

Quantification of a major bovine allergen by a two-site immunometric assay based on monoclonal antibodies

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A two-site immunometric assay based on monoclonal antibodies (mAb) was developed for the measurement of a 20-kDa major allergen of cow. The sensitivity of this assay was (BDA20) 1 ng/ml. It was used to measure airborne allergen concentrations in 10 Finnish cowsheds. The mean concentration of the BDA20 measured at two stationary sites was 280 ng/m³. Concentrations varied more than 10-fold among cowsheds (54–804 ng/m³). The mean intertest coefficient of variation was 8.2%, and the mean intratest variation 4.1%.

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Dairy farmers are exposed to various types of dust in their work environment; as a consequence, work-related respiratory symptoms are common among them (14). Airborne dust may induce respiratory disorders through various mechanisms. One important pathway is an immune response to organic material in the dust. Efforts are being made to assess the correlation between the level of specific sensitizing materials in the ambient air of the farming environment and respiratory symptoms. For persons working in cowsheds, cow-derived material is one of the major sensitizers (14, 16). It caused almost 40% of the 352 cases of occupational asthma reported in Finland in 1989 (8). We have previously quantified bovine epithelial dust with an immunoassay based on ELISA inhibition (18) and later with a more sensitive double-antibody sandwich ELISA (17). These studies have shown an association between the level of bovine-derived material in the air of the cowshed and farmers' humoral responses (19).

We have identified a 20-kDa protein (BDA20) as the major allergen of cow (20, 21). More than 90% of cow-reactive farmers had IgE response to this allergen. The aim of this study was to develop a quantitative immunoassay specific for this major allergen. A panel of monoclonal antibodies (mAbs) produced against bovine allergens was found to include three antibodies which recognize two different epitopes of BDA20. From these antibodies, we have developed a new two-site immunometric assay. The

new assay was shown to be highly specific and reproducible in measuring the allergen in cowshed samples.

Material and methods

Antigen preparation

Bovine, horse, cat, and dog dander allergen extracts (BDA, HDA, CDA, and DDA, respectively) were prepared essentially as described previously (20). The freeze-dried raw materials were purchased from Allergon AB (Ängelholm, Sweden). The materials were 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 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 36600 g for 30 min, the supernatants were dialyzed (mol. wt. cutoff 3500) for 24 h at 4°C against distilled water, lyophilized, and stored at –20°C. The protein concentrations of the antigen preparations were measured according to the method of Lowry et al. (11) with bovine serum albumin as a standard.

Monoclonal antibodies

Balb/c mice were immunized three times intraperitoneally with 100 µg BDA at 2-week intervals. Freund's complete adjuvant was used in the first

immunization and Freund's incomplete adjuvant in the subsequent immunizations. The mice were killed 4 days after the last immunization. mAbs were produced according to Galfre et al. (7). Briefly, spleen cells of immunized mice were fused with the NSI-1 mouse myeloma cell line with 50% polyethylene glycol (mol. wt. 1500). Fused cells were dispersed to several 96-well plates and were cultured in Dulbecco's minimal essential medium supplemented with glutamin, penicillin, streptomycin, 10% fetal calf serum (Gibco), and $1 \times$ hypoxanthine-aminopterin-thymidine. Hybridoma supernatants were tested with ELISA and immunoblotting methods, as described previously (22). Three hybridomas secreting antibody to the 20-kDa component were cloned three times by the limiting dilution method. The antibodies were purified in a protein-A Sepharose column by the method of Ey et al. (4).

