

Immunological and physical properties of allergen solutions

Effects of nebulization

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Lyophilised birch pollen allergen extracts, reconstituted with different diluents (H₂O, saline, Albumin diluent* (AD)) were investigated to determine whether the allergen activity and quality of the extracts deteriorated by nebulization with different nebulizers (Pari, Wright, and Sandoz). Allergen activity was measured by IgG₁ RAST inhibition technique and allergen quality was analysed by crossed immunoelectrophoresis (CIE). The distribution of particle sizes of aerosols of different allergen solutions was determined by a TSI Aerodynamic Particle Sizer. A significant difference ($P < 0.05$) in allergen activity was found between the AD and H₂O diluents before and after using a Sandoz nebulizer and a Wright nebulizer equipped with a small chamber. This suggested greater allergen activity in AD-diluted solutions, and the pattern was repeated with the other two nebulizers, but was not statistically significant. The samples diluted with saline showed no significant differences in quality after nebulization except for the impacted aerosol in which one of the precipitates was slightly diminished. In the AD-diluted sample one of the precipitates disappeared from the impacted aerosol and from the nebulization chamber after 2 min nebulization. To elucidate whether the extract proteins had been bound by albumin, samples were submitted to CIE with rabbit anti-human albumin in an intermediate gel. By this procedure one precipitate was transformed to a precipitate in the intermediate gel, indicating that one or more proteins in the extracts may associate with albumin. No significant difference in output was observed between the nebulizers. The particle size distribution curves for each diluent (saline, AD) were identical for the Wright nebulizer with 99% of the dry particles distributed within 0.5-2.0 µm. Although allergen solutions reconstituted with different diluents were not deteriorated in different nebulizers, it may be that the addition of human albumin to an allergen solution may induce one or more proteins in the extracts to associate with it. This could reduce allergen adsorption over periods longer than this study. The use of different diluents did not change the distribution of particle size generated by the nebulizer tested.

Key words: aerosols; allergen activity; allergen quality; allergen stability; nebulizer output; particle size distribution; RAST inhibition.

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In the aetiological diagnosis of bronchial asthma, inhalation provocation tests are essential for determining the extent of specific or non-specific airway reactivity (2, 9). In these tests, solutions of allergen extracts and pharmacological irritants are nebulized and administered to the patients (13, 19). To improve reproducibility, sensitivity,

and specificity of these tests, standardization of the procedures is essential, and as several factors influence the response to a bronchial challenge, each factor must be described and controlled.

The technical and methodological factors include aerosol generation and inhalation (2, 9), preparation and storage of test solutions, mea-

surement of the response, and expression of the results. The non-technical factors include patient characteristics such as baseline airway caliber, medication, environmental factors and medical diseases.

The first part of the present study was designed to investigate whether different types of nebulizers and diluents used to reconstitute lyophilised allergen extracts might cause a decline in the allergenic activity of the aerosol. The second part was designed to determine the distribution of the particle sizes of aerosols obtained after reconstitution with different diluents.

MATERIAL AND METHODS

Allergen extracts

Betula verrucosa (birch) pollen extracts 100,000 biological units per ml (BU/ml) (Pharmalgen®, Allergologisk Laboratorium A/S (ALK), Copenhagen, Denmark), standardized according to the Nordic guidelines, were used (6). Allergen was obtained as lyophilised extract, and the same batch was used in all experiments. Extracts for testing were reconstituted and diluted immediately before use in 1) Albumin diluent® (AD) (0.03 % human albumin, 0.9 % saline, 0.4 % phenol) obtained from ALK; 2) sterile isotonic saline (saline); 3) sterile H₂O (H₂O). The latter two diluents were delivered from the hospital pharmacy.

Allergen quantitation

Allergen activity was measured using a RAST inhibition technique (17) modified from the IgG subclass RAST developed by Djurup (7). 500 µl of the birch allergen extract diluted to 500 BU/ml were added to the test tubes (Maxisorp®, polystyrene tubes, 70 × 11 mm, NUNC, Roskilde, Denmark), and the tubes were incubated overnight at room temperature. After coating, the tubes were washed three times with phosphate buffered saline (PBS).

