

Evaluating the penetration of *Cladosporium* spores into the human respiratory system on the basis of aerobiological sampling results

A. RANTIO-LEHTIMÄKI

Department of Biology, University of Turku, Finland

The penetration of *Cladosporium* spores and spore aggregates into human airways was studied using three different spore sampling methods: 1) a Burkard spore trap for determining the aggregation degree of *Cladosporium*; two samplers, simulating the human respiratory system, 2) a 6-stage Andersen 2000 sampler, and 3) a new size-selective bioaerosol sampler (SSBAS), designed specifically for immunochemical and chemical analyses. The aggregation degree of *Cladosporium* spores varied between 1.0 and 1.3 spores per dispersal unit. Grouping seems to be of little if any importance to the penetration ability of *Cladosporium* spores into the respiratory tract. The distribution of spores in the Andersen and SSBAS differed significantly in the largest size class (spores $> 7 \mu\text{m}$ in diameter); with the Andersen sampler only 10.8 % of the spores were detected in stage 1, compared with 43 % with the SSBAS. On the Andersen culture plates 95 % of all colonies were detected in stages 1-4, where particles $> 2.1 \mu\text{m}$ in diameter are trapped. In the SSBAS altogether 99.4 % of spores were found in the first two filter stages (cutoff point approx. $2.5 \mu\text{m}$ in diameter). Conclusions regarding the penetration of spores to the lungs on the basis of aerobiological results should always be based on the use of properly calibrated spore traps.

Key words: aerobiology; fungus spores; lungs; spore samplers.

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CLINICAL ASPECTS

Very little is known about the behavior in the human respiratory tract of small particles containing allergenic material. By using various sampling methods, certain conclusions have been reached as to the point in the respiratory tract at which these particles can penetrate, but almost none of these experiments has been performed with a properly calibrated sampler. The present study concerns the spores of cladosporia and their penetration ability using different sampling devices. Many sampling methods overestimate the percentage of spores reaching the alveoli and secondary bronchi. A new sampler (SSBAS), which has been properly calibrated, shows that only 0.6 % of all *Cladosporium* spores are able to reach stages where particles $< 2 \mu\text{m}$ in diameter are able to penetrate.

Cladosporium is the most frequent airborne spore type in most climatic regions, the only exceptions being the subarctic (18) and very dry areas (6). Also, in southern Finland it is the principal fungus spore causing allergic symptoms. According to Koivikko (14), 7.1 % of mould-allergic children in Finland ($n = 1300$)

react (in a + + + skin prick test) (1) to *Cladosporium* extracts. Furthermore, since the extracts of *Cladosporium herbarum* commercially available are of variable and often poor quality, this figure may be an underestimate.

The spore size of *Cladosporium* varies over a wide range, between $3-70 \times 2-56 \mu\text{m}$.

In many respects the literature is still inadequate and controversial regarding the relationship between inhaled fungal spores and the origin of lower respiratory tract allergic disease (2, 9, 10, 17, 20). It has been established, on the basis of experimental inhalation studies with dry and aerosolized particles, that a small fraction of pollen grains (up to 40 μm in diameter) can penetrate into the respiratory tract (16). Thus spores, which are generally smaller, are certainly also present in tracheobronchial secretion. On the other hand, the mechanism whereby the allergenic material emerges from the intact spores has not been determined. There have been relatively few studies of provocation challenge tests with mould extracts, and the results have often been ambiguous because of the lack of standardized antigens and inhalation challenge techniques.

Dispersal units of several *Cladosporium* spores are often observed in microscopic analyses of aerobiological samples. As well as to each other, spores are often attached to various particles, such as soot. The point of penetration in the respiratory tract is determined by the aerodynamic size of the dispersal unit as a whole.

The aggregation degree of cladosporia has been investigated earlier (5, 8, 12). In these studies, it was found to vary between 1.3 and 3.6 spores per dispersal unit.

