**Validation of Strasbourg Environmental Exposure Chamber (EEC) ALYATEC® in mite allergic subjects with asthma.**

**Abbreviations**

CV: Coefficient of Variation

Der p1: Dermatophagoides pteronyssinus

Dpt: *Dermatophagoides* *pteronyssinus*

EEC: Environmental Exposure Chamber

ELISA:  Enzyme-Linked Immunosorbent Assay

ISO: international standards

LAL: Limulus amebocytes lysate

MMAD: Mass Median Aerodynamic Diameter

PBS: Phosphate Buffer Saline

VOC: Volatile Organic Compound

EEC technical characteristics

1 Airflow control

The airflow is totally controlled inside the exposure room, due to the ventilation system and the use of two airlocks for entry and exit. The air pressure of the exposure room is maintained at a slightly lower level than the airlocks and the surveillance room, to prevent allergen loss from the exposure chamber. During exposure, volunteers wore protective clothing typically used in clean rooms to minimize the influx of additional particles into the chamber.

Airflow was generated with two air handling units that contained high efficacy filters (AFPRO HEPA H14 filters, Alkmaar, Netherlands). One filter was for blowing air (ROBATHERM®, RM 06/12, Obernai, France) to ensure 100% fresh air inside the exposure room. The other filter was for air extraction (ROBATHERM®, RM 09/12, Obernai, France), which ensured that the exhausted air was free of allergens. The nebulization system comprised four generators coupled with a nebulization device, which contained four ultrasonic nozzles and peristaltic pumps that tightly controlled the liquid flow instilled in the nebulizer (SinapTec®, Lezennes, France). The nebulized solution was mixed in a chamber developed and patterned by Alyatec®, before it diffused through six outlets. These outlets were positioned at 10 cm below the ceiling and at 210 cm above the floor (Figure S1).

2 Airborne particles and allergen measurements

Particle counters (LightHouse Worldwide Solutions, CA, USA) continuously monitored the number and size of particles during nebulization. Ten particle counters were distributed inside the exposure room, next to the head supports of the armchairs (green boxes, Figure E1). Particle counters were located every two seats at 1.07 m above the floor and at an average distance of 35 cm from the volunteer’s mouth.

Airborne allergen concentrations were sampled with 5 glass fiberFilters (Millipore® AP4003705) placed next to the particle counters, one for every four seats. At the end of the exposure, filters were collected, allergens were eluted, and concentrations were measured with an ELISA assay (Indoor biotechnology® Charlottesville, USA), as published previously (S1). The detection limit for airborne allergen measurements was 0.2 ng/m3 (S2).

3 Quality control processes

3.1 Airflow system and environmental conditions

All the parameters assessed were in conformity with ISO 8 standards, after an evaluation by an independent company (AIR QUALIF®, Dorlisheim, France, certificate number 150316AL1).

After each exposure, the nebulization system was cleaned according to a controlled process. Allergen and volatile organic compound (VOC) contaminations within the EEC were assessed by performing measurements in the toilets, in the supervision room, and in the exposure room after the cleaning process was completed. Measurements were performed in the exposure room at seven different positions, including on the armchair; at two different places in the supervision room; and in the toilet. Endotoxin levels were assessed by measuring the total endotoxins trapped on the five glass fiber filters deployed in the exposure room. Assessments were performed with a colorimetric test, as previously described (S3,S4).

Air flow and air extraction were measured before each exposure test with an anemometer (Testo 417, TESTO®, Forbach, France) with interassay-coefficients of variation (CVs) of 8.5% and 3.7%, respectively (n=58), data not shown). Moreover, before each allergen exposure, all the environmental parameters (air pressure, temperature, relative humidity) were checked and adjusted to fit values defined by the exposure protocol.

The nebulization system was cleaned with a nebulization of 300 mL ultra-pure water, prepared with the purification unit, PURE LAB FLEX 3 (ELGA LAB WATER, VEOLIA, Antony, France). The quality of the ultra-pure water was assessed by an independent company (Eurofins labs, Courtaboeuf, France). Moreover, the air exchange rate was continuously maintained at 7 exchanges per hour, 24 h per day, 7 days per week. Between each allergen exposure, the surfaces of the chamber were systematically cleaned to remove large particles on the seats, the walls, and the floor.