Cross-reactivity and epitope specificity

Cross-reactivity of the mAbs against horse, cat, and dog dander extracts was studied by ELISA inhibition as follows. The ELISA plate was coated with BDA20 at a concentration of 0.5 µg/ml (coating buffer: 0.05 M carbonate buffer, pH 9.6). Hybridoma supernatant and several dilutions of the inhibiting extracts were combined in a test tube and were then added to the ELISA plate. Phosphate-buffered saline (PBS) containing 0.5% (v/v) Nonidet P40 (BDH Chemicals Ltd, Poole, UK) and 0.1% (w/v) casein was used as a diluent. BDA20 and BDA were used at concentrations varying from 3.9 to 2000 ng/ml, and HDA, CDA, and DDA from 97 to 200 000 ng/ml. Dilutions of mAbs were selected by preliminary test. After 1 h at 37°C, the plate was washed and incubated with the diluent for a further 30 min at 37°C. Peroxidase-conjugated antimouse Ig (DAKO) at a dilution of 1:1000 was added, and the plate was incubated for 1 h at 37°C. After washing, the substrate solution (tetramethylbenzidine, 0.1 mg/ml; Merck, Darmstadt, Germany) and urea hydrogen peroxide (BDH Chemicals Ltd), 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 with a Titertek Multiskan spectrophotometer (Flow Laboratories).

Epitope specificity was studied by coating the ELISA plate with BDA20 at a concentration of 1 µg/ml. Biotinylated mAb1 diluted 1:25 and hybridoma supernatant containing mAb1, mAb2, and mAb3 at various dilutions (1:2–1:1024) were combined in a test tube and added to the plate. After 1 h at 37°C, the plate was washed and blocked for 30 min at 37°C with the diluent. Vectastain ABC Elite reagent at 1:500 dilution was added to the plate.

The plate was incubated for 30 min at room temperature. After washing, the substrate solution was added and the reaction was completed as described above.

Affinity purification of BDA20

BDA20 protein was purified as described by Ylönen et al. (22). Briefly, purified mAb1 was dialyzed overnight against coupling buffer (0.1 M NaHCO_3 , pH 8.3, containing 0.5 M NaCl). Two grams of CNBr-activated Sepharose 4B was washed with 1 N HCl in a sintered glass filter for 15 min and then briefly with the coupling buffer. The gel and the antibody solution (30 mg/20 ml) were combined in a stoppered vessel and rotated overnight at 4°C. Excess antibody was washed out with the coupling buffer, and the remaining active groups were blocked with 0.1 M Tris-HCl, pH 8.0, at room temperature for 2 h. The gel was washed with three cycles of alternating pH with 0.1 M acetate buffer, pH 4, containing 0.5 M NaCl, followed by a wash with 0.1 M Tris, pH 8.0, containing 0.5 M NaCl. The gel (7 ml) was then transferred to a Bio-Rad Econo-Pac column, and BDA was passed three times by gravity through the column. After washing with 50 ml 0.1 M Tris, pH 8.0, and 50 ml 0.01 M Tris, pH 8.0, bound protein was eluted with 100 mM glycine, pH 2.5. The eluate was collected in 1-ml fractions and was neutralized immediately with 100 µl 1 M Tris, pH 8.0. Absorbance was measured at 280 nm, and protein-containing fractions were pooled, dialyzed overnight against PBS, and lyophilized.

Biotinylation

mAbs were biotinylated with a succinimide ester of biotin (2). Antibodies were diluted in 0.1 M sodium borate buffer, pH 8.8, to contain approximately 1 mg/ml antibody. Ten milligrams of *N*-hydroxy-succinimide biotin was dissolved in 1 ml dimethyl sulfoxide, and this solution was added to the mAb solution at 250 µg ester per mg antibody. The mixture was incubated at room temperature for 4 h. Next, 20 µl of 1 M NH_4Cl per 250 µg of ester was added, and the solution was incubated for 10 min at room temperature. The solution was dialyzed overnight against PBS and was stored at -20°C.

Air sampling in cowsheds

Air samples were obtained as described previously (19). Briefly, samples of airborne particulate material were collected from cowsheds during the winter of 1985–6 by a volumetric filter method according to the Finnish standard (5), with a constant airflow of 2–20 l/min through an MF-Millipore AAWP 03700

(pore size 0.8 μm) or comparable filter. Stationary samples were collected during the two working periods of 1 day. Samples were taken from feeding and manure passages. No interfering air was allowed to flow around the samplers. After collection, the filters were stored at -20°C . For measurement of the total concentration of the dust, filters were first weighed and then cut into pieces and suspended in 5 ml of 0.15 M PBS, pH 7.2. The suspension was sonicated four times (2.5 min each time) to release the particulate material from the filter. After centrifugation at 36 000 g for 45 min, the supernatant was collected and stored at -70°C .

