

A new method for simultaneous immunodetection and morphologic identification of individual sources of pollen allergens

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Background: Exposure to outdoor allergens has commonly been estimated by collecting airborne particles with a Hirst-type spore trap and then using morphologic criteria to identify the intact pollen grains and fungal spores that are recognized as allergen sources. Several antibody-based blotting or fixation methods have also been developed that enable the counting of amorphous airborne particles carrying allergen, but none of these methods allow the ready association of the released allergen with the morphologically identifiable particle of origin. A method has been developed that uses pressure-sensitive adhesive tape to sample the airborne particles and then allows the immunoidentification of the specific particles that are the allergen sources.

Objective: Our purpose was to visualize and immunostain the particles carrying pollen allergen that are collected with a volumetric spore trap.

Methods: A Burkard sampler was used to collect airborne particles onto pressure-sensitive adhesive tapes. The particles were permanently fixed between the tape and a protein-binding membrane when the tape was laminated with the membrane. Allergens that elute from the particles onto the membrane were detected with a range of antibodies. Both the particle and associated immunostained allergen were viewed through the transparent tape for final microscopic identification.

Results: Polyclonal and monoclonal antibodies and IgE from allergic patients stained allergens in the periphery of particles collected on the tapes. Individual pollen grains and paucimicronic particles were seen with halos of immunostained allergen surrounding them. When IgE was used, the density of immunostaining in the halo surrounding *Lolium perenne* pollen grains was found to be proportional to the level of *Lolium*-specific IgE. The method is highly sensitive and can be used to detect different airborne particles that carry allergen. Both the particle and the immunostaining can be subjected to a range of simple measurement techniques.

Conclusion: Individual particles carrying allergens and antigens were visualized. These particles included intact pollen grains, paucimicronic particles, and fungal spores. (*J Allergy Clin Immunol* 2000;105:725-31.)

Key words: Aeroallergen, airborne pollen, Lol p1, Lol p 5, paucimicronic, mAb antibodies, Hirst spore trap

Traditional measurements of exposure to allergens carried by pollen grains and fungal spores involve collection of an air sample and identification of the potential aeroallergen sources on the basis of the morphologic characteristics of the particles viewed under a microscope. However, counts of pollens and spores do not always reflect the allergen content of the air or correlate with allergy symptoms.¹⁻⁵ Under certain circumstances more than half of airborne allergen is not associated with intact pollen grains or spores but is carried on paucimicronic particles of a size that may readily enter the lung.^{2,6,7}

Several different methods for identification of aeroallergens associated with particles have been developed; however, they have either been not robust enough for outdoor air sampling applications⁸ or have not preserved the direct association between the individual allergen prints and the particles that were their origin.^{4,5,9-11} In such studies the source of the allergen and its characteristics such as size, shape, morphologic features, and species of origin cannot be determined. Studies on allergen in size-fractionated samples have enabled the allergen distribution between different sized particles to be estimated,^{2,7,12-14} although without visualization of the sources these estimations are confounded by the possibility of larger particles bouncing their way through cascade impactors.^{7,12-14}

A new method for the capture, staining, and subsequent identification of particles carrying the allergens is demonstrated here. The technique has been developed for a commonly used volumetric air sampler that is the benchmark for aerobiology studies.

MATERIAL AND METHODS

Adhesive tapes

Three water-based (Adhesives Research, Glen Rock, Pa, and JAC Australia, Sydney, Australia) and 1 solvent-based (Avery Dennison, Painesville, Ohio) acrylic pressure-sensitive adhesive tapes were used.¹⁵ These met the selection criteria of remaining stable and tacky for more than 2 weeks when exposed under different climatic conditions, were optically clear, and were no more than 75 μ m thick, and thus did not interfere with visualization of particles when viewed through the adhesive. When wet, the tapes were required to remain clear and adhere to the protein-binding membrane during the 48 hours in aqueous reagents required for the immunostaining steps. In other experiments¹⁵ we have found the acrylic tapes to be twice as efficient as the standard (petroleum jelly/paraffin/toluene) adhesive for collection of small particles (1-

