

Immune reactivity of cow-asthmatic dairy farmers to the major allergen of cow (BDA20) and to other cow-derived proteins. The use of purified BDA20 increases the performance of diagnostic tests in respiratory cow allergy

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Summary

Background Cow dust is one of the most important inducers of occupational allergic diseases in Finland. For example, in 1991 it accounted for almost 40% of the new occupational asthma cases.

Objective This study compares the performance of the purified major cow allergen (BDA20) and crude bovine epithelial extract (BEA) in diagnostic tests and examines the role of milk allergy-associated bovine proteins (bovine serum albumin, α -lactalbumin, β -lactoglobulin, casein) in respiratory cow allergy.

Methods The humoral responses of cow-asthmatic and healthy farmers to the various components of BEA were analysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The levels of specific IgE and IgG antibodies were quantified with enzyme-linked immunosorbent assays (ELISAs). The cellular responses were analysed with antigen-specific lymphocyte proliferation tests.

Results The specific anti-BDA20 IgE measurement was found to be best in distinguishing between the asthmatic farmers and their healthy colleagues. It proved possible to determine a cut-off value that gave the analysis a specificity and sensitivity of 100%; the distinction between the two groups was highly significant ($P < 0.0001$). In the lymphocyte proliferation analysis, cow asthma was more closely associated with reactivity to BDA20 than to BEA. In the measurement of anti-BDA20 and anti-BEA IgG antibody levels, considerable overlap between the groups was observed, suggesting that these antibodies are not directly involved in cow allergy. When proteins associated with milk allergy were used as test reagents, no statistically significant differences could be observed between the groups, except for anti-casein IgE antibodies the level of which, however, overlapped considerably between the farmer groups.

Conclusion These findings suggest that purified BDA20 is better than BEA for diagnosing cow asthma and that proteins associated with milk allergy are of only marginal significance in this disease.

Keywords: antibody, cow allergy, diagnosis, immunoblotting, lymphocyte proliferation, purified allergen, SDS-PAGE

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Introduction

For persons working on dairy farms, cattle-derived matter is a major sensitizer leading to allergic respiratory and skin diseases. Cow dust was reported to account for almost 40% of the 352 cases of occupational asthma recorded in 1991, placing cow dust first in the list of work-related sensitizers in Finland [1,2].

We have previously shown that the 20 kDa and 22 kDa components of bovine epithelium are the major cow allergens, the prevalence of immunoglobulin (Ig)E reactivity being the highest (97%) against the 20 kDa allergen [3]. The important allergens in cow milk are different from dander allergens [4,5], and their significance to the respiratory sensitization of dairy farmers is largely unknown.

The aetiological diagnosis of allergic diseases is normally based on the measurement of allergen-specific IgE, skin-prick and provocation tests. The aim of this study was to find out whether the purified 20 kDa allergen (BDA20) would be better than crude bovine epithelial antigen preparation (BEA) in diagnosing cow-derived asthma with *in vitro* methods. Furthermore, the role of proteins associated with milk allergy in sensitizing dairy farmers was studied. It was observed that BDA20 was preferable to BEA in diagnostic tests for cow asthma. As expected, milk-derived proteins seemed to have only a minor role in cow-derived asthma, although in individual cases some of these proteins may contribute to the development of respiratory allergy.

Materials and methods

Subjects

The subjects of this study were 11 randomly selected dairy farmers (seven men, four women) with clinically diagnosed cow asthma and 14 healthy dairy farmers (seven men and women). The mean age of the asthmatic subject group and the control group were 46 years (range 27–56) and 40 years (range 30–54), respectively. The asthmatic subjects were patients of the Pulmonary Clinic of Kuopio University Hospital. The bovine origin of their asthma had been confirmed with either skin-prick test with cow allergen (Allergologisk Laboratorium, ALK, Denmark), with inhalation test (automatic dosimeter nebulization, Spira Elektro 2, Finland [6]) using cow epithelial antigen (ALK) or with RAST (Pharmacia). For a person to be classified as being asthmatic to cow-derived material, 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 results 2+ or higher (3+ being the

histamine control equivalent), the RAST score of 2 or higher (scale 0–4) and in the inhalation test, a decrease of 15% or higher in flow expiratory volume in 1 second (FEV₁) values. The control subjects were dairy farmers randomly selected by a trained nurse from healthy farmers who had their regular health examination at the occupational clinic of a rural community near Kuopio. A standardized questionnaire was presented to the farmers, and if no symptoms or signs indicating hypersensitivity or other chronic diseases were reported, the farmer was considered healthy.