Serum from a patient who had been undergoing birch immunotherapy for 3 years was used for IgG₄ RAST inhibition. The allergen sample was diluted 1:10, mixed with serum diluted 1:40

in incubation buffer (sodium phosphate 0.1 mol/l, sodium chloride 0.15 mol/l, sodium acid 10 mg/l, EDTA 7.4 g/l, bovine gammaglobulin 5 g/l, and normal rabbit serum 10 % (w/v), pH 7.0) including polyethylene glycol 6,000 2 % (w/v), and incubated overnight at 4°C. The next day, the incubation mixture was centrifuged (1800 g/20 min), and the supernatants tested in duplicate in the IgG₄ RAST: 500 µl were added to the allergen coated test tubes and incubated for 3 h at room temperature. After three washings, 500 µl of anti-IgG₄ monoclonal antibody (Unipath, Bedford, England, diluted 1:32,000) were added to the tubes and incubated for 3 h. After washing, the tubes were incubated overnight with 500 µl of iodine-labelled rabbit anti-mouse IgG (DAKO, Copenhagen, Denmark), and on the following day washed and counted in a gamma-scintillation counter. Counts per minute corrected for background were used for further calculations.

The coefficient of variation was 11.7 % between duplicate determinations of the same allergen sample, and 10.4 % between duplicate samples. To eliminate day-to-day variations, all samples from each nebulizer were processed on the same day.

Analysis of allergen quality

CIE was performed according to Højby (16, 25) using 1 % agarose (HSA-grade, Litex, Denmark) in Tris-barbital-buffer (0.024 M sodium barbital, 0.073 M Tris and 0.01 M sodium acid pH 8.6). In the first dimension 10 or 20 µl of antigen were applied and processed for 30 min at 10 V/cm. The second dimension was processed for 18 h at 2 V/cm using 12 (or 24) µl/cm² of rabbit antiserum against birch allergen extract (prepared according to Harboe & Ingild (15)). For detection of human serum albumin (HSA), an antibody against HSA (DAKO, Copenhagen, Denmark) was applied (30 µl/cm²) to an intermediate antibody gel. The plates were washed, pressed and dried in hot air (16) and stained with Coomassie blue.

Nebulizers

Four nebulizers used for routine bronchial provocation tests were selected: 1) a Pari nebulizer

No. 16 (Paul Ritzan Pariwerke, Stanberg am See, Germany). The nebulizer and the nebulization chamber are made entirely of polycarbonate material; 2) a Sandoz 1500 jet nebulizer (Sandoz Products, Rugby, Warwickshire, England). The nebulizer is made of polycarbonate material; and 3) and 4) a Wright jet nebulizer (28). The nebulizer is made of methylmethacrylate, whereas the nebulization chamber is made of glass. The latter nebulizer was used with a standard reservoir 3) and with a newly constructed small reservoir 4) made of methylmethacrylate (20). The same nebulizers were used throughout the study. The Wright and Sandoz nebulizers were operated by compressed air at 1.3 bar at a flow of 12.5 l/min. The Pari nebulizer was operated by a pressure of 1.3 bar at a flow of 5 l/min.

The output from each nebulizer was determined by weighing on a Mettler balance the complete nebulizer before and after 2.0 min nebulization of 2 ml diluent. Ten determinations were made for each diluent (AD, saline, H₂O) and each nebulizer and mean $\pm$ SD is given as result (Table 1).

Analyses of aerosol particles

The aerosol was analysed during 100-s sampling. Electrostatic charge on aerosol particles was neutralized by a KR⁸⁵ radioactive source (TSI model 3054) (5). The liquid droplets were then evaporated in a diffusion dryer and led to a mixing chamber for dilution with dry air (TSI diluter 3302). The aerodynamic diameter could subsequently be analysed in an aerodynamic particle sizer, model APS 3300. The velocities of the individual particles in the air stream were measured by a two-slot laser velocimeter focusing a photomultiplier tube. The particle passing through the two beams produces two electrical impulses. The time interval between the two impulses is inversely proportional to the particle velocity, and directly proportional to particle aerodynamic diameter. Aerosols were examined with regard to dry particles, the relationship between the size of dry particles and saturated droplets according to (10). The aerodynamic particle sizer was calibrated with Latex particles (dia-

meter 0.51 μ m, 0.794 μ m and 1.1 μ m (Standard Particle Unisphere, Serva), 2.02 μ m and 5.05 μ m (Duke Scientific Latex)). Particle size distribution was determined three times for each nebulizer and for each solution. Results are reported as number of particles versus log aerodynamic diameters (17 logarithmic steps which equalized 99% cumulative number of particles determined).