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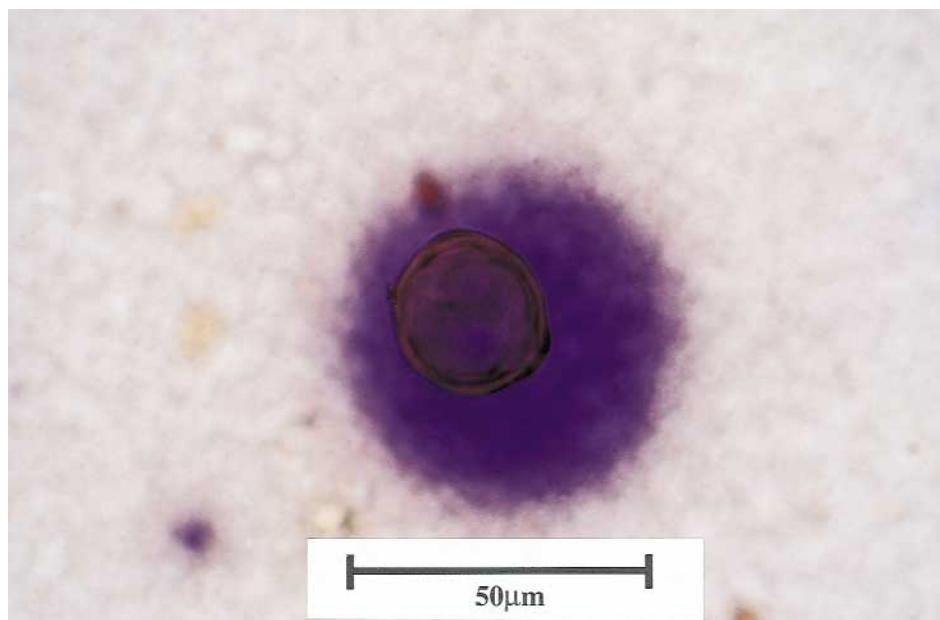


FIG 1. Pollen grain with surrounding halo immunostained with polyclonal antibody.

Abbreviations used

BCIP/NBT:	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
DDW:	Deionized distilled water
PBS-Tween:	PBS plus 0.05% Tween 20
PVDF:	Polyvinylidene difluoride

20 μ m in diameter); thus they may be suited to capture of paucimicronic particles. However, the capture efficiency of the acrylic tapes for larger fungal spores (*Alternaria* like) and a range of pollens appear to be about 50% of that of the standard material.¹⁵

Air sampling

Air sampling was performed with a Burkard 7-day volumetric spore trap operated at a suction rate of 10 L/min (Burkard Manufacturing, Rickmansworth, UK). Because the purpose was to collect samples of aerospora for methods development and not a standardized representation of exposure, the sampler was placed on the ground in a suburban backyard of Sydney that was surrounded by park land. Sampling was performed throughout the spring and early summer (September to December).

Immunostaining of adhesive tapes

After collection of particles, a dry polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Life Science) was pressed down firmly onto the particle-coated adhesive tape. The adhesive and PVDF sandwich was immersed in 80% ethanol until wet, rinsed 3 times in deionized distilled water (DDW), and left in PBS overnight to allow allergens to elute from the particles. Nitrocellulose membrane (0.45 μ m, Millipore, Bedford, Mass) was also suitable for capture of eluted allergens. The next day the samples were blocked with 5% skim milk (Diploma, Bonlac Foods, Melbourne, Australia) in PBS for 45 minutes and then rinsed 3 times (for 1 minute each) with PBS plus 0.05% Tween 20 (PBS-Tween). All incubations were carried out on an orbital shaker. The blocked membrane-adhesive sandwich was immunostained with either poly-

clonal or monoclonal antibodies or IgE from human sera (see below). All antibody dilutions were in 2% skim milk in PBS-Tween. Unless otherwise noted, all washes between each incubation consisted of 6 rinses, a 30-minute soak, and another 6 rinses in PBS-Tween. Negative controls (ie, samples not incubated with the primary antibody) were also treated as below.