Preparation of bovine epithelial antigen

Bovine epithelial antigen (BEA) was prepared essentially as described previously [7]. The freeze-dried gamma-irradiated raw material of cow dander 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 then extracted for 24 h at 4°C in phosphate buffered saline (PBS), pH 7.0 (1:10 w/v; NaN₃ 0.05%), with agitation. After centrifugation at 36 600 g for 30 min at 4°C, the supernatant was dialysed (MW cut-off 3500 Da) for 24 h at 4°C against PBS, pH 7.0, sterilized by filtration (0.2 µm), lyophilized and stored at –20°C. The protein concentration of the BEA preparation (11.1 mg/mL) was measured according to the method of Lowry *et al.* [8] using bovine serum albumin as a standard.

Production and purification of monoclonal antibodies to BDA20

The anti-BDA20 monoclonal antibody producing hybridoma was developed as described previously [9]. Larger amounts of the antibody were produced in the peritoneal cavities of BALB/c mice [9]. The ascites fluid was purified in a HiTrap Protein G affinity chromatography column (Pharmacia Biotech, Sweden). In brief, ascites fluid was centrifuged and filtered through a 0.45 µm filter. The pH of the ascites was adjusted to pH 8.0 by adding a 1:10 volume of 1.0 M Tris, pH 8.0. The HiTrap Protein G column was equilibrated with 5 bed volumes of start buffer (50 mM Tris, pH 8.6), and the ascites fluid was circulated three times through the column. The column was washed with 10 column volumes of start buffer and eluted with 100 mM glycine-HCl buffer, pH 2.7. The eluate was collected in 0.5 mL fractions and a 1:10 volume of 1.0 M Tris, pH 8.0, was used to bring the pH of the eluate back to neutral. Absorbance was measured at 280 nm and the protein-containing fractions were pooled. Buffer exchange to coupling buffer (0.2 M NaHCO₃/0.5 M NaCl, pH 8.3) was

done in a NAP-25 gel filtration column (Pharmacia Biotech, Sweden).

Affinity purification of BDA20

A HiTrap NHS-activated affinity column (Pharmacia Biotech, Sweden) for purifying BDA20 from BEA was prepared according to the instructions of the manufacturer. Briefly, a HiTrap NHS-activated 1 mL column was washed with 6 mL of ice-cold HCl and immediately after this, 4 mL of the purified antibody in coupling buffer (10 mg/mL) was injected into the column. The antibody solution was pumped back and forth with two syringes at room temperature for 30 min. The coupling solution was washed away with 3 mL of coupling buffer. Excessive active groups in the column were deactivated by washing the column with three cycles of 0.5 M ethanolamine/0.5 M NaCl, pH 8.3, 6 mL at a time, followed by 6 mL of 0.1 M acetate/0.5 M NaCl, pH 4. Finally, 6 mL of PBS, pH 7.0, was injected into the column. BEA solution diluted at 1:3 was passed through the column three times. After washing with 10 mL of PBS followed by 10 mL of PBS with higher salt concentration (0.5 M), the bound protein was eluted with 100 mM glycine-HCl buffer, pH 2.5. The BDA20 containing eluate was collected in 0.5 mL fractions and neutralized with 50 μ L of 1.0 M Tris, pH 8.0. Absorbance was measured at 280 nm, and the protein-containing fractions were pooled. Tris and glycine were removed with NAP-25 gel filtration columns. The final concentration of BDA20 was adjusted to 1 mg/mL with PBS, after which it was filtered through a 0.22 μ m filter and then stored at -70°C . The purity of the preparation was verified with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis and immunoblotting

The IgE-reactive components of BEA were detected as described previously [10]. Briefly, the antigens were first separated in SDS-PAGE in a 12.5% gel using the discontinuous buffer system of Laemmli [11]. The separated components were transferred to a nitrocellulose membrane (Millipore 0.45 μ m) according to the procedure of Towbin *et al.* [12]. After transfer, the nitrocellulose membrane was blocked in PBS, pH 7.3, containing 0.1% casein, 0.05% Tween 20, and 0.05 w/v NaN_3 at 37°C for 1 h. The membrane was cut into strips and incubated in sera from the cow-asthmatic and control subjects, diluted 1:5 in blocking buffer, at room temperature overnight. The strips were then washed in Tween-PBS for 4×5 min and incubated overnight with I^{125} -labelled anti-human IgE (Phadebas

RAST). After washing, the reactive bands were detected with autoradiography.