In the design of a new aerosol sampler, one of the main principles is the comparability of results to human lung deposition rates (e.g. the Andersen sampler (3)). According to Hidy (11) an ideal aerosol sampler would consist of two consecutive stages. Particles deposited in the head and tracheobronchial regions would be collected in the first stage, particles capable of reaching the alveolar region in the second stage. Within the 0.1–3.5 μm range, there is a relatively low and remarkably constant alveolar zone deposition rate of approximately 20 % for normal nose-breathing subjects. These were also the basic principles in the design of our new size-selective bioaerosol sampler. It may never be possible to design a trap that truly simulates human respiration; thus lung deposition experiments must be performed by other means.

MATERIAL AND METHODS

Three complementary spore sampling methods were used in this study. The Burkard trap (Burkard Manufacturing Co.) is widely used for the monitoring of airborne biological particles. The suction capacity is approx. 10 l/min, corresponding roughly to normal human breathing. The Burkard trap was used to collect samples for visual analysis of the total number of airborne cladosporia and calculations of aggregation. Identification of various *Cladosporium* species on the basis of single spores is not always possible. The Burkard trap has not been calibrated in controlled conditions, thus its precise sampling efficiency for different particle sizes has not been determined. A general sampling efficiency coefficient of 70 % was used; one even as low as 60 % has been recommended (21).

The 6-stage Andersen 2000 sampler (3) is a viable sampling method, and the identification of colonies is much easier than that of single conidia as in the Burkard samples. The Andersen sampler theoretically divides particles into 6 size-classes as follows: stage 1, ≥ 7.0 ; stage 2, 4.7–7.0; stage 3, 3.3–4.7; stage 4, 2.1–3.3; stage 5, 1.1–2.1; stage 6, 0.65–1.1 μm . These stages serve the purpose of stimulating human airways, as shown in Fig. 1. Particles are inertially impacted onto the agar collection surfaces. Maximum collection efficiency is achieved with a constant airstream of 28 l/min.

Andersen results are always underestimates, since some spores fail to germinate, overgrowth is relatively common and antagonistic effects cause germination failure.

The third sampler, the size-selective bioaerosol sampler (SSBAS), was designed and constructed especially for immunochemical analysis, but it is also well suited for taking samples for direct microscopic analysis. It was designed and manufactured by the Technical Research Centre of Finland in 1985 and calibrated by Kauppinen et al. (13). Since then the sampler has been tested experimentally (19). Theoretically, and also evidentially on the basis of the calibration experiments, this apparatus separates particles into size-classes as follows: stage 1: $\geq 7 \pm 0.4$ μm ; stage 2: $\geq 2.4 \pm 0.2$ μm ;