Polyclonal primary antisera

The choice of polyclonal antisera was determined by the specificity required. An inhouse rabbit antiserum raised against rye grass extract was used at 1:2000 for 1.5 to 2 hours, followed by washing. The binding of rabbit antibodies was detected with goat antirabbit IgG alkaline phosphatase conjugate (Sigma Chemical, St Louis, Mo) used at 1:500; incubation times and washing conditions were as above. The color was developed with either 1-step 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Pierce, Rockford, Ill) or fast red TR/naphthol AS-MX (Sigma). Color development was stopped by washing the membranes in DDW. The PVDF membrane was cleared with ethylene glycol/glycerol solution (9:1),¹⁶ which slightly improves particle and halo definition.

Monoclonal primary antibodies

Samples were incubated with affinity-purified mAbs raised against *Lolium perenne* that were developed in house. Their production and characterization has been described elsewhere.¹⁷ Briefly, mAb No. 4 and No. 17 react strongly to antigens of *L perenne* pollen, weakly to those of *Festuca elatior*, and not to antigens of other temperate or tropical grasses tested.¹⁷ In Western blotting they recognized bands of molecular weight 28 and 30 kd and in competitive ELISAs they inhibit IgE binding, so therefore they are most likely specific for either Lol p 1 or Lol p 5. mAb 4 was used at 0.2 μ g/mL and mAb 17 was used at 2 μ g/mL; the samples were incubated for 1.5 to 2 hours and thereafter washed as above. The samples were then incubated in sheep antimouse IgG alkaline phosphatase conjugate (Sigma) at 1:500; incubation times and washing conditions were as used above. The substrates used were either 1-step BCIP/NBT or fast red TR/naphthol AS.