Antibody measurements with ELISA

The measurement of allergen-specific IgG and IgE antibodies with enzyme-linked immunosorbent assay (ELISA) has been described previously in detail [3]. In brief, for measuring IgE specific for BDA20, BEA, bovine serum albumin (BSA; Sigma; A-4378), α -lactalbumin (Sigma; L-5385), β -lactoglobulin (Sigma; L-0130) and casein (BDH Ltd, UK; 44020), microwell plates were coated at a concentration of 5 μ g/mL of each protein in the coating buffer. After blocking the plates with diluent (containing casein) they were stored frozen. For measuring casein-specific antibodies, human serum albumin (Sigma, A-8763) was used as a blocking agent. Serum samples were tested at a dilution of 1:10 in duplicate. For the detection of specific IgE, goat anti-human IgE (Cappel, USA; 0201-0061) was used. After adding biotinylated rabbit anti-goat IgG (Cappel, USA; 8606-3712) and Vectastain ABC Elite Kit (Vector Laboratories, USA; PK-6100) reagents (avidin DH and biotinylated horseradish peroxidase H), colour reaction was developed and measured as reported previously. The results were presented as optical density values (OD) minus the reagent blank value (eight wells/plate). For measuring specific IgG antibodies, the plate was coated as described above but using proteins at a concentration of 0.5 μ g/mL. The plate was incubated with sera diluted at 1:400 (anti-casein IgG) or at 1:200 (other specific IgG) at 37°C for 1 h and was then washed. Next, the plate was incubated with peroxidase-conjugated rabbit anti-human IgG (1:1000; Dako, P 214) at 37°C for 1 h. Colour reaction was then developed and measured as above.

Lymphocyte proliferation test

Peripheral blood mononuclear cells (PBMC) were separated from 15 mL of heparinized peripheral blood with Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation. The peripheral blood was diluted 1:2 in PBS, pH 7, and layered over 3 mL of Lymphoprep in 10 mL glass test tubes. After centrifugation at 550 g at room temperature for 30 min, the layer containing mononuclear cells was removed, washed twice with PBS and suspended in complete RPMI 1640 (Gibco) (containing 5% AB serum, 2 mM L-glutamine [Gibco], 20 μ M 2-mercaptoethanol, sodium pyruvate [Gibco] and non-essential aminoacids [Gibco] according to the manufacturer's instructions, penicillin [100 IU/mL] and streptomycin [100 μ g/mL]) and then counted

by Trypan blue exclusion. PBMCs ($10^5/0.2\text{ mL}$) were stimulated in triplicate in round-bottomed microtitre plates (Nunc Delta, A/S Nunc, Denmark) with the proteins mentioned in Antibody measurements at a concentration of $50\text{ }\mu\text{g/mL}$. This concentration of the proteins was determined to be appropriate in preliminary tests. Wells containing phytohemagglutinin (Wellcome; 1.25%) and no stimulator were included as controls. The plates were incubated in a humidified 5% CO_2 incubator at 37°C for 5 days. After pulsing for 16 h with 0.5 mCi of ^3H -thymidine (specific activity 2.0 Ci/mmol ; Amersham International plc, UK) per well, the radionuclide uptake was measured with scintillation counting (LKB Wallac 1214 Rackbeta, Pharmacia, Finland). The results are expressed as a stimulation index (SI) obtained by dividing the counts per minute (cpm) in the presence of a stimulator with the cpm without a stimulator (medium alone).

Results

SDS-PAGE and immunoblotting

The sera of the asthmatic and the healthy dairy farmers were first characterized for their IgE antibody spectrum

against the crude extract of bovine epithelium (BEA) with SDS-PAGE and immunoblotting analysis. The prevalence of IgE antibodies in the sera of asthmatic patients reacting with the different allergen components of BEA is shown in Fig. 1. The sera of the patients showed strong IgE reactivity especially to the 20 kDa (BDA20) and 22 kDa components. Two patients showed a weak reaction and two patients did not have BEA-specific IgE in their sera detectable by immunoblotting. The sera of the non-asthmatic control persons did not exhibit IgE reactivity to BEA (data not shown).

Antibody measurements with ELISA

The results of the specific anti-BDA20, anti-BEA and anti-casein IgE measurements of the asthmatic and the control subjects are presented in Fig. 2. There was a statistically significant difference between the patients and control subjects for each of the three antigens (Mann-Whitney U -test; $P < 0.0001$, $P = 0.0001$ and $P < 0.05$, respectively). In the analyses of anti-BSA, anti- α -lactalbumin and anti- β -lactoglobulin IgE antibody levels, no statistically significant differences could be observed between the two groups ($P > 0.05$; data not shown).