Protocol

In Part I qualitative and quantitative deterioration, if any, of allergenic activity was compared for each type of nebulizer and diluent.

To elucidate the quantitative recovery of allergenic activity, birch allergen diluted to a final concentration of 10,000 BU/ml with AD, saline, and H₂O, respectively, was nebulized twice for 2 min using each of the four nebulizers described above. Four samples were drawn during each run from 1) the vial in which the lyophilised extract was reconstituted, 2) from the nebulization chamber before, 3) after 1 min, and 4) after 2 min of nebulization. The allergenic activity in these samples was determined by RAST inhibition.

CIE was used to verify whether the individual proteins in the allergen solution were nebulized and still present in the aerosol. Using a Finn pipette, four samples of each diluent (AD and saline) were drawn: sample 1 from the original vial, sample 2 from the nebulization chamber before nebulization, sample 3 from the impacted aerosol, and sample 4 from the nebulization chamber after nebulization. The aerosol was produced by the Wright nebulizer. The impacted aerosol was collected by leading it into a spiral tube which induces the liquidized aerosol to impact on the wall (Fig. 1).

Part II describes the distribution of particle size. We wanted to see whether changes in diluents altered the distribution of particle size for a jet nebulizer (Wright). The allergen solutions tested were diluted with AD and saline.

Statistics

Mean and standard deviations are given. The 95% confidence interval was calculated by means

Table 1

Output determined for three different nebulizers and the Wright nebulizer equipped with a standard chamber and a small chamber. Three different diluents have been used ($P > 0.05$)

	Output			
	Pari	Sandoz	Wright standard chamber	Wright small chamber
H ₂ O				
$\mu\text{l} \times \text{min}^{-1}$)				
SD	222	333	147	136
	60	10	10	20
Saline				
$\mu\text{l} \times \text{min}^{-1}$)				
SD	221	341	146	132
	40	20	10	10
Albumin diluent*				
$\mu\text{l} \times \text{min}^{-1}$)				
SD	253	345	145	129
	80	10	10	10

of t-distribution (1). Logarithmic transformation was carried out when appropriate. Least-square

regression was used for analysis of linear relationship. $P < 0.05$ was considered significant.

RESULTS

Part I

Fig. 2 shows the allergen activity for each diluent (AD, saline, H₂O), nebulized by the four nebulizers before and after nebulization for 1 and 2 min, using the RAST inhibition technique. Four dots represent two experiments with two determinations. No significant differences were observed between the two experiments and two determinations for each diluent. Statistically significant changes ($P < 0.05$) in allergen activity were found between the solutions diluted with H₂O and AD for two of the nebulizers: Wright equipped with a small chamber, and Sandoz, but the pattern, although not statistically significant, was seen for all nebulizers with solutions diluted with AD, indicating greater allergen activity in these solutions than in those diluted with H₂O and NaCl before and after nebulization. No statistically significant differences were observed between any of the samples drawn from the original vials and nebulization chambers ($P > 0.05$).

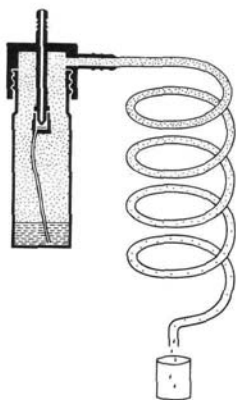


Fig. 1. Device for collecting the impacted aerosol by leading the aerosol into a spiral tube which induces the aerosol to impact on the wall.