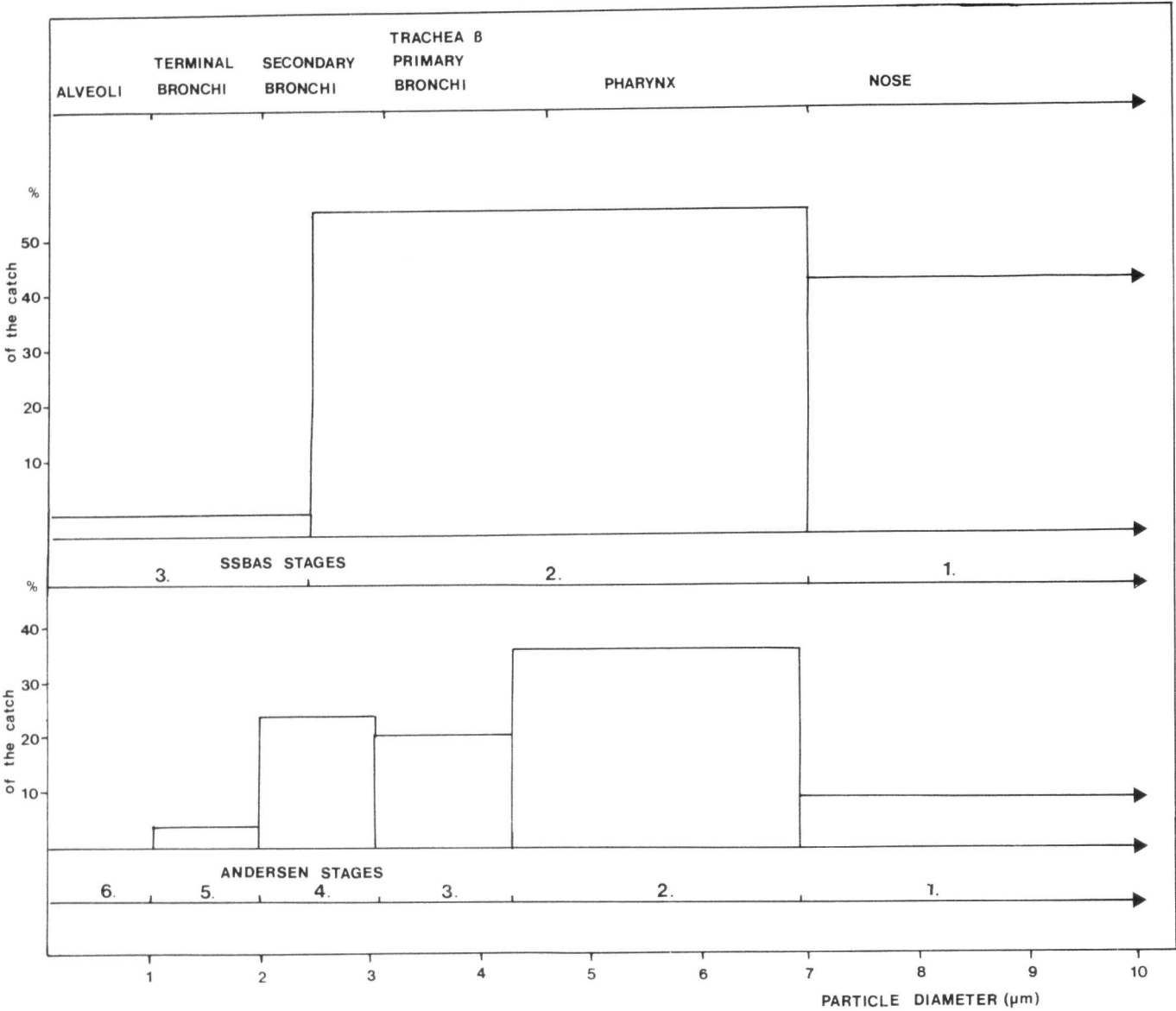


Fig. 1. Retention of *Cladosporium* spores in six stages of Andersen sampler and three stages of SSBAS compared with the corresponding stages of the human respiratory system.

particles smaller than 2.5 μm in diameter are trapped in stage 3 and particles of molecular size, which penetrate all three filter stages are condensed at a low temperature (−20°C). The pore size of filters used (Nuclepore Membrafil) in stage 1 was, 0.7 μm, in stages 2 and 3, 0.45 μm.

The fourth sample is thus in a liquid state, when melted. Air is drawn in at a rate of 18.52 l/min. These cutoff points were selected for two reasons: 7 μm is approximately the size limit for nasal retention when inhaled through the nose. Secondly, in urban air aerosol particles generally have two peaks, one below 10 μm in diameter and another about 0.2 μm in diameter (11).

The Finnish Aerobiology Group has one continuously operating Burkard trap in Turku, on the roof of the University buildings (15 m above ground level). All background counts presented in this study come from this sampler (22). Andersen samples were taken in Turku outdoors twice a month in 1983–84. The air volume per sample was 72 l. The culture medium used was YPGA (4% glucose, 0.5% peptone, 0.5% yeast extract). Colonies were analysed after 4 days, 1 week, 2 and 4 weeks. Each dispersal unit forms only one colony, Andersen counts are therefore given in CFU (colony forming units)/m³ air. No Andersen samples were taken simultaneously with SSBAS sampling.