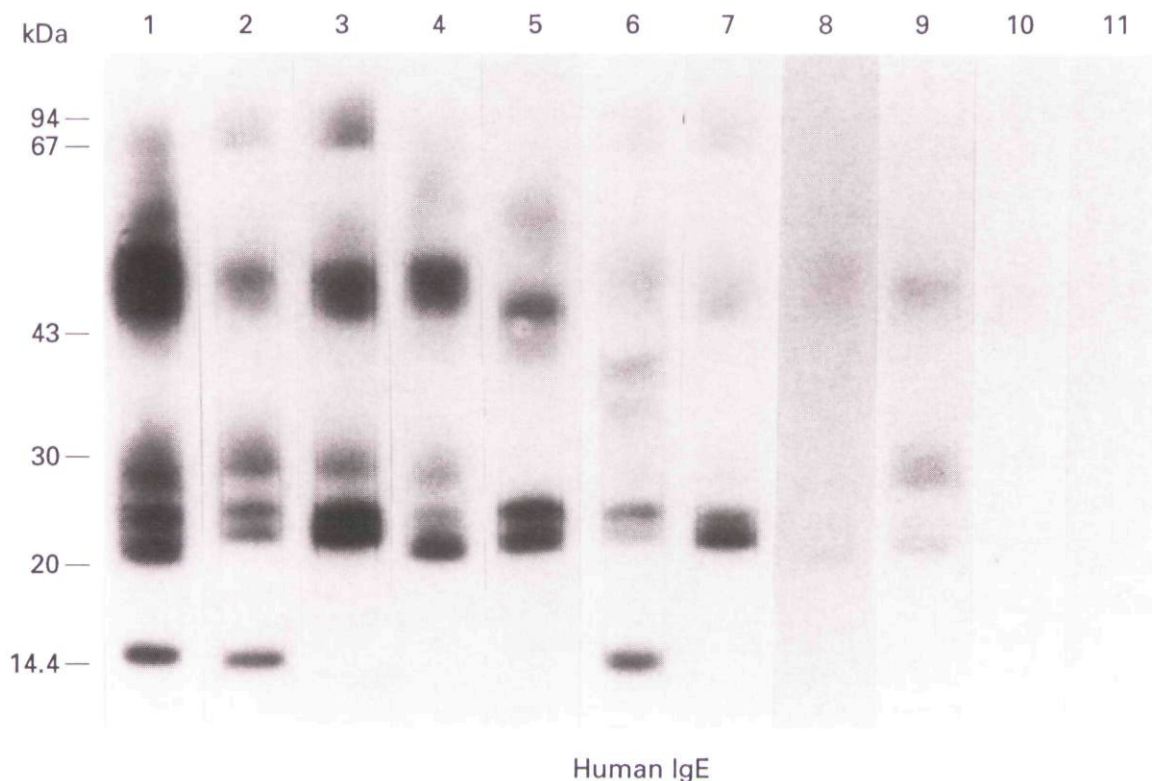


Fig. 1. Cow-asthmatic patients' IgE reactivity to the various components of BEA in the immunoblotting analysis.