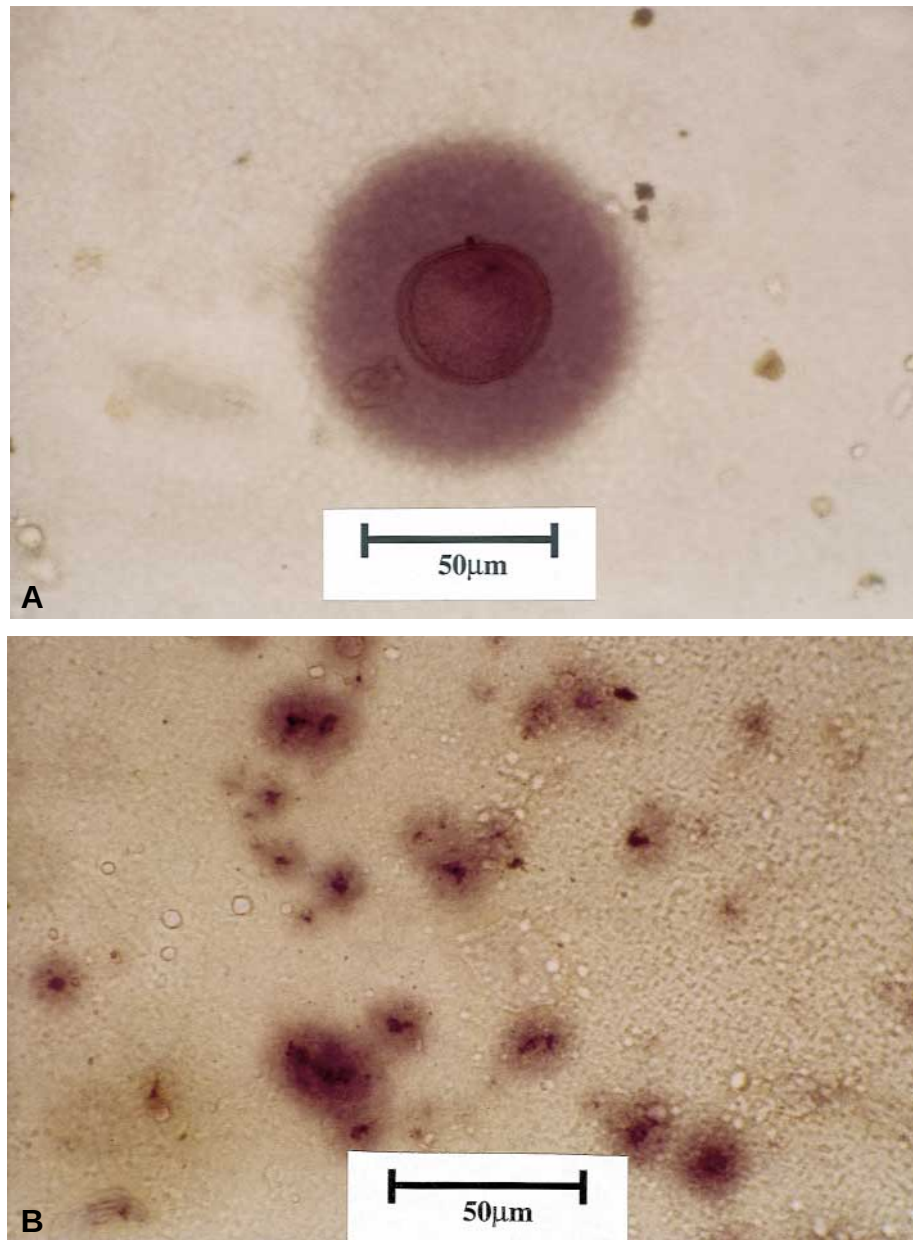


FIG 2. A, Pollen grain with surrounding halo immunostained with mAb No. 17 (Lol p 1 or Lol p 5). **B,** Paucimicronic particles with surrounding halo immunostained with mAb No. 17.

Human IgE primary antisera

This system was used to detect the carriers of rye grass pollen allergens with atopic human serum of the appropriate known specificity previously established by in vivo assay (Unicap, Pharmacia-Upjohn, Uppsala, Sweden). In all cases class III atopic serum was used undiluted and incubations were overnight, with gentle rocking. After this primary antibody incubation, the samples were washed and incubated with biotin-labeled, goat antihuman IgE (KPL, Gaithersburg, Md), which was diluted 1:500 for 1.5 to 2 hours. The conjugate was ExtrAvidin (modified streptavidin) alkaline phosphatase conjugate (Sigma) diluted 1:500 and incubated for 1.5 to 2 hours. The substrates used were either 1-step BCIP/NBT or fast red TR/naphthol AS.

Quantification of immunostaining

To determine the relationship between immunostaining and the level of specific IgE in patient serum, a series of experiments were undertaken. To standardize the source of allergens, commercially available *L perenne* pollen was used (Allergon, Engelholm, Sweden). This was sprinkled onto pressure-sensitive adhesive tape (Avery Dennison, Painesville, Ohio) with use of a 35-μm mesh dispenser to gain an even spread of pollen grains across the membrane. The allergen was overlaid with PVDF membrane and immunostained as described by the above protocol for IgE. The source of IgE was 11 human sera of known specific IgE to *L perenne* as determined by an in vitro specific IgE assay (Unicap, Pharmacia-Upjohn). For each serum the brightness intensity of the immunos-

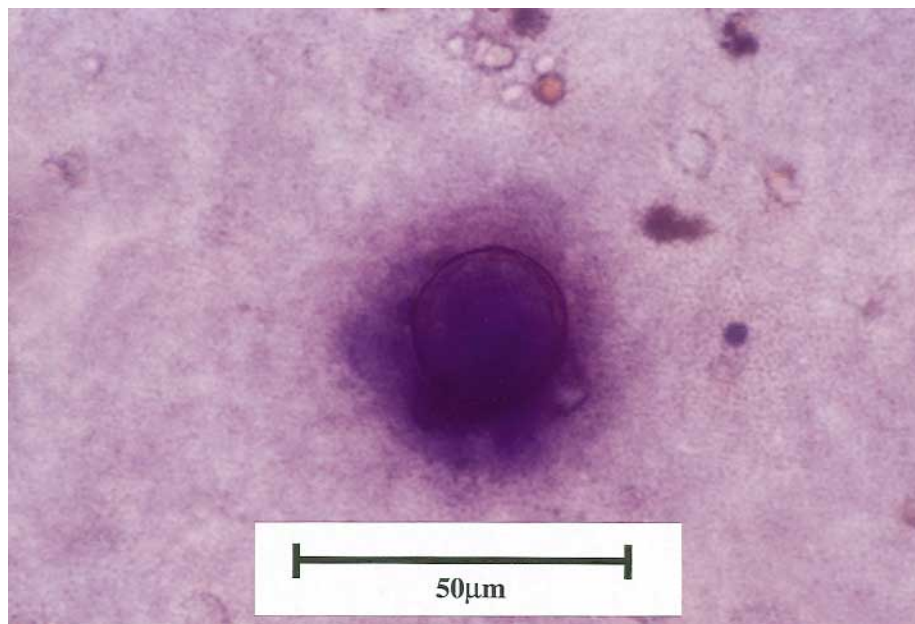


FIG 3. Pollen grain with surrounding halo immunostained with pooled neat rye grass-specific human sera (radioallergosorbent class 3).