The SSBAS-samples were collected during 1985–86, from March to October, at least once a week (24 h samples). *Cladosporium* spore frequencies in the SSBAS samples were calculated microscopically for each stage. The filters were cleared with immersion oil and an area of 0.4 cm² on each filter was analysed. The frequencies were transformed to correspond to particle counts in a cubic metre of air. In evaluating the aggregation degree, only spores which were still attached to each other were considered as groups. On the other hand, very small buds, which may not have been potential dispersal units, were counted, because of the difficulty of judging microscopically when a cell is sufficiently mature to constitute an independent potential dispersal unit.

The aggregation degree of *Cladosporium* on the Burkard tapes was calculated monthly (4 days' random sample per month) in 1985. The random field method introduced by Mäkinen (15) was used. (The same system of analysis has been used for 10 years by the Finnish Aerobiology Group in its daily pollen and spore forecasts.) The counts were transformed to correspond to spore frequencies/m³ air.

The aerodynamic diameters of the most frequent *Cladosporium* species (Table 1) were calcu-

lated by the simple method introduced by Fuchs (7). The arithmetic mean of the maximum and minimum length of a spore was calculated as a diameter of the dispersal unit. This may differ greatly from the Stokes equivalent radius (7).

Andersen results and SSBAS results were compared with simultaneous Burkard counts using the BMDP1R program (4).

RESULTS

During May–September 1985, the aggregation degree of *Cladosporium* spores in the Burkard samples varied between 1.0 and 1.3 cells per dispersal unit (monthly averages shown in Table 2). The percentage of single spore dispersal units varied from 84 to 91 % (Table 2), and *Cladosporium* spore counts can thus be used as such (without transformation for calculating penetration ability) without generating any significant error. When *Cladosporium* frequencies were at their highest in late August, the aggregation degree was at its lowest.

In spring and early summer the most numerous cladosporia were *Cladosporium herbarum* (spore size 5–23 × 3–8 µm) and *C. cladosporioides* (spore size 3–11 × 2–5 µm); in late summer and

Table 1

Spore sizes of various *Cladosporium* species probably occurring in aerobiological samples in Scandinavian countries and calculated retention stages of these spores for SSBAS and Andersen sampler

	Spore size (µm)	Aerodynamic size (µm)	Calc. ret., SSBAS	Calc. ret., Andersen
<i>C. allii-cepae</i>	30–90 × 11–22	20–56	1	I
<i>chlorocephalum</i>	3.5–34 × 3–9	3–21	1–2	I–IV
<i>cladosporioides</i>	3–11 × 2–5	2–8	1–3	I–IV
<i>cucumerinum</i>	4–25 × 2–6	3–15	1–2	I–IV
<i>elatum</i>	4–25 × 2–5	3–15	1–2	I–IV
<i>herbarum</i>	5–23 × 3–8	4–15	1–2	I–III
<i>macrocarpum</i>	9–28 × 5–13	7–20	1–2	I
<i>ornithogali</i>	20–70 × 10–15	15–42	1	I
<i>phlei</i>	15–36 × 7–14	11–25	1	I
<i>sphaerospermum</i>	3–4.5	3–4.5	2	III–IV
<i>staurophorum</i>	5–18 × 1.5–3	3–10	1–2	I–IV
<i>tenuissimum</i>	3–25 × 3–6	3–15	1–2	I–IV
<i>variabile</i>	5–30 × 3–13	4–21	1–2	I–III
<i>C. state of Amorphototheca resinae</i>	3–12 × 2–4	2–8	1–3	I–IV
<i>C. state of Mycosphaerella dianthi</i>	25–55 × 8–17	16–36	1	I
<i>C. state of M. macrospora</i>	35–70 × 13–25	24–47	1	I

autumn *C. macrocarpum* (spore size 9–28 × 5–13 µm) was more abundant. Analysis of the Burkard samples showed no significant difference in grouping between the various *Cladosporium* species.

