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Publisher: Taylor & Francis

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Grana

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/sgra20>

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Available online: 03 Sep 2009

To cite this article: George F. Schäppi, Philip E. Taylor, Cenk Suphioglu & R. Bruce Knox (1997): A new approach to the investigation of allergenic respirable particles using a modified Andersen Impactor, *Grana*, 36:6, 373-375

To link to this article: <http://dx.doi.org/10.1080/00173139709362631>

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A new approach to the investigation of allergenic respirable particles using a modified Andersen Impactor

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Schäppi, G. F., Taylor, P. E., Suphioglu, C. & Knox, R. B. 1997. A new approach to the investigation of allergenic respirable particles using a modified Andersen Impactor. – Grana 36: 373–375. ISSN 0017–3134.

Pollen allergens have been shown to occur in fine particles much smaller than pollen grains. As such allergenic particles have a high probability of entering the lower human airways, it is important to further investigate their origin and their biological and immunological properties. Accordingly, we have modified the particle collection surface of an Andersen Impactor using adhesive coated microscope slides to enable identification by high resolution microscopic analysis and immunodetection of allergenic particulates.

George F. Schäppi, Philip E. Taylor, Cenk Suphioglu & R. Bruce Knox, Pollen & Allergen Research Group, School of Botany, The University of Melbourne, Parkville, Victoria 3052, Australia. Professor R. Bruce Knox passed away suddenly on August 30, 1997. The remaining authors wish to dedicate this paper to his memory.

(Manuscript accepted 10 October 1997)

Within the atmospheric aerosol, pollen allergens occur in particle size fractions much smaller than pollen grains. We found that particles in the range of 0.6 to 10 µm contained grass pollen allergens, for example the major timothy (*Phleum pratense*) pollen allergen Phl p 5 (Schäppi et al. 1996). The major birch (white birch: *Betula verrucosa* Ehrh. = *B. pendula* Roth.) pollen allergen Bet v 1 has been recorded in aerosol fractions smaller than pollen grains, even when no birch pollen grains were present (Rantio-Lehtimäki et al. 1994). Unlike pollen grains, these small allergenic particles have a high probability of entering the lower human airways (Wilson et al. 1973, Bates et al. 1966), constricting the bronchi and causing an attack of asthma.

The origin of allergen loaded respirable particles has been open to debate. We have found that grass pollen grains rupture in rainwater by osmotic shock, each grain releasing about 700 allergen loaded starch granules (Suphioglu et al. 1992). Free Group 1 allergen molecules dispersed in the aerosol are also able to bind to other particulate matter (Knox et al. 1997). Yet we have observed that birch pollen, unlike grass pollen, does not osmotically rupture in water. Several hypotheses have been proposed concerning the origin of allergen loaded respirable particles from birch: sedimented pollen has been suggested to release its allergens when wetted and the allergenic material again becomes airborne after drying (Rantio-Lehtimäki et al. 1994) or orbicules may act as carriers for birch pollen allergens (El-Ghazaly et al. 1995). Recently, we discovered that birch pollen, after moderate rainfall (<1 mm per day), germinates, particularly on birch leaves. Pollen tubes (up to 300 µm in length) are produced, which after at least 5 and up to 48 hours burst and release allergen loaded starch granules (Schäppi et al. 1997). These findings are supported by recent studies showing predominant localization of Bet v 1 in starch granules by immunogold labelling (El-Ghazaly et al. 1996).

It is important to further characterize these allergenic

particles. Here, we take birch as an example, but the technique is applicable to many other systems. For white birch, pollen grains are large particles, 21–22 µm in diameter (Mäkelä 1996). Candidates for fine particles include starch granules, 0.5–2.5 µm in diameter and also orbicules, 0.5–0.8 µm in diameter (as determined from figures in El-Ghazaly et al. 1995). Accordingly, we have developed a new sampling technique by modifying an Andersen Impactor to enable identification by high resolution microscopic analysis and immunodetection of allergenic particulates.

MATERIALS AND METHODS

Sampling of respirable particles with a modified Andersen Impactor

The Andersen 1 ACFM six-stage Particle Sampler (Andersen Samplers Inc., Atlanta GA, USA) is a cascade impactor which is designed to simulate the penetration depth of particles in the lungs. The sampler intakes air through an upright orifice at a rate of 28.3 l per min which allows maximum collection efficiency. This constant flow is provided with a continuous-duty vacuum pump. The air passes through six stages, each perforated with 400 orifices consecutively decreasing in size in each stage. A glass petri dish containing 27 ml of agar is usually placed under each stage as sampling media. Particles impact on the sampling media according to their aerodynamic diameter (Table I). The original setup is designed to measure the concentration and size distribution of airborne bacteria and fungi.