Table 2 gives the CIE test results of the four samples of allergen solutions. The four samples diluted with saline showed three precipitates. Samples 1, 2, and 4 showed identical patterns, whereas sample 3 (the impacted aerosol) showed diminution of one of the precipitates.

The four samples diluted with AD showed precipitates in the samples from the original vial

5, and from the nebulization chamber 6 before nebulization, with the exception of one of the precipitates which disappeared in samples 7 (the impacted aerosol) and 8 (the nebulization chamber after nebulization).

To investigate whether the extract proteins had been bound by albumin, sample 4 taken from the nebulization chamber after 2 min nebulization of both diluents (AD and saline) was subjected to CIE with rabbit anti-human albumin in an intermediate gel 4a (Fig. 3). This procedure transformed one precipitate from both solutions to a precipitate in the intermediate gel, indicating that both dilutions contained albumin, whereas the AD-diluted allergen contained a larger amount of albumin 8a (Fig. 3). That one precipitate disappeared from the anti-allergen antibody gel may indicate that one or more proteins in the extract are associated with albumin.

Part II

There was no significant difference in output function between the three nebulizers as a function of diluent (Table 1). The particle size distribution curves measured for dry particles of each allergen dilution (AD and saline) were identical for the Wright nebulizer (Fig. 4).

DISCUSSION

An aerosol is a mechanically stable but thermodynamically labile system containing water in a close temperature- and humidity-dependent equilibrium with the water vapour of the surrounding air. Thus, great difficulties are encountered when determining allergen activity in the aerosol droplets. We determined the allergen activity of the allergenic extracts (solutions) remaining in the nebulizers during nebulization. The quality of aerosolized allergens was examined by determining the relative distribution between individual components in a sample from the aerosol. This sample was collected in an impaction device (Fig. 1) constructed so as to reduce the risk of allergen denaturation.

Using IgG₄ RAST inhibition technique, the activity of allergen solutions diluted with three

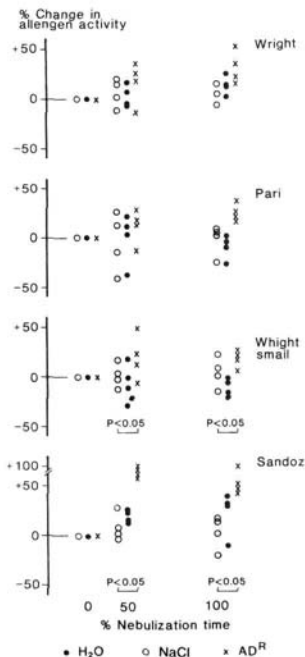


Fig. 2. Allergen activity before and after 1 and 2 min nebulization by IgG₄ RAST inhibition technique for each diluent nebulized by four different nebulizers (Wright, Pari, Whight small chamber, and Sandoz). A statistically significant change ($P < 0.05$) in the allergen activity before and after nebulization was found between the solution diluted with AD and H₂O for two nebulizers, Wright small chamber and Sandoz (analysis of variance with a 95% confidence interval for mean).

Table 2

Four samples of allergen solutions diluted by either saline or AD described by CIE

Samples from:		Diluent	Intermediate gel	Precipitate No.		
				1	2	3
1.	Tube	saline	empty	+	+	+
2.	Nebulization chamber before nebulization	saline	empty	+	+	+
3.	Impacted aerosol	saline	empty	±	+	+
4.	Nebulization chamber after nebulization	saline	empty	+	+	+
5.	Tube	AD	empty	+	+	+
6.	Nebulization chamber after nebulization	AD	empty	+	+	+
7.	Impacted aerosol	AD	empty	-	+	+
8.	Nebulization chamber after nebulization	AD	empty	+	+	+
4a.	Nebulization chamber after nebulization	saline	anti-alb*	+	+	-
8a.	Nebulization chamber after nebulization	AD	anti-alb**	-	+	-

