ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN - POLONIA
VOL.LX, SUPPL. XVI, 436 SECTIO D 2005

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Immunological methods of birch pollen allergens detection

Immunologiczne metody wykrywania alergenów pyłku brzozy

Pollen grains – male plant gametes are necessary for plant reproduction. They usually have regular shapes, resembling a sphere or spheroid. Dimensions of a live pollen grain varies – it can expand or shring depending on amount of water absorbed. Dimensions of dead and emptied pollen grains seem to be constant an between 5 to 200 µm. Pollen grains of anemophilous plants have 17 to 58 µm in diameter. Material obtained from aqueous pollen extraction contains a complex mixture of proteins, glycoproteins, stains, carbohydrates and low molecular substances. Many substances among these are allergens responsible for evoking clinical symptoms in sensitive persons. Most authors claim that pollen allergens are located above all in the inner layer – intine and come out to the surface through pores (3,5). When pollen grain comes into contact with surface of nasal or pharyngeal mucosa, some of the enzymes from intine, released first by the grain, can facilitate penetration by allergens into mucosa (1,2,4,6,7).

Albeit some studies exhibited presence of antigens in other parts of a plant, like root, stalk and leaves, pollen grain remains the most important source of allergens. It is supposed that apart from pollen grains, in most anemophilous plants small (0.5 – 2 µm) granularities composed from sporolenines, located in anther wall, are also vectors for allergens. These granularities were found on the inner wall of pollen grains of most anemophilous plants. Due to their dimensions, they can easily reach lower airways. In analysis of bronchoalveolar lavage (BAL) fluid from patients after 4-hour exposure in peak pollen release period we found fine grains of sporopolenines which we recognized in that time as fragments of damaged pollen walls (6).

AIM

The aim of the study was to assess the usefulness of immunodiffusion, counter-current immunodiffusion and latex test in determination of concentration of birch pollen allergens.

MATERIAL

The initial material for the study included fresh birch obtained directly from blossoming plants by putting plastic bags onto single inflorescences.

METHOD

Birch (*Betula verrucosa*) allergen solution was obtained according to own technology using fresh birch pollen grains from inflorescences blossoming in heightened temperature. Birch twigs from the trees, being observed for over several years, were cut off when the inflorescences were well-shaped and soft. The time of twigs with inflorescences cutoff and transfer of these into a chamber with 18°C temperature was chosen as the date 2 days before forecast of birch pollen release start. For birch pollination start forecast the cumulative temperature method was used, summing up temperatures over 5°C in consecutive days. Each inflorescence was secured in a sealed bag, being concurrently a case for collected pollen grains. Immunized rabbit serum was obtained by birch pollen allergen administration (20 µg of allergen with incomplete Freund adiuvant) subcutaneously triple with a week interval.

Immunodiffusion test - was performed on the agarose (agar gel BASICA SHE with EEO above 0.3, in TAE buffer). The gel was poured over 45mm Petri dishes. After the gel set, five 4 mm wells were cut (1 in the center and 4 located radially 10mm from the central well). The bottom of each well was covered with a drop of gel. Serum from rabbit immunized previously with birch pollen allergen was introduced into the central well. 4th remaining wells were filled with examined aqueous solutions obtained by washing microtubes in which substances from examined air samples sedimented. Petri dishes prepared in such manner were incubated for 48hrs in a humid chamber in 37°C, then in 20°C over 5 days. The samples were subsequently stained with Coomassie Brillant Blue G-250. Some of the samples were stained with amide black. After the staining, precipitation lines were evaluated.

Counter-current electroimmunodiffusion test - was performed on the agarose (agar gel BASICA SHE with EEO above 0.3, in TAE buffer, 2.5hrs. 4,5 V/cm). The gel was poured over microscope slides so as to form 2mm gel layer. In the center 3mm wells were cut in two rows, two wells in each row. The wells were separated with a 4mm space. The bottom of each well was covered with a drop of gel. Serum from rabbit immunized with birch allergen was put into the wells on the side of anode. Into the first well non-diluted serum was put, into the second one – diluted by half. Into cathode-side wells the examined the obtained dilutions from washing Burkard apparatus samplers were put (after analyzing of 1, 5 and 10 cubic meters of air). Electroimmunodiffusion test was performed in an apparatus built by the author, at 300V. Electrodes of the device and paper straps which conducted voltage to the agar gel dishes were immersed in TAE buffer. In the next tests a veronal buffer (pH 8,6) was also used. Staining process was identical to immunodiffusion procedure. After the staining, precipitation lines were evaluated.

Latex test - Latex agglutination is a convenient and quick test. Positive result can be observed almost immediately after mixing of antigen with latex coated with globulins. Agglutination reagent was globulin-covered and albumin-stabilized latex with sodium azide as a preservative. The reagents are highly stable, and diagnostic lab does not require special equipment. Latex test was conducted with self-coated polystyrene latex. Coating was performed in a water bath at 37°C over 2hrs with constant mixing. Further, latex was centrifuged and suspended in a solution of glycine-buffered saline (GBS) by Fisher Diagnostic (pH 8.2) Such homogenic suspension of 1% globulin-coated latex was mixed with the equal volume of GBS solution with sodium azide and 0.3% fatty acids-free bovine albumin. To obtain minimal concentration of an allergen that can be detected with latex agglutination, a dilution series of a test allergen used for intranasal provocation (solutions were prepared from freeze dried allergen). The determination was performed on a dish from black glass with separate areas preventing solutions mixing. On successive areas, 0.02 ml of latex reagent and 0.02 ml of examined antigen dilution were put. After 5 minutes, results were read out. Circular agglutination was considered a positive result.