We have modified the particle collection surface (Fig. 1). Microscope slides were coated in adhesive (10% Dow Corning in xylene) and placed in the centre of the glass petri dish. The surface of the slide was adjusted to the recommended height of the original collection surface in order not to alter the distance between the jet orifices and the collection surface. To avoid particles bouncing off the impaction surface the remainder of the petri dish surface was covered with the recommended collection substrate (agar). The air sampler was operated during the birch pollen season 1996 below birch (white birch: *Betula verrucosa* Ehrh. = *B. pendula* Roth.) trees on the campus of the University of Melbourne in 8 h intervals and

Table I. Aerodynamic diameter of particles collected by stages 1–6 of the Andersen impactor.

Stage	Aerodynamic diameter of collected particles
1	7 µm and above
2	4.7–7 µm
3	3.3–4.7 µm
4	2.1–3.3 µm
5	1.1–2.1 µm
6	0.65–1.1 µm

Particle size cut off diameter at 50% collection efficiency for spherical particles with unity mass density according to the manufacturers specifications.

the slides replaced each 8 h. The exposed microscope slides could then be further examined.

Microscopic investigation of particles

The microscope slides were mounted in Calberla's stain, which stains pollen exines red, and viewed by brightfield light microscopy.

Detection of starch granules

The microscope slides were mounted in iodine/K iodide (IKI) reagent which stained starch particles blue-black in colour. They were viewed by brightfield light microscopy.

Immunoprinting of the major birch allergen Bet v 1

20 min were allowed for allergen transfer from the particles attached to microscope slides to a polyvinylidene difluoride (PVDF) membrane, wetted 1 min in methanol and rinsed in phosphate buffered saline (PBS) before use. After blotting, the membranes were blocked (10% w/v skim milk powder in PBS) for 1 h at room temperature, washed once in PBS containing 0.05% Tween (TPBS) and twice in PBS only, 5 min each with vigorous shaking. The membranes were then incubated for 2 h with monoclonal antibody 3B4F11D6 specific to Bet v 1 (Allergopharma J. Ganzer KG, Reinbek, Germany), diluted 1:500 in antibody buffer 0.5% BSA in PBS. After washing once with TPBS and twice in PBS alone for 5 min each, the membranes were then incubated with affinity isolated anti-mouse Ig raised in sheep which was horse radish peroxidase conjugated (diluted 1:200) for 2 h followed by washing in TPBS and PBS. The membranes were then developed in peroxidase substrate (0.06% 4-chloro-1-naphtol, 0.006% hydrogen peroxide and 20% methanol in PBS) at 37°C for 10 min and development was stopped by washing in distilled water. The membranes were then examined

under light microscopy for antigen positive purple spots and photographically recorded.

Scanning electron microscopic investigation of respirable particles

To observe respirable particles for electron microscopy, glass slides were broken into small pieces and mounted onto stubs with carbon dag. They were sputter coated with gold for 3 min and then transferred into a Jeol-J840 Scanning Electron Microscope.

RESULTS

Microscopic investigation of particles

On stage 1 (fraction: 7 µm and above), birch pollen grains were present (Fig. 2a) on the microscope slide exposed. Stages 2 and 3 collected a range of irregular-shaped particles. Mainly on stage 4 and 5, but also on stage 6 (fractions: 0.65–3.3 µm), starch granules were present (stained blue-black in colour) (Fig. 2b).

Immunoprinting of the major birch allergen Bet v 1

The slides examined by immunoprinting of Bet v 1 showed antigen positive purple spots on stages 1 and 4, with less activity detectable on the other stages. On stage 1, the spots resembled pollen grains in overall dimensions, while on stage 4 (Fig. 2c) the spots were in general smaller than the irregular shaped portions of cytoplasm, suggesting that Bet v 1 is confined to the surface of starch granules within. This could be determined by comparison of Bet v 1 immunoprints and IKI-stained slides, showing the co-localisation of starch granules and Bet v 1 in air samples (Fig. 2b and 2c; IKI-stained and likewise Bet v 1 positive particles are indicated by an arrow).

Scanning electron microscopic investigation of respirable particles

On stage 4 (fraction: 2.1–3.3 µm), spherical particles morphologically similar to starch granules had collected and were viewed by scanning electron microscopy (Fig. 2d). Unlike grass pollen (Suphioglu et al. 1992), the birch starch granules were frequently surrounded by other organelles and so had an irregular shape. A range of morphologically different particles collected on stage 6 slides (fraction: 0.65–1.1 µm) (Fig. 2e). Particles were present which resembled orbicules (see El-Ghazaly et al., 1995), which in the anther are seen as short-spined spheres of sporopollenin. Electron microscopy investigations of the exposed slides from different stages showed that the deposition characteristics of the sampler could be largely maintained.