Two-site immunometric assay

MicroELISA plates (Nunc-Immuno Plate Maxisorp F96 Cert., Roskilde, Denmark) were coated overnight with mAb3 at a concentration of 2 $\mu\text{g}/\text{ml}$. After washing, the plates were blocked for 2 h at 37°C with the diluent (PBS containing 0.5% (v/v) Nonidet P40 and 0.1% (w/v) casein) and then stored frozen at -70°C . Before use, the plates were allowed to reach room temperature and were then washed. BDA20 was added to each plate at twofold dilutions varying from 0.12 to 250 ng/ml. Air filter extracts were added to the plate at dilutions varying from 1:5 to 1:160, and the plate was incubated overnight at room temperature. After incubation, the plate was washed four times and was then further incubated with the diluent for 30 min at 37°C . Biotinylated mAb1 at a concentration of 0.5 $\mu\text{g}/\text{ml}$ was added, and the plate was incubated at 37°C for 2 h. After incubation and washing, the biotinylated antibody was detected as described above.

Results

Immunization of Balb/c mice with crude bovine dander extract and fusion of the spleen cells with mouse myeloma cells resulted in three hybrid cell lines secreting antibody against the 20-kDa major allergen of cow (BDA20). Epitope specificities of the three mAbs were studied by ELISA inhibition, as described in Methods. mAb1 and mAb2 were found to share the same specificity, whereas mAb3 reacted with a different epitope of BDA20 (Fig. 1). Species specificity of the mAbs was confirmed by ELISA inhibition with horse, dog, and cat dander extracts. Fig. 2 shows the inhibition curves when the binding of mAb1 or mAb2 (used in the final assay) against BDA20 was inhibited with cow and horse dander extracts. The inhibition curves observed with cat and dog dander extracts were similar to those in Fig. 2 showing no inhibition.

A double-sandwich ELISA involves the use of two antibodies, one fixed on the solid support to

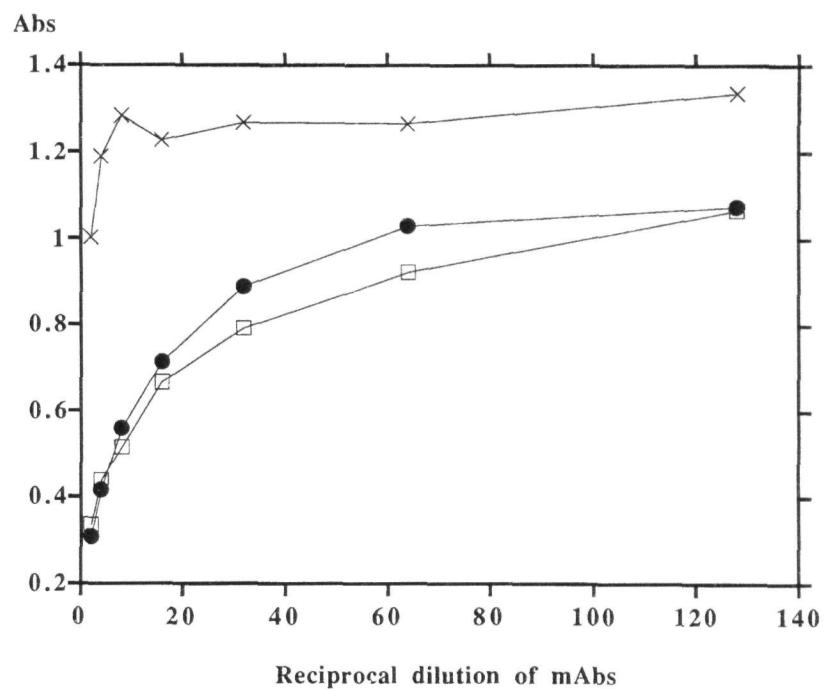


Fig. 1. Epitope specificity of three BDA20 specific monoclonal antibodies. Plate was coated with BDA20, and biotinylated mAb1 competed for binding sites with several dilutions of mAb1 (●), mAb2 (□), and mAb3 (x).