Immunochromatographic test for allergen detection – theoretical assumptions.
In clinical routine, immunochromatographic tests are used for detection specific proteins of Rotaviruses and Adenoviruses. Use of these tests is simple. One needs only to dilute a sample of examined substance in a buffer (it is ready for use). The specificity of the test is ensured by the use of monoclonal antibodies against specific for Rotavirus and Adenovirus protein units, conjugated with particles

of gold. Test strips are covered with monoclonal immunoreagents specific for Rotavirus and Adenovirus. Performance of currently available tests is very simple. Liquid sample and conjugate migrate together along the strip due to capillarity. When the mixture reaches first specific monoclonal reagent on the strip (test area), Rotaviruses or Adenoviruses present in the sample are blocked and this immunological reaction produces a blue-red stripe. Then, the mixture migrates on along the strip, until it reaches another, non-specific, murine reagent (control area), where another blue-red stripe appears. Control stripe serves as control for proper sample propagation and successful chromatography. Providing proper test conduction, control stripe appears every time.

Bulk manufacturing of quick immunochromatographic tests for basic allergens detection seems possible at present state of knowledge, and allergologist community is interested in the usage of these types of tests in everyday practice (1,8).

RESULTS

Lowest birch pollen allergen concentration which produced precipitation lines in immunodiffusion and electroimmunodiffusion assays, equaled approx. 35 EU/ml. It corresponds to about 150 birch pollen grains in $1m^3$ of air. The lowest birch pollen allergen concentration detected by latex agglutination equals 0.012 mg/ml which corresponds to about 3µg in a sample.

Due to various standardization methods of test allergens which are used by individual manufacturers, it is very difficult to relate these results to standard units used by manufacturers. To do this, much more measurements are needed.

CONCLUSIONS

1. The results of our evaluations allow to claim that detection thresholds for birch pollen allergens with immunodiffusion and counter-current electroimmunodiffusion are comparable.
2. Using immunodiffusion, results are available up to several times quicker.
3. Sensitivity of latex test is much lower, than immunodiffusion and electroimmunodiffusion, but still sufficient for detecting of clinically relevant concentrations of an allergen (sufficient to evoke symptoms in susceptible individuals).
4. To estimate amount of allergen in a specific quantity of pollen grains, more measurements are needed.

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9. This work was financially supported by grant No NZC/W1/2002-2004 from Medical University of Warsaw

STRESZCZENIE

W praktyce alergologicznej stosowane są aerobiologiczne metody badania stężenia ziaren pyłku roślin i spor będących źródłem alergenów. W przypadku wydostania się alergenu poza ziarno pyłku lub zarodnik może dochodzić do sytuacji gdy stężenie faktycznie unoszącego się w powietrzu alergenu nie koreluje ze stężenie nośnika czyli ziaren pyłku roślin czy zarodników. Zdarza się to przede wszystkim w trakcie opadów deszczu i w wyniku działania na ziarna pyłku i zarodniki zmiennych czynników fizyko-chemicznych. Autorzy przedstawiają wyniki wstępnych prac mających na celu sprawdzenie inowacyjnej metody pomiaru stężenia alergenów brzozy brodawkowatej w powietrzu. W badaniach wykorzystano pomiar cząstek zawieszonych w powietrzu atmosferycznym aparatem firmy Burkard przeznaczonym do badań immunologicznych, wyposażonym w mikrodołki. Autorzy przedstawiają metodykę pomiarów stężenia alergenów brzozy *(Betula ver.)* z wykorzystanie metody immunologicznej z przeciwciałami poliklonalnymi przeciwko alergenom brzozy. Wyniki badań pozwalają na stwierdzenie, że próg detekcji alergenów pyłku brzozy jest zbliżony przy zastosowaniu metodą immunodufuzji i elektroimmunodyfizji przeciwprądowej.

SUMMARY

Current allergology practice uses established aerobiological methods for determination of pollen grains and spores concentration, being allergens source. When allergen leaves pollen grain or spore, a discrepancy between actual concentration of airborne allergen and concentration of the carrier, i.e. pollen grains or spores, may occur. It takes place mostly during rains and when pollen grains and spores are exposed to varying physicochemical conditions. The authors present findings from preliminary studies which are aimed to verify an immunological method of determination birch allergens in the air. In this study, immunological analyzer by Burkard utilizing microwell technology was used for determination of particles suspended in atmospheric air. The authors present methodology of measurement of birch allergen (*Betula ver.*) concentration using an immunological method with the usage of policlonal antibodies anty-birch's pollen. The results of our evaluations allow to claim that detection thresholds for birch pollen allergens with immunodiffusion and counter-current electroimmunodiffusion are comparable. The results of our evaluations allow to claim that detection thresholds for birch pollen allergens with immunodiffusion and counter-current electroimmunodiffusion are comparable.