tained halo around 50 individual pollen grains was measured. The halo intensity is a measurement of the amount of light absorbed by the stain that is distributed over the entire area occupied by the halo; therefore all intensity measurements were measured as integrated OD according to the method of De Lucca et al (1999).¹⁸

Statistical analysis

The size of halos surrounding intact pollen grains and nonpollen particles was compared with ANOVA. Regression analysis was used to determine the relationship between the level of anti-*Lolium* specific IgE in serum and the density of immunostaining surrounding *L perenne* pollen grains.

RESULTS

This technique allows the immunostaining of a wide variety of sources of outdoor allergens in such a way that the association between the particles, which are the origin of the allergen, and the allergen itself is visually apparent. Immunostaining was performed on all tapes that resulted in halos of allergenic protein surrounding individual pollen grains and other particles collected onto the tapes. For all the adhesives the allergenic particle was clearly visible in association with its allergen at up to 1000× magnification under transmission microscopy.

For grass pollens, the darkest halos were produced by staining with polyclonal rabbit sera (Fig 1), followed by mAb antibody (Fig 2, A) and then IgE (Fig 3). The background on the membranes was highest for IgE. The controls with no primary antibody were negative.

The nonspecificity of the polyclonal rabbit antibody raised against rye grass was apparent; it was found to stain *Acacia* pollen grains and *Cladosporium* spores but not *Alternaria* spores (Fig 4, A and B). Indeed, this anti-serum stained a range of pollens of sizes ranging from 12

to 55 μm (Table I), whereas the halos of immunostaining were, on average, 2 to 3 times this size (17.5-225 μm).

In addition to intact pollen grains, all sera (monoclonal and human IgE) also stained paucimicronic particles (Fig 2, B) and larger particles that were clearly not intact pollen grains. Overall, these varied in size from 1.25 to 60 μm, whereas their halos were up to 10 times the size of the particle (15-130 μm). Halos surrounding nonpollen particles were significantly larger than halos surrounding pollen grains (ANOVA, $P < .05$).

The weather conditions associated with the appearance of paucimicronic particles were predominantly dry conditions with occasional periods of light rain (less than 1 mm in 24 hours) on only 3 of the 14 sampling days. There were no thunderstorms around the period of sampling.

The density of immunostaining in a halo, as measured by the intensity of light transmitted through each halo, was positively correlated to the levels of rye grass-specific IgE in the serum (Fig 5). The serum samples had a range of *Lolium*-specific IgE from <0.18 kU/L to 1178 kU/L. Immunostaining was not observed for the serum with the lowest level of specific IgE or for serum with 2.31 kU/L, which came from a patient with no history of rye grass allergy but who was skin prick test positive to rye grass. With the exception of this serum, *L perenne*-specific IgE was detected for all sera with a specific IgE titer of 0.36 kU/L and above.

DISCUSSION

Measurement of airborne plant and fungal allergenic particles has traditionally been based on Burkard volumetric samplers, morphologic identification, and counting. The technique described here retains this technology,