catch the antigen, and the other to detect the bound antigen. Aliquots of the three mAbs were biotinylated for the detection phase, and all paired combinations of mAbs were tested for their efficacy to detect BDA20. The highest level of sensitivity was obtained with the combination of mAb3 as catching antibody and mAb1 as detecting antibody. Confirming the epitope specificity tests, a combination of mAb1 and mAb2 proved to be nonreactive. Checkerboard titrations were done for antibody concentrations, coating and incubation times, and temperatures. In the final test, coating with the catching antibody (2 $\mu\text{g}/\text{ml}$) was done overnight at 4°C , and

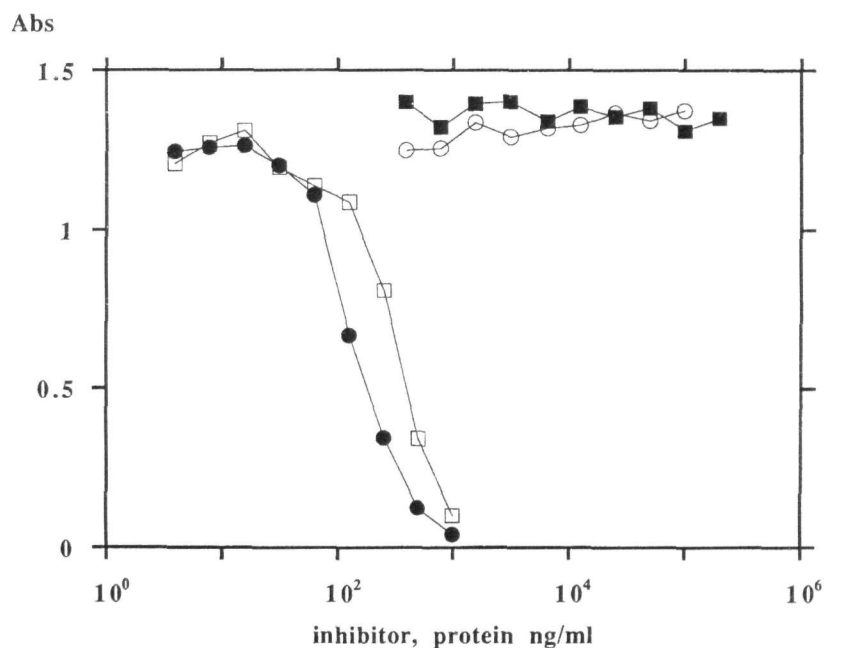


Fig. 2. ELISA inhibition curves. Binding of mAb1 and mAb3 to BDA20 was inhibited with cow dander (BDA) and horse dander (HDA). mAb1: BDA (●), HDA (■); mAb3: BDA (□), HDA (○).