* A small precipitate appeared in the intermediate gel

** A large precipitate appeared in the intermediate gel

different diluents and nebulized by four different nebulizers was investigated. There was significant difference in allergen activity before and after nebulization between H₂O and AD diluents for two of the nebulizers. To verify whether individual proteins in the allergen aerosol were still present after nebulization, CIE was performed on samples from the allergen solutions diluted with AD and saline, and from the impacted aerosol. This showed that a precipitate disappeared from the anti-allergen antibody gel in the sample diluted with AD, indicating an association of one or more proteins in the extract with albumin. The small precipitate appearing in the intermediate gel when diluted with saline (Table 2, Fig. 3) may be ascribed to albumin present either in the original extract or as a cross-contamination in the nebulizer. Analysis of a freshly prepared allergen solution with saline from another vial of the same batch revealed the presence of minute amounts of albumin, even when analysed by an alternative method (nephelometry). In either case the presence of albumin in the intermediate gel binds one or more proteins.

Methods for quantitative determination of allergenic activity in extracts are numerous: skin testing (8), determination of single antigens by radioimmunological methods, and RAST inhibition, being the principal (12, 18, 26). These methods can be supplemented with evaluation of individual components in an allergen mixture by CIE (18), or other electrophoretic techniques,

such as SDS-polyacrylamide gel electrophoresis or isoelectric focusing (14).

RAST inhibition measures total allergen activity (3). As we wanted an estimate of the total allergen activity of the extracts, we used inhibition of IgG-subclass RAST. The serum used for inhibition was rich in IgG₄ antibodies due to long-term specific immunotherapy, and this subclass is known to express higher titres and to cover more components than IgE in an allergen extract (27). Further determination of airborne birch pollen antigens by IgG₄ inhibition RAST has shown a close correlation to the pollen count of that season (17). RAST inhibition has frequently been used in other studies and has proved reliable for assessing the stability of allergen extracts (26). CIE was used to determine individual allergens in the extracts, a method which is less sensitive than the IgG₄ RAST inhibition. However, both techniques demonstrated the presence of allergens before and after nebulization, and the CIE further demonstrated the presence of allergens in the impacted aerosol, although one allergen component was reduced in the saline-nebulized aerosol and in the aerosol and remaining extract diluted with AD.

The decaying mechanisms of allergenic extracts may be adsorption, thermal denaturation or enzymatic auto-digestion. The generation of aerosol droplets in the nebulizers might cause denaturation or aggregation of the allergens. As this mechanism combined with adsorption was

suspected to occur more rapidly than thermal or enzymatic degradation, our investigations focused on this aspect. Adsorption to the wall of the container becomes more important with highly diluted extracts (22), as the adsorbed fraction of the active material will be proportionally higher (24). Small volumes will enhance the adsorption effect because of the higher surface/volume ratio. This is stressed by the fact that intense or sustained shaking enhances adsorption (12), while gentle and temporary shaking does not (23). In this study different nebulizers constructed from different materials were used. Samples were

drawn with the same device, but only small volumes enhancing the absorption effect were used, and significant differences were observed between AD and H_2O as diluents; a non-significant pattern equalizing this was also obvious (Fig. 2).

Adsorption of proteins to a solid phase is highly dependent on the material and surface characteristics of the solid phase. Concentration of allergens remaining in the nebulization chambers showed no statistically significant decline in any of the four set-ups during nebulization (Fig. 2), perhaps indicating that in spite of differences in the construction materials, no significant adsorp-