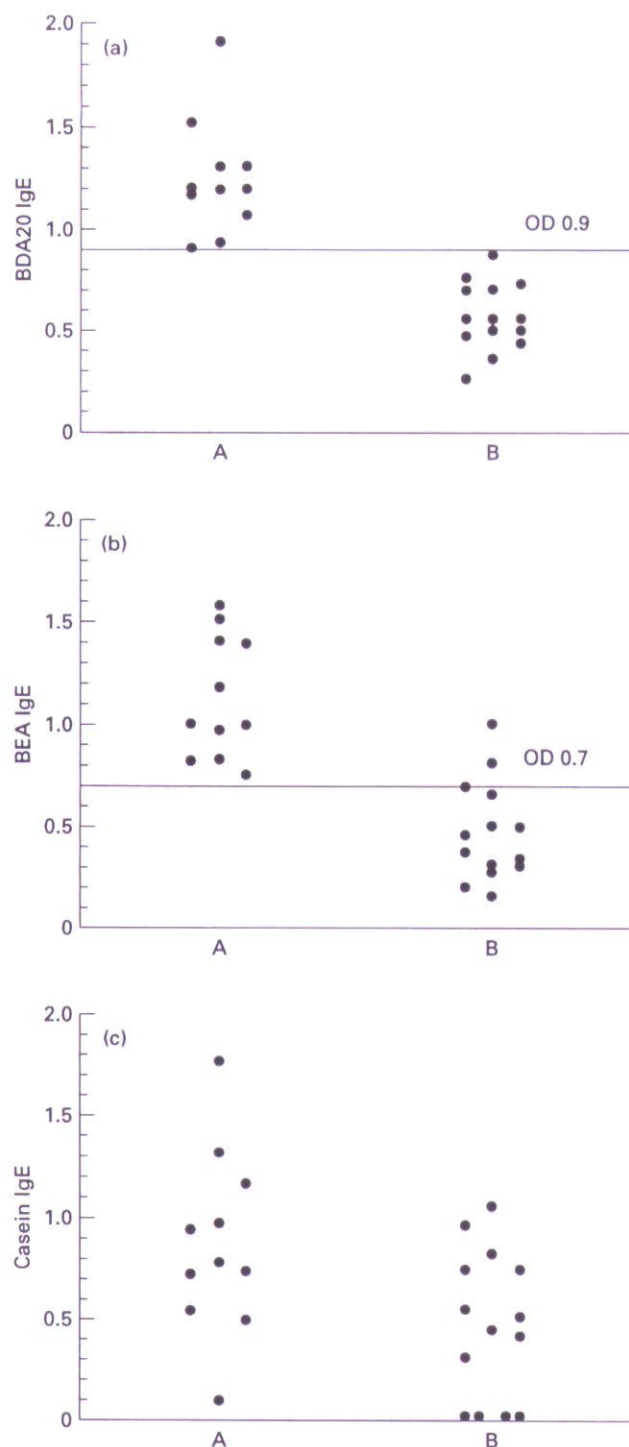


Fig. 2. Distribution of anti-BDA20 (a), anti-BEA (b) and anti-casein (c) IgE levels in the sera of cow-asthmatic (A) and healthy farmers (B). For anti-BDA20 and anti-BEA measurements, the cut-off values allowing statistically the most significant distinction between the groups are indicated.

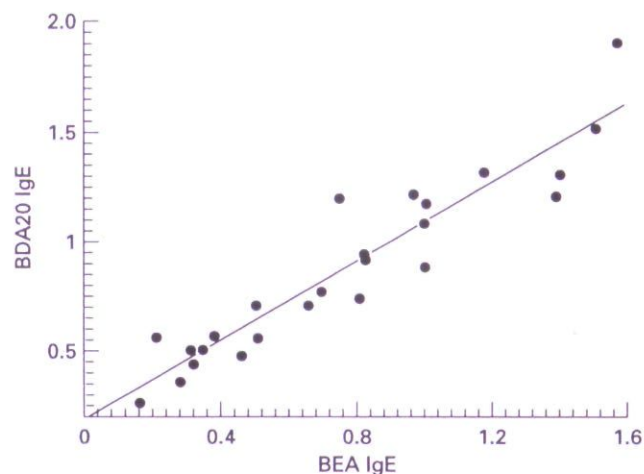


Fig. 3. Correlation of anti-BDA20 and anti-BEA IgE levels of asthmatic and healthy farmers (with a regression line).

The analysis of the association of the anti-BDA20 and anti-BEA IgE levels of the patients and control subjects are shown in Fig. 3. The levels of these antibodies showed a high correlation (Spearman Rank Correlation; ρ 0.94, $P < 0.0001$). As demonstrated in Fig. 3, there were no cases exhibiting a high IgE level to only one of the antigens. Interestingly, both the anti-BDA20 and the anti-BEA IgE antibody levels correlated significantly with the anti-casein IgE antibody level (ρ 0.56, $P < 0.01$ and ρ 0.50, $P < 0.05$, respectively). When this association was analysed separately for the cow-asthmatic and the healthy farmers, it was observed to be weaker among the cow-asthmatic farmers (ρ 0.38, $P = 0.23$) than among the healthy farmers (ρ 0.52, $P = 0.06$). A positive correlation was also observed between anti-casein IgE and anti-BSA IgE levels (ρ 0.45, $P < 0.05$). Highly significant correlations were discovered between anti-BSA IgE levels and anti- α -lactalbumin and anti- β -lactoglobulin IgE level (ρ 0.91, $P < 0.0001$ and ρ 0.82, $P < 0.0001$, respectively) as well as between anti- α -lactalbumin and anti- β -lactoglobulin IgE levels (ρ 0.89, $P < 0.0001$).