DISCUSSION

The modification of the collection surface described in this short communication has several advantages. The collected particles are ready for further investigations without the need of any extraction steps. As they are impacted in a well dispersed pattern, their presentation on the slides makes straightforward qualitative and semi-quantitative investi-

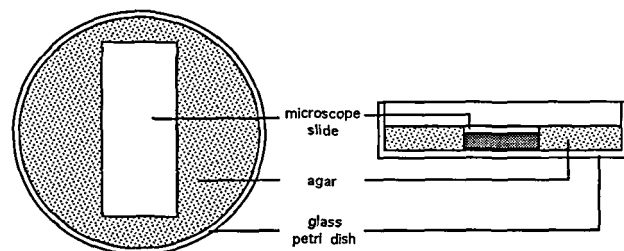
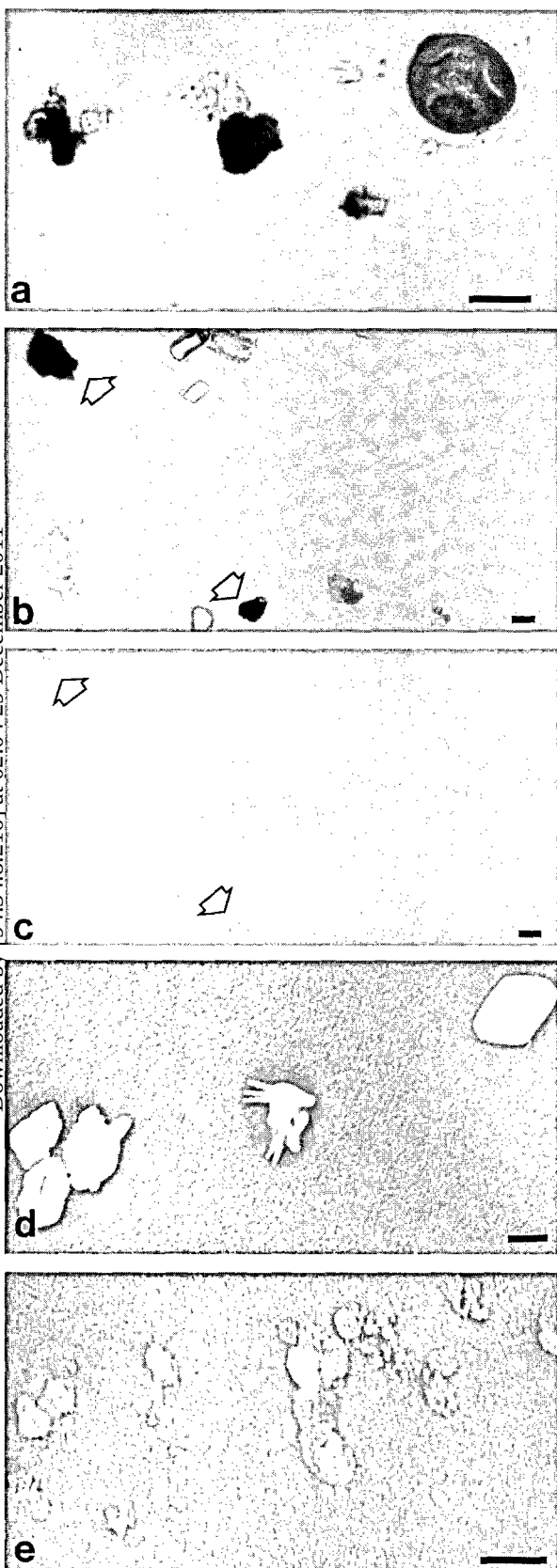


Fig. 1. Modified particle collection surface of the Andersen Impactor with an adhesive coated microscope slide placed in the centre of the glass petri dish.



gations possible. Subsequent applications of various investigation techniques to the same sector of a sample allows instructive comparisons of distribution patterns. As the agar surrounding the slides can still be incubated, and colonies of bacteria and fungi counted, the proposed modification allows multipurpose particle collection. The changes to the method are simple and cost effective but can enable a greater range of techniques to be applied for investigations in technically difficult areas of aerobiology.

ACKNOWLEDGEMENTS

We thank Dr. B. Weber (Allergopharma J. Ganzer KG, Reinbek, Germany) for providing antibodies, the Swiss National Science Foundation, the Australian Research Council and the Australian National Health and Medical Research Council for financial support.

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Fig. 2. Exposed collection slides investigated by various techniques: (a) Brightfield light microscopy of a slide mounted in Calberla's stain; stage 1, bar represents 10 µm. (b) Brightfield light microscopy of a slide mounted in IKI reagent staining starch particles blue-black in colour; stage 4, bar represents 1 µm. (c) Corresponding immunoprint of the major birch allergen Bet v 1; stage 4, bar represents 1 µm. (d) Scanning electron micrograph (SEM); stage 4, bar represents 1 µm. (e) SEM; stage 6, bar represents 1 µm.