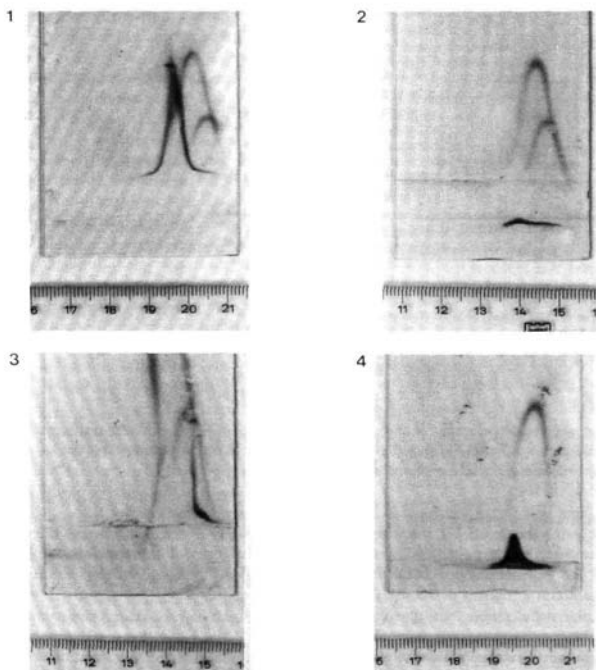


Fig. 3. Analysis of allergen quality performed by crossed immunoelectrophoresis. 1 = 4 (Table 2), 2 = 4a (Table 2), 3 = 8 (Table 2) and 4 = 8a (Table 2).

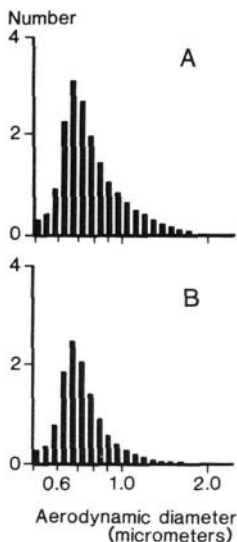


Fig. 4. The particle size distribution for dry particles: a) saline solution, b) Albumin Diluent® solution. Allergen birch 100,000 BU/ml. Wright nebulizer. Sampling time 100 s. No significant difference between curves ($P > 0.05$).

tion to the walls of the chambers occurred. However there was greater allergen activity in the AD-diluted than in the H₂O-diluted solution, which might explain this solution's lower allergen adsorption to the wall.

As the same pattern was observed in all four set-ups, although not significant, this might be of significance over a longer period. Allergen activity might also be concentrated because of the decreased amount of diluent in the nebulization chamber after nebulization; but allergens were detected even in impacted aerosols (Table 2, Fig. 3). Albumin is virtually safe and non-immunogenic for man (4). That one precipitate disappeared from the anti-allergen antibody gel in the extract to which AD was added, may

confirm that adsorption is prevented by the association of one or more proteins with albumin in the allergen extract. The percentual adsorption of allergens to the wall of the container is dependent on the total protein content of the extract; the observed better stability of horse dandruff extract compared with timothy pollen extract (11) may be related to its higher protein content. This might indicate that high amounts of allergenic or non-allergenic protein may prevent adsorption of allergens. The importance of this mechanism is stressed by the beneficial effect of AD, polysorbates (Tween 80), and glycerol, which prevent adsorption of allergens to glass surfaces (23, 24). This is a problem especially in highly diluted allergen preparations, and storage for more than a few hours.

Further studies are needed to identify the relative importance of the albumin-associated birch pollen, i.e. whether it is a major allergen or one of the intermediate allergens.

The output from the nebulizers was unaffected by whether the diluent chosen was AD or saline. The aerodynamic diameter of the particles generated from the two solutions was identical with equal distribution curves (Fig. 4). The optimal aerodynamic particle size will result in maximum deposition at the receptor site, and for dry particles the optimal aerodynamic diameter for tracheo-bronchial and pulmonary deposition is $< 1 \mu\text{m}$ (9, 10, 21) and $< 6 \mu\text{m}$ for saturated droplets (1, 14, 28). For the nebulizers used in this study, 99% of the particles were distributed within the optimal limits (9, 13, 19) (Fig. 4). This applied for each diluent used, although during jet nebulization the HSA-containing diluent (AD) foams in the nebulizer vial. This has no effect on the distribution of particle size or on the activity of the allergen extract aerosol.

This study investigated whether allergen activity in an allergen solution reconstituted with different diluents was deteriorated by nebulization with different nebulizers. This appeared not to be the case, but addition of HSA (AD) to the allergen solution might induce one or more proteins in the extract to associate with the HSA, thus decreasing adsorption of the allergens to the vial surface, as a statistically significant increase in allergen activity was found in the AD-diluted solution with added

HSA, compared with the H₂O-diluted solution. No such significant difference was found between the solutions diluted with NaCl and AD, but this was tested over a very short period. Moreover, we showed that the use of two different diluents does not change the distribution of particle size generated by the nebulizers.

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