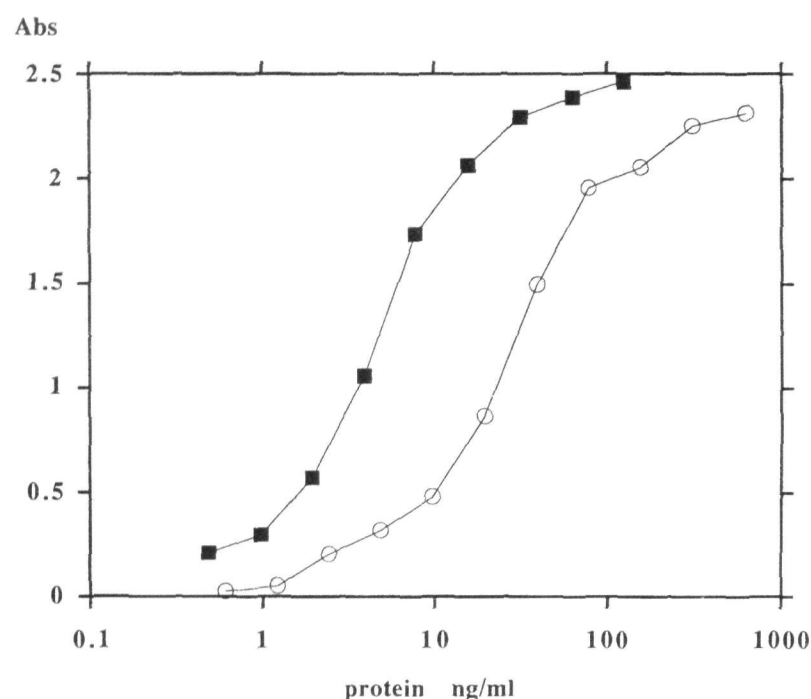


Fig. 3. Comparison of reactivity of BDA20 (■) and crude BDA (○) in two-site immunometric assay.

the incubation with the BDA20-containing specimen overnight at room temperature. After that, the reaction was continued as described in Methods.

After the test conditions were defined, the efficacy of the test for measuring airborne allergen concentrations in cowsheds was examined. Nineteen dust samples collected at two stationary sites of 10 cowsheds were measured by the two-site immunometric ELISA for the presence of BDA20. The mean BDA20 concentration was 266 ± 223 ng/m³ (SD) in the feeding passage and 295 ± 211 ng/m³ (SD) in the manure passage. Total dust concentrations were 2.04 ± 1.90 mg/m³ (SD) and 1.62 ± 1.20 mg/m³ (SD), respectively. As shown in Fig. 3, the sensitivity of the

assay was approximately 1 ng/ml, and about six times more crude BDA than BDA20 was needed to obtain a similar absorbance. The mean intratest coefficient of variation of the assay calculated from values of two samples tested four times at two different dilutions was $4.1 \pm 0.2\%$ (SD). The mean intertest coefficient of variation was calculated by measuring seven samples three to six times on different days. It was discovered to be $8.2 \pm 4.7\%$ (SD).

A difference between slopes of the standard curve and of the specimens may lead to inaccuracies in the calculated concentrations. Therefore, the slopes of the BDA20 standard curve and twofold dilution curves of three of the samples were compared. As shown in Fig. 4, the straight parts of the four curves were remarkably parallel.

Discussion

Several variations of immunochemical methods have been used to determine airborne allergen concentrations, and especially to measure amorphous and unviable materials, because these can rarely be quantified by other methods. Examples include the methods used to estimate levels of airborne allergens derived from plants (1), insects (9, 12), fungi (3), and mammals (10, 13, 15). mAbs have an established role in a number of immunoassays, providing them with high specificity and good reproducibility. The need to develop an advanced method to detect bovine allergens in cowsheds was one of the reasons for us to establish a panel of mAbs against the main bovine dander allergens.

As expected, the new two-site immunoassay for BDA20 proved to be more reproducible and slightly more sensitive than our earlier assays for bovine dander allergens based on polyclonal antibodies. The intertest coefficient of variation of the new test proved to be clearly lower (8.2%) than that of the polyclonal double-antibody sandwich assay (20%) (17). The low end of the linear part of the standard curve gives an indication of the sensitivity of the test. For the new test, the sensitivity limit was 1 ng/ml, as compared with 3 ng/ml of the polyclonal antibody-based assay (unpublished result). Our results are in accordance with those of other reports emphasizing the sensitivity and reproducibility of monoclonal-based immunoassays for measuring allergens. For example, in a mite major allergen assay, sensitivity was 5 ng/ml (12) and in a *Fel d I* assay, as high as 0.25 ng/ml (10).