For diagnosing cow asthma by using anti-BDA20 or anti-BEA IgE tests, it is necessary to determine a cut-off value for the antibody level. Different cut-off values were tested (stepping by 0.1 OD) to find the value for which the significance of distinction between the groups was statistically the highest possible (Fisher's Exact Test). In the analysis of the anti-BDA20 IgE antibody level, the most significant ($P < 0.0001$) distinction between the groups was obtained at the OD value of 0.9 (Fig. 2a). Both the sensitivity and specificity of the analysis were 100%. Anti-BEA IgE measurements were analysed accordingly, and the most significant distinction

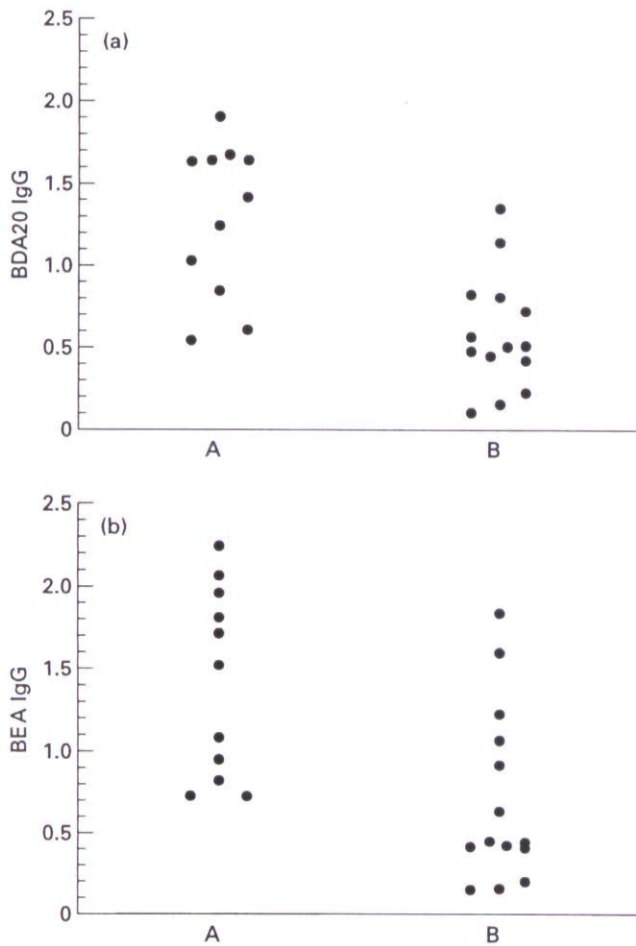


Fig. 4. Distribution of anti-BDA20 (a) and anti-BEA (b) IgG levels in the sera of cow-asthmatic (A) and healthy farmers (B).

($P < 0.0001$) between the asthmatic and healthy farmers was obtained at the OD value of 0.7 (Fig. 2b). In this case, the sensitivity was 100% and specificity 86%.

The results of the anti-BDA20 and anti-BEA IgG measurements are shown in Fig. 4. In both analyses, antibody level differences between the groups were statistically significant (Mann–Whitney U -test; $P < 0.001$ and $P < 0.01$, respectively). As is evident, however, the overlap of antibody level ranges between the asthmatic and the non-asthmatic dairy farmers was considerable. When the levels of IgG specific to other antigens were analysed, no statistically significant differences could be found between the two groups of farmers ($P > 0.05$; data not shown).

Lymphocyte proliferation test

The proliferative responses of PBMCs to bovine-derived antigens were detected both in the asthmatic and the

