

Short communication

Dispersion of horse allergen in the ambient air, detected with sandwich ELISA

Background: The objective was to establish an ELISA to detect horse allergen in ambient air and settled dust.

Methods: Monoclonal antibodies (mAbs) were produced against extracts of horse antigen. Two mAbs were selected and used in a sandwich ELISA. By the aid of portable pumps, air samples were collected in one stable and in the ambient air surrounding this stable. Furthermore, settled dust was collected by wiping spots with pieces of fabric, at sites within 500 m of the stable.

Results: Extracts of horsehair could be extensively diluted and still be positive. Extracts of cat and dog allergen failed to be detected. Furthermore, the mAbs were shown to detect an IgE-binding component. This was demonstrated by an ELISA using mAbs as capture antibody and sera from horse-allergic subjects as secondary antibody with readout depending on anti-IgE antibody. The sera with the highest RAST class to horse were positive in this ELISA. Airborne levels of horse allergen were over 500-fold higher in the stable than just outside the stable and over 3000-fold higher than at a residential building located only 12 m from the stable. Similarly, an inverse correlation was found between the distance to the stable and levels of “outdoor settled” horse allergen ($r = -0.9$, $P < 0.001$).

Conclusions: We have developed a sensitive, horse-allergen-specific, mAb assay allowing detection of low levels of horse allergens. Raised levels of horse allergen were found outdoors only in the close vicinity of the stable.

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The antigen responsible for allergic reactions to horses has been studied for the last 50 years (1–6). Horses are held to release large amounts of allergen, causing severe allergic reactions. However, studies supporting this anecdotal notion are scarce. Furthermore, Miles et al. found increased skin prick test (SPT) reactions to horse to be associated with patients with an atopic status and severe asthma, compared with patients sensitized to other allergens (7). A national Swedish official report of 1989 on allergic diseases and risk factors recommended a distance of 500 m between homes or schools and day-care centers and stables to protect susceptible individuals from exposure to horse allergen (8). Until then, a minimum distance of 200 m was the general recommendation in Sweden, mainly to protect nearby residences from odor and insect-related problems. However, many stables are located considerably closer to dwellings than that.

The recommendation of 1989 has given rise to many questions by public planning authorities, owing to the fact that objective knowledge of the dispersion of horse allergen in the environment has been lacking. Due to

methodological problems, the questions have remained unanswered until recently: it has simply not been possible to measure environmental horse allergens.

The aim of this study was primarily to test a newly developed ELISA with monoclonal antibodies (mAbs) to horse allergen, and to make a preliminary study of the dispersion of horse allergen in ambient air and settled dust.

Material and methods

ELISA

The mAbs were produced by four intraperitoneal immunizations of BALB/c mice with extracts of horse allergen. Mouse spleen cells were subsequently fused with mouse myeloma cell-line SP2/0, as described by Galfre et al. (9). After initial screening, two hybridomas were selected and subcloned twice. The two mAbs selected, which did not react with other allergen extracts such as cat and dog, were designated 103 and 14G4, respectively (MabTech AB, Stockholm Sweden). mAb 103 was the first, coating mAb. The assay was set up as a sandwich ELISA. mAb 103 (4 µg/ml) was added to 96-well Maxisorp microtiter plates (Nunc Roskilde, Denmark) at the amount of 100 µl/well. The plates were incubated overnight at

4°C. Subsequently, they were aftercoated for 1 h in phosphate-buffered saline (PBS) and 0.2% bovine serum albumin (BSA), 200 µl/well. The plates were washed three times, and standard dilutions and dilutions of samples were added, and allowed to incubate overnight at room temperature. Standard and samples were set up in duplicates. Next, biotinylated mAb 14G4 (1 µg/ml, 100 µl/well) was added and incubated for 60 min. After incubation with streptavidin-alkaline phosphatase (ALP) (mAbTech AB, Stockholm Sweden) diluted 1/1000, the substrate (*p*-nitro-phenyl-phosphatate disodium: Sigma, St Louis, MO, USA), diluted in 1 M diethanolamine buffer, pH 9.8, was added. The absorbance was measured with an ELISA plate reader (LabSystem Multiscan, Helsinki, Finland). As a positive sample, an extract of horsehair/dander from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK) was used.

