation. This finding is in agreement with that of Prahl, (11) who also found his four major allergens of cow hair and dander extract from other cow-derived sources like urine.

An unexpected finding in our study was that two allergic patients failed to show reactivity in IgE immunoblotting (patients 4 and 10). According to clinical data, they were considered allergic to cow allergens. The RAST result of patient 4 was negative but nasal challenge was positive and skin prick test gave maximal reac-

tion. The nasal challenge of patient 10 was negative but both the RAST score and skin prick test gave values of two. The immunoblotting result of patient 4 could be explained by the negative RAST result. A possible reason for the result of patient 10 might be the epithelial antigens used in tests. In the RAST and skin prick test, commercial antigens were used, but the challenge and immunoblotting studies were done using BEA.

Our results indicate that urine might also be responsible for sensitizing dairy farmers to cow-antigens. In some cases, the IgG response seems to be stronger against urine than epithelial antigens: When SDS and mercaptoethanol treated antigens are used, antigen components of <25 kD seem to be the most important immunogens. The non-allergic group usually showed negative or faint reactions against these antigens, while all allergic patients showed detectable bands in this area. In both extracts a component at 20 kD was found to be the major allergen.

ACKNOWLEDGEMENT

We thank the Allergy Research Foundation (Finland) for supporting this study.

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