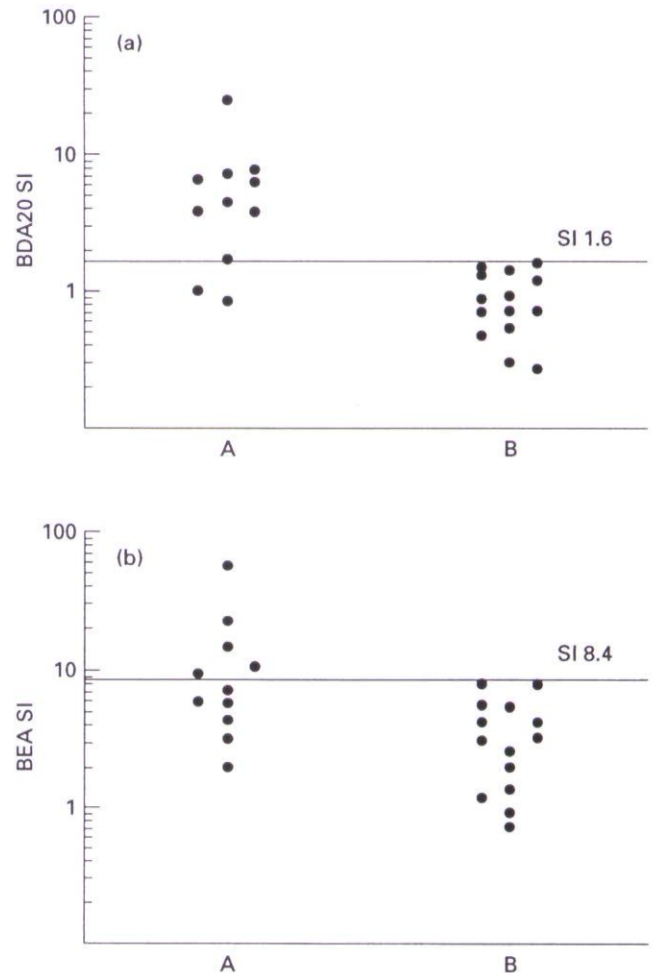


Fig. 5. Distribution of the proliferative responses (indicated as stimulation indexes [SI]) of PBMCs to BDA20 (a) and BEA (b) of cow-asthmatic (A) and healthy farmers (B). For both measurements, the cut-off values allowing statistically the most significant distinction between the groups are indicated.

non-asthmatic farmers. In the BEA analysis, the ranges of cpm of the non-asthmatic farmers were from 521 to 6704 (mean 2135 cpm) and in the BDA20 analysis from 182 to 904 (mean 468 cpm). The respective ranges of the asthmatic subjects were from 1316 cpm to 14 573 cpm (mean 4469 cpm) and from 535 cpm to 6336 cpm (mean 2067 cpm). The cpm values of the asthmatic and non-asthmatic farmers obtained from control wells containing no antigen ranged from 228 to 3332, the mean value being 610.

The stimulation indexes (SI) of PBMCs to BDA20 and BEA are shown in Fig. 5. When these results were analysed statistically, significant differences were found between the asthmatic and non-asthmatic subjects (Mann–Whitney U -test; $P < 0.001$ and $P < 0.01$, respectively). In the analyses of responses to other antigens

statistically significant differences were not found ($P > 0.05$; data not shown).

Cut-off values for distinguishing cow-asthmatic farmers from healthy farmers were tested similarly as described for IgE measurements. When the indexes of BDA20 stimulation were analysed, the most significant distinction ($P < 0.0001$) between the groups was obtained with the SI values of 1.6 (Fig. 5a) and 1.7. In these cases, the sensitivity of the analysis was 82% and the specificity 100%. The most significant distinction ($P < 0.01$) between the groups in the analysis of BEA stimulation indexes was obtained by values ranging from 8.0 to 9.5 (Fig. 5b). In these cases, the sensitivity of the test was 45% and the specificity 100%.

Discussion

Bovine dander contains a number of allergens, of which the 20 kDa and 22 kDa components can be defined as major allergens [10]. Although the number of patients having IgE reactive to these allergens (Fig. 1) was lower than in our previous study [3], the results of this study clearly confirm the significance of the 20 kDa and 22 kDa allergens in respiratory cow allergy. The great majority of cow-allergic patients, but none of the control subjects, had IgE antibodies reactive with these allergens in immunoblotting. As to the 20 kDa component (BDA20), this conclusion is further supported by the fact that even those cow-asthmatic dairy farmers who had a negative immunoblotting result had more BDA20-specific IgE in their sera than their non-asthmatic colleagues did (Fig. 2).

Several cow proteins other than those derived from dander can be allergenic. Hypersensitivity to casein, α -lactalbumin and β -lactoglobulin is associated with milk allergy [4]. The difference in the principal route of exposure between dander and milk proteins is the obvious explanation why different proteins derived from the same animal source seem to be associated with different allergic diseases. This was also demonstrated in this study. The levels of IgE and IgG antibodies as well as the proliferative responses of PBMCs to bovine epithelial antigen (BEA) and BDA20 were significantly higher in the group of cow-asthmatic subjects than in the control group, whereas no differences could be observed between the groups in the levels of anti-BSA, anti- α -lactalbumin and anti- β -lactoglobulin IgE and IgG antibodies or in the responses of PBMCs to these proteins. No difference was observed between the asthmatics and non-asthmatics in IgG levels and PBMC responses to casein, either.