Extracts of horse dander

Horse dander was obtained by grooming three horses. An amount of 100 mg horse dander was mixed with 10 ml PBS supplemented with 0.2% BSA and 0.1% Tween 20, followed by end-over-end rotation for 1 h. Subsequently, the samples were centrifuged at 400 g, and the supernatants were collected and analyzed in ELISA, as described above for the NIBSC horse extract.

Test of environmental air and dust samples

Seventeen air samples were collected in one stable and in the ambient air in the nearby surroundings (≤ 40 m) of this particular stable, located mainly south, southeast of the stable. Samples were also collected at a reference area over 500 m from any stable. All samples were collected over 2 days with similar weather conditions: sunny, dry, relative humidity of 55–70%, and a north to northwest wind direction (i.e., in the direction of the residential quarters) with a wind speed below 2.5 m/s.

Methods for collecting samples

Air samples were collected, outdoors and in the stable, as described elsewhere (10, 11). Briefly, samples were obtained with I.O.M. dust samplers (SKC Ltd, Blandford, UK), equipped with 25-mm Floropore Membrane Filters (Millipore AB, Sundbyberg, Sweden), with a pore size of 1 µm. Portable pumps with an airflow of 2 l/min were used (Casella, Casella London Ltd, UK). The pumps were allowed to run for 3 h, and the airflow was measured before and after sampling. Effective sampling time and average airflow were used to calculate the total volume of sampled air. The filters with airborne dust were left in the cassettes and kept at -20°C until extraction.

At the same locations as previously described, and also at sites within 500 m of the stable, settled dust was collected from hard surfaces (50×100 mm) at spots sheltered from rain, at a height of approximately 1 m from the ground. This was done to estimate the long-time allergen deposition, independent of temporary local wind conditions. The areas were wiped with pieces of fabric (compress) for 30 s. The compresses were then put into polyethylene bags and brought to the laboratory.

Extraction of samples

Air samples. The filters used for air samples were extracted after 1 h of end-over-end rotation in 2 ml of buffer (PBS supplemented with 0.2% bovine serum albumin and 0.1% Tween 20). Measured levels of horse allergen were expressed in units/m³ air, calculated from the NIBSC standard for extract of horsehair and dander. One unit is equal to 1 ng protein of NIBSC extract of horsehair and dander. The detection limit of the ELISA was 5 units/ml of the NIBSC.

Settled dust. Allergens were extracted from the piece of fabric in 10 ml of PBS supplemented with 0.2% BSA and 0.1% Tween 20. The piece of fabric was discarded and the buffer centrifuged. The supernatant was collected and frozen at -20°C until analyzed. The measured allergen levels in the samples, respectively, are given as total amount of horse allergen (U), by the ELISA method given above.

Statistical analysis

Statistical analyses were not performed to compare differences between airborne samples, due to the small number of samples. For settled dust, the correlations between allergen levels and the distance to the stable were computed with Spearman rank correlation, and *P* values of <0.05 were considered significant.

ELISA with mAbs and human sera

To detect whether or not the coating mAb detects an IgE-binding protein, we performed the following experiment. Microtiter wells were coated as above with mAb 103 and allowed to incubate with extract of horse allergen (100 ng/ml). Subsequently, sera, diluted 1:10, from seven subjects were added. The sera were chosen to give a spectrum of different RAST classes to horse. Three sera had RAST class 6, one had class 4, two had class 3, and one was negative in the RAST for horse. In the next step, a rabbit anti-human IgE serum (MIAB, Uppsala, Sweden) was added at a dilution of 1:5000. Subsequently, a rabbit anti-goat-Ig ALP (DAKO A/S, Glostrup, Denmark) diluted 1:2500 was added. Finally, the substrate was added as in the ELISA described above, and the absorbance was measured with an ELISA plate reader.