The Andersen results (Fig. 2) were clearly lower than the Burkard counts, but the results were mutually correlated ($n = 48$; $R = 0.436$; $P < 0.01$). The same was true of the SSBAS (Fig. 3) and Burkard results ($n = 42$; $R = 0.637$; $P < 0.001$), and here the frequencies were clearly lower for the SSBAS.

The first size-class (> 7 µm in diameter, corresponding in theory to nasal deposition) is the same for the SSBAS and the Andersen sampler. On the basis of the Andersen results 10.8 % of cladosporia is retained in the nose; the figure yielded by the SSBAS is over 43 %. The second size-class in the Andersen sampler was clearly dominant: 37.8 % of all colonies were detected in stage 2. This correlated closely with “the average size” of a single *Cladosporium* spore (Table 1) and with a dispersal unit consisting of one spore and one bud, which was the main type of *Cladosporium* spore group on the Burkard tapes.

In the Andersen sampler over 84 % of cladosporia were caught in stages 2–4.

The next, almost identical cutoff points for these two samplers were 2.1 µm (Andersen) and 2.5 µm (SSBAS) in diameter. In the human respiratory tract these particles reach the terminal bronchi and alveoli. Based on the Andersen results, up to 5.0 % of *Cladosporium* spores are < 2.1 µm in diameter and reach the lower respiratory tract: on the basis of the SSBAS results only 0.6 % of cladosporia ever reach the terminal bronchi and alveoli.

DISCUSSION

In Burkard samples, no calculations were made for spores attached to other particles (soot, etc.) and thus forming large dispersal units. The aggregation percentages found here are lower than those in previous studies (5, 8, 12). It is possible that the spores are separated when impacted on the tape, but other studies referred to have made use of similar samples.

It seems probable that the Burkard sampler overestimates the spore counts of *Cladosporium*-size (at least when a correction coefficient of 0.7 is used). For example, for *Betula* pollen-size (approx. 25 µm in diam.) the SSBAS is more effective than the Burkard sampler (19).

The high percentage of single *Cladosporium* cells in air and the small annual variation in their concentrations (22) makes it possible to compare the retention of particles in Andersen and SSBAS even if samplings have not been made simulataneously.

Obviously, particles penetrate deeper in the Andersen stages than they should theoretically, error limits having been determined. SSBAS was experimentally calibrated with ammonium fluorescein particles of different sizes (13). The maximum wall losses at stages 1 and 2 were approx. 12 and 5 %, respectively. Almost all cladosporia spores were detected in these two stages. Therefore, the highest possible error in *Cladosporium* spore counts in SSBAS-samples is below 10 %.

Approximately 80 % of the smallest size-class reaching the alveoli will be exhaled immediately (11). On a day when the *Cladosporium* frequency is 17,000/m³ air (the highest daily average in

Table 2
Percentages of *Cladosporium* spore aggregates in Burkard samples in 1985

	Mean size of aggregate spores	1-spored DU	2-spored DU	3-spored DU	4-spored DU
May	1.11	84.2	15.8	–	–
June	1.31	84.1	11.4	1.8	2.5
July	1.11	89.9	9.4	0.7	–
August	1.14	91.2	6.1	1.4	1.2
September	1.16	88.7	5.6	2.8	2.8

DU: dispersal units.

Burkard samples during this study), the spore retention rate in the alveoli would be approx. 200/day according to the Andersen results but only 23 according to SSBAS results.

The mechanism provoking allergic symptoms is still unclear. We have quite extensive knowledge about antigens in, for example, *C. herbarum*, but know very little about how the spores function in the human respiratory system. How are the antigens released, how quickly, and how important is it to know at which point in the respiratory tract the spores are able to penetrate?

Our new sampler, the SSBAS appears to be a most useful tool for such studies. The sampler can also be used in immunochemical analyses because the samples are on filters and, in the molecular size-class, in the liquid phase.

Aerobiologists should be more critical in their selection of samplers. They should demand calibrated machines and competent background knowledge.

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Address:

Auli Rantio-Lehtimäki

University of Turku

Department of Biology

SF-20500 Turku

Finland

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