One of the principal findings of this study was that the purified allergen, BDA20, can be successfully used as a

reagent in humoral and cellular analyses of respiratory cow allergy. Even though the BDA20 preparation was purified only by a single step of affinity chromatography (a purity level of 90% [9]) its use offered great advantages. The performance of both IgE measurements and lymphocyte proliferation tests increased considerably when BDA20 was used instead of BEA. The observation that the specificity and sensitivity reached the value of 100% in the measurement of specific anti-BDA20 IgE, the distinction between the two groups being highly significant ($P < 0.0001$), convincingly demonstrate the significance of BDA20 in respiratory cow allergy. In other words, analysing the anti-BDA20 IgE level of a patient appears to be sufficient for a reliable laboratory diagnosis of respiratory cow allergy. This is also illustrated in Fig. 3; subjects having a high anti-BEA IgE level but not a high anti-BDA20 IgE level were not detected.

It was interesting to find that there was a significant difference between the two groups in the level of the IgE antibody to one of the important milk proteins, casein. Although the significance of this difference was not statistically high ($P < 0.05$), this observation may suggest that certain proteins associated with milk allergy may also have a role in occupational respiratory cow allergy. In fact, this view is supported by reports according to which milk proteins, including casein, if inhaled may provoke respiratory sensitization [13,14]. Since bovine dander has been shown to contain casein or material crossreacting with it [5], it is possible that in certain cases casein or similar material can be an allergen contributing to the development of respiratory cow sensitization. It may be mentioned that three out of the 11 cow-asthmatic patients had clearly elevated levels (over the mean of control persons plus 2 SD) of specific IgE to various combinations of milk-derived proteins (data not shown). This may suggest that in addition to casein, other proteins associated with milk allergy (BSA, α -lactalbumin and β -lactoglobulin) may also contribute to the development of respiratory sensitization. On the whole, however, it is the cow dander proteins, not the milk-derived proteins, that seem to be associated with respiratory cow allergy. Additional evidence for this view can be obtained from correlation analyses showing that the highest correlations of specific IgE levels were observed between BDA20 and BEA on one hand and between different milk-derived proteins on the other hand. This indirectly points to the route through which sensitization has mainly developed. As for the positive correlation observed between anti-BDA20 and anti-casein IgE antibodies, we believe that it does not reflect structural similarities between these two proteins. When we compared the cDNA sequence of BDA20

with those of caseins in the EMBL and SWISS-PROT libraries no homologies were discovered (manuscript submitted). It may be concluded that in respiratory cow allergy casein is not a very significant aero-allergen, most probably a minor allergen. This is supported by the observations that anti-casein IgE values overlap strongly between the farmer groups and that the correlation between anti-BDA20 and anti-casein IgE is stronger among non-asthmatic than asthmatic farmers.

The asthmatic dairy farmers had significantly higher levels of anti-BDA20 and anti-BEA IgG antibody in their sera than their non-asthmatic controls (Fig. 4). However, since there was a considerable overlap in both antibody levels between the two farmer groups, it can be supposed that other factors, such as the level of exposure, may be involved in the development of IgG responses as well. In fact, we have previously shown that the level of exposure to cow- and swine-derived materials correlated positively with the level of specific IgG to these materials [15,16]. Because of the strong overlap of anti-BDA20 and anti-BEA IgG measurements, these tests are not useful in distinguishing cow-asthmatic farmers from non-asthmatic ones.

The positive PBMC stimulation results with BDA20 were encouraging in that they show that the T-cell epitope mapping of BDA20 will be feasible. On the other hand, for diagnosing cow asthma the results of PBMC responses were less useful than those of IgE measurements. This was especially true for BEA measurements, in which the sensitivity of the test remained low (at best 45%). By using BDA20 as a test reagent, sensitivity could be increased up to 82%. The fact that lymphocyte proliferation test does not give as clear-cut results as specific IgE measurements for distinguishing cow-asthmatic subjects from non-asthmatic ones is not a surprise since PBMC responses are not exclusively related to allergy. It has been shown previously, and indicated indirectly by our specific IgG measurements, that non-allergic persons may have allergen-specific T-lymphocytes in their peripheral blood [17]. It is important, however, to notice that in the lymphocyte proliferation test, better performance was obtained by using BDA20 than BEA as a test reagent. As for the specific immunotherapy of cow-induced asthma, this refers to a possibility that BDA20 may be a more appropriate choice than BEA.

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