BIAcore analysis

The mAbs were tested by pairwise map analysis with BIAcore (Biospecific Interaction Analysis) Biosensor (Pharmacia & UpJohn, Uppsala, Sweden), essentially as previously described for rat allergen (12). Detection was through surface plasmon resonance in which real-time interactions between molecules are monitored as shifts in the reflection angle of a light beam over the gold-plated chip onto which the studied molecules are bound. The shift is recorded as a signal in resonance units over time in a sensogram. The analysis was performed as follows. Rabbit-anti-IgG1 was bound to the dextran surface of the sensor chip. mAb 103 or 14G4 was added, followed by blocking of unoccupied sites with control mouse IgG1. Extract of horse allergen was added and then a second antibody (103 or 14G4). If the second antibody binds the antigen, it indicates that the two tested antibodies recognize different epitopes on the same molecules or that the epitope is repeated.

Results

An initial ELISA experiment showed that higher sensitivity was achieved with mAb 103 for coating and 14G4 as the second mAb (data not shown). As standard, NIBSC horsehair/dander antigens were used. The detection limit was set to 5 ng/ml protein of the NIBSC.

An epitope-mapping experiment suggested that the two mAb, 103 and 14G4, recognized different epitopes on the same molecule. Thus, binding could take place when one mAb was used as capture antigen and the other as detection antibody, but it was abolished when the same antibody was used twice.

Testing of the sensitivity and specificity by use of

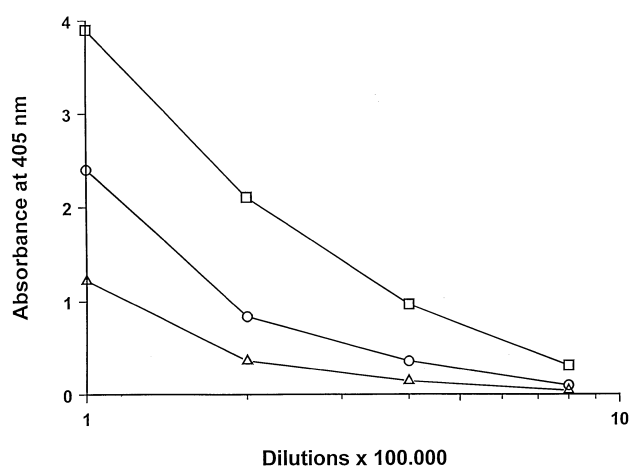


Figure 1. Three extracts of horse dander assessed in sandwich ELISA described. x-axis: dilution of three extracts; y-axis: absorbance at 405 nm.

extract from three horses showed that these extracts could be diluted several hundred thousand times and still be positive in the test (as depicted in Fig. 1). In contrast, extracts of cat and dog dander were not positive (data not shown).

Seven human sera were employed in an ELISA to determine whether or not the mAbs detected an allergen. The three sera with RAST class 6 to horse displayed IgE activity to the horse antigen bound by mAb 103. In contrast, the remaining sera with a lower RAST class to horse, as well as a serum which was RAST class negative, failed to react.

In the two indoor air samples from the investigated stable, horse allergen was found at amounts of 591 560 and 286 400 units/m³ air, respectively (mean 438 980 units/m³). The content of horse allergen in an outdoor air sample collected just outside the stable entrance was 1140 units/m³, whereas that detected outside the closest residential building (12 m away) was 150 units/m³ (mean). Thus, airborne levels of

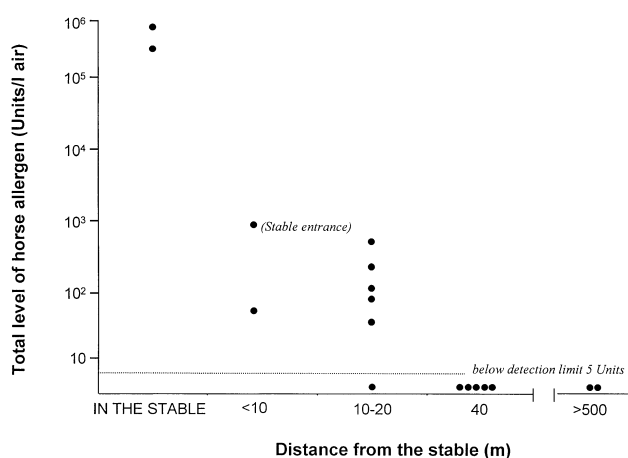


Figure 2. Airborne levels of horse allergen (units/l air) collected in stable and outdoors at different distances from stable.