In our test, the intertest coefficient of variation of 8.2% was twice as high as the intratest variation. The relatively high variation seemed to be associated with certain samples. This was possibly due to the fact that some samples contained more particulate material, and, as a consequence, the results varied

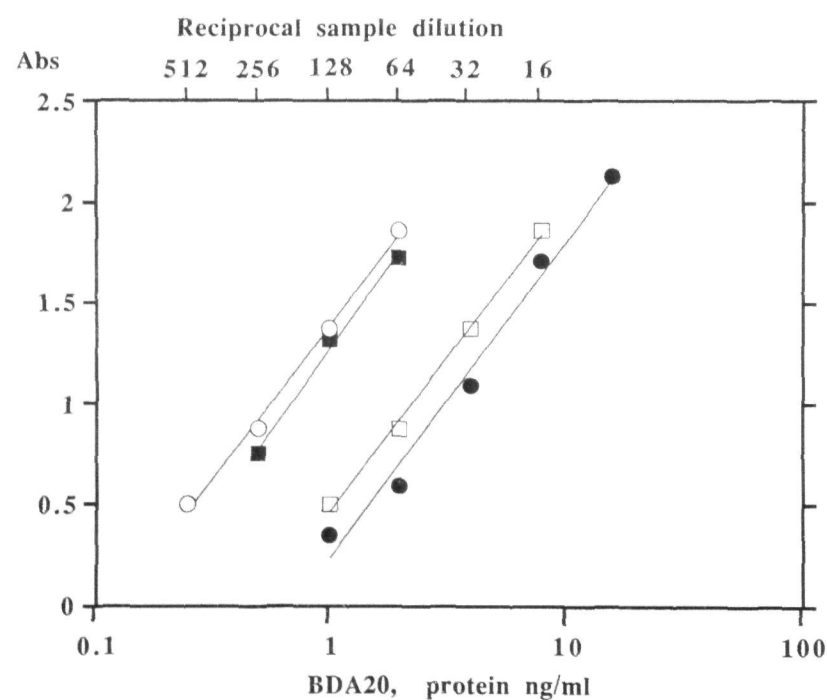


Fig. 4. Linear parts of BDA20 standard curve (●) and dilution curves for three samples (■, □, ○) in two-site immunoassay.

from test to test. This effect was not apparent in the intratest variation, which was only 4.1%. A similar intratest variation was detected in the *Fel d I* assay (10).

One of the problems encountered in immunoassays is the nonparallelism between the standard antigen preparation and sample dilution curves. This has been interpreted as an indication of partial antigenic differences between the standard antigen and the antigens present in the sample (6, 23). We originally observed this phenomenon when we measured bovine antigens by ELISA inhibition method (18). However, as shown in Fig. 4, when dust samples are measured by the new test, this does not seem to be a problem: the sample dilution and standard antigen curves are parallel. Most probably, this is due to the higher specificity of the new immunoassay, and indicates that only BDA20 is being measured, not related or altered molecules. The cross-reactivity of the catching and the detecting mAbs against heterologous animal danders was also tested. With cat, dog, and horse danders, no cross-reactivity was found.

The feasibility of the new immunoassay in practice was assessed by determining the BDA20 content of dust samples collected from 10 cowsheds. When BDA20 concentrations were compared among cowsheds, a considerable variation was observed (54–804 ng/m³). A similar variation in total dust and specific dust concentrations among different cowsheds was also detected previously by Virtanen et al. (19). In previous measurements made by polyclonal-based, double-sandwich ELISA, BDA concentrations measured from stationary sites varied from 2.1 to 13.0 µg/m³ (five cowsheds). Since BDA20 represents approximately 15% of the total protein concentration of BDA (Fig. 3), these results agree with our new results. This is the first time that an allergen-specific assay was used to measure airborne allergens in cowsheds. The closest comparable results have been obtained from laboratory-animal housing areas. Mouse urinary prealbumin concentrations in the disturbed air varied between 94 and 134 ng/m³, depending on the sampler. In the disturbed air of the rat or rabbit rooms, rat urinary prealbumin and rabbit AgR1 concentrations varied between 536 and 1358 ng/m³ and 3626 and 4897 ng/m³, respectively (13).

Measurement of airborne allergen concentrations is not the only application of the new BDA20 assay. It could also be useful in quantitating and standardizing allergen extracts prepared for skin prick tests, provocation assays, etc. Experiments along these lines are in progress.

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