To measure contamination, airborne allergen concentrations were sampled every 30 min with a glass fiber (Millipore® AP4003705) placed on the vacuum pump. Samples were quantified with an ELISA assay (Indoor biotechnology® Charlottesville, USA), as published previously (16). VOC levels were assessed with the Photo Ionization Detector, ppbRAE3000 (RAE Systems®, San Jose, USA), fitted with a 10.6 eV lamp, which detected a wide variety of VOCs (from 1 to 10,000 ppb). To measure airborne endotoxin during Dpt exposure, filters were centrifuged, then eluted in 5 mL of PBS-Limulus Amebocyte Lysate (LAL) solution (Coamatic® Chromo-LAL, Cape Cod, USA). A sample (100 µL) of the eluted solution was incubated with Chromo-LAL (Coamatic® Chromo-LAL, Cape Cod, USA) and the endotoxin concentration was measured with spectrophotometry at 405 nm (Versamax Microplate reader, Molecular devices®, California, USA).

3.2 Particle monitoring

All particle counters were annually calibrated by an independent company, in accordance with the ISO 21501-4 standard (Gométrologie, Créteil, France). Before exposure, the flow rate of each particle counter was adjusted with the GFM37 flowmeter (Aalborg®, Orangeburg, USA) to a tolerance of 5%. There was no modification in the background particle number when volunteers entered and moved around inside the exposure room (data not shown).

The Mass Median Aerodynamic Diameter (MMAD) of all particles was calculated in real-time by each particle counter. Moreover, we also calculated MMAD of the particles carrying allergens with an Andersen MK II cascade impactor with 8 stages (Thermo Scientific®, Waltham, USA), coupled with ELISA assay.

3.3 Allergen monitoring

Before nebulization, allergen concentration in saline was measured with an ELISA assay (Indoor biotechnology®, Charlottesville, USA). Before each exposure, the flow rate of each vacuum pump that had a glass fiber filter attached was measured with a GFM37 flowmeter (Aalborg®, Orangeburg, USA) and we assessed reproducibility (CV of 2.4%, n= 125 flow rate measures, data not shown). We also assessed the reproducibility of filter elution among four different technicians with four different Der p1 concentrations.

We assessed the reproducibility of filter elution among four different technicians with four different Der p1 concentrations. First, 100 µL of a solution containing a determined concentration of Der p1 was dropped on glass fiber filter. Then, filters were placed in a 5mL syringe, incubated overnight, and eluted in 1 mL of PBS Tween solution (Fischer Scientific, Fair Lawn, USA). The allergen concentrations in the eluted solutions were assessed with ELISA assays (IndoorBiotechnologies). The inter-assay CVs were 11.5, 10.4, 6.7, and 11.6%, respectively, for Der p1 concentrations of 15.63 ng/mL, 31.25 ng/mL, 62.5 ng/mL, and 125 ng/mL (n=4 trials for each concentration, data not shown).

**Références**

S1. Chapman MD, Ferreira F, Villalba M, Cromwell O, Bryan D, Becker W-M, et al. The European Union CREATE project: a model for international standardization of allergy diagnostics and vaccines. J Allergy Clin Immunol. 2008;122(5):882‑889.e2.

S2. de Blay F, Heymann PW, Chapman MD, Platts-Mills TA. Airborne dust mite allergens: comparison of group II allergens with group I mite allergen and cat-allergen Fel d I. J Allergy Clin Immunol. 1991;88(6):919‑26.

S3. Sohy C, Lieutier-Colas F, Casset A, Meyer P, Pauli G, Pons F, de Blay F. Dust and airborne endotoxin exposure in dwellings in the Strasbourg metropolitan area (France). Allergy. 2005;60(4):541-2.

S4. Doyen V, Pilcer G, Dinh PH, Corazza F, Bernard A, Bergmann P, Lefevre N, Amighi K, Michel O. Inflammation induced by inhaled lipopolysaccharide depends on particle size in healthy volunteers. Br J Clin Pharmacol. 2016 Nov;82(5):1371-1381. doi: 10.1111/bcp.13052.

**Figure legend**

**Figure S1**

A schematic view of the exposure room illustrates the strategy for achieving a homogeneous spatial distribution of nebulized particles. Particle counters are represented with green boxes and air/nebulization are represented with green arrows. Air is extracted through five air intakes (white boxes R1 to R5). Red values indicate the ratios between the total number of particles measured at the adjacent counter, relative to the mean total number of particles measured by all the counters on 35 separated experiments.

**Figure S1**