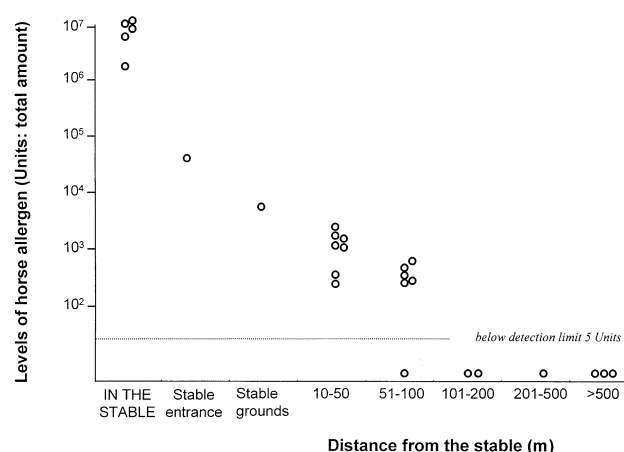


Figure 3. Levels of horse allergen (units*) in settled dust collected in one stable and at different distances from stable (at places sheltered from rain). *Total amount of horse allergen per piece of fabric.

horse allergen were over 500-fold higher in the stable than just outside the stable and over 3000-fold higher than at a residential building located only 12 m from the stable. Horse allergen could not be detected in air samples collected at 40 m, nor in the reference area over 500 m from stables (Fig. 2).

In outdoor settled dust (collected at places sheltered from rain), the total amount of horse allergen in samples was also substantially lower than in samples from inside the stable. A negative correlation was found between analyzed levels of horse allergen in settled dust and the distance to the stable ($r=0.9$, $P<0.001$). At distances not less than 100 m from the stable, horse allergen could not be detected (Fig. 3).

Discussion

We have generated horse-allergen-specific mAbs and designed a sandwich ELISA. Our purpose in this study was to test the utility of this assay in environmental analysis. To this end, we collected both air samples and settled dust from a stable and its vicinity. In this way, the dispersion of the horse allergen from a stable into ambient air could be assessed. As expected, the levels of horse allergen decreased with increasing distance from the stable. To obtain a detailed and reliable dispersal pattern, one would need a much larger investigation. Several aspects have to be taken into account. First, data from several stables should be studied. Moreover, weather conditions, such as wind speed and direction, differ locally even over a short time (due to microclimate and turbulence spectra). Therefore, air samples should be collected over a prolonged period of time, to reflect true long-time dispersion. Furthermore, local "topographic" features, such as buildings and trees, should be considered. It seems

clear that the total levels of airborne, but also settled, horse allergen in dust collected just outside the stable entrance and in the close vicinity of the studied stable were considerably lower than the corresponding levels in indoor samples of the stable. This may indicate that horse allergen is mainly to be found airborne in the vicinity of the animal. This has been discussed by other authors, in this context, as occurrence of horse albumin in the environment (13). However, one may not totally exclude the possibility of long-distance dispersion of animal allergens outdoors in special weather conditions before more investigations have been performed.

We are not aware of any studies of the occurrence of the dispersion of horse allergen in the environment. In contrast, several investigations have dealt with the characterization of horse allergens. For example, Gregoire et al. have developed mAbs to the horse allergen Equ c 1 (3). In that study, Equ c 1 was cloned and sequenced. The amino-acid sequence revealed Equ c 1 to be a member of the lipocalin superfamily. Lipocalins are small, mainly extracellular proteins carrying small, hydrophobic molecules (14). Lipocalins have been found in urine, saliva, tears, and

serum. The homology between Equ c 1 and rodent urinary protein, also a member of the lipocalin family, was 49–51%. Interestingly, an allergen belonging to the lipocalin family has been detected also in bovine dander (15).

Using sera from subjects RAST positive to horse allergen, we demonstrated that the mAbs used in the present study detected an IgE-binding protein. We are presently in the process of further characterizing the allergen recognized by these mAbs.

In conclusion, the ELISA method to detect horse allergen has a sensitivity that allows measurements of low levels of allergen in environmental samples. In the vicinity of a stable, the outdoor levels of horse allergen were increased, but allergen dispersal seemed to be limited, as horse allergen could not be detected over 100 m from this particular stable.

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