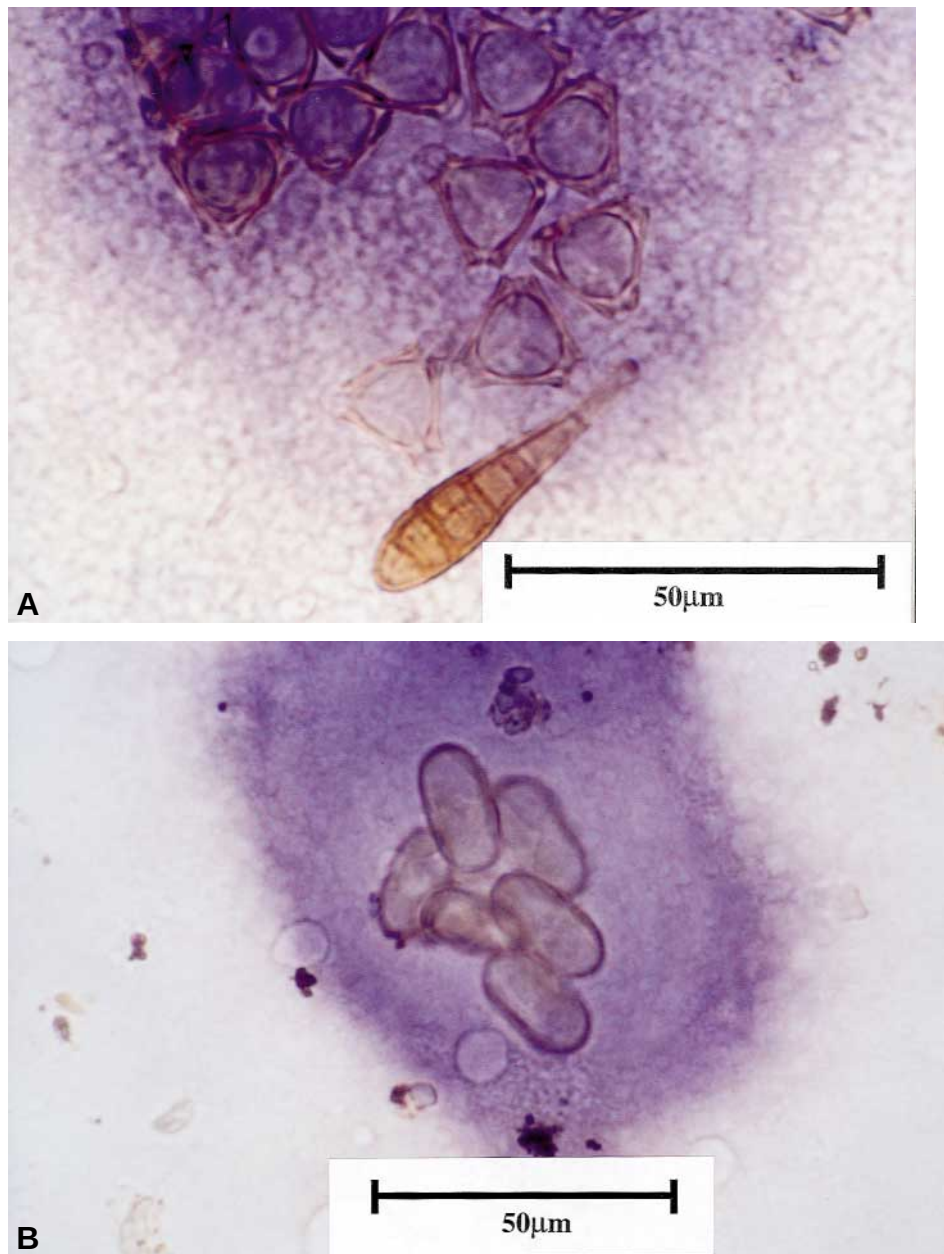


FIG 4. A, *Acacia* pollen grains with surrounding halo immunostained with polyclonal antibody. *Alternaria* spore is not stained with this antibody. **B,** *Cladosporium* spores with surrounding halo immunostained with polyclonal antibody.

used as a benchmark in aerobiology, and extends it to include the detection of specific allergens and the associated airborne particles. The use of acrylic adhesive tapes has eliminated many of the problems associated with immunostaining of traditional air-sampling adhesives. Previous studies have described immunoblotting methods for the detection of specific airborne allergens⁹ and counting of allergen spots.^{4,10,11} In the current study the protein-binding membrane remains adhered to the particle-laden tape; thus both the particle and eluted protein are seen and can be subjected to a range of analytic procedures. Morphologic analysis can be used to identify

intact spores and pollen grains, whereas the bound allergen can be subjected to a range of immunoprobings procedures. The latter can include antibodies of different known specificities or polyspecific antibodies of human or animal origin. The density of the immunostained halos can be measured by image analysis.

The results exemplify the importance of the specificity of the sera used and provide a way of validating and exploring this. The inhouse rabbit antisera that was raised against rye grass extract not only bound to other unrelated pollens but also to *Cladosporium*, but not *Alternaria*, fungal spores. The development of species-specific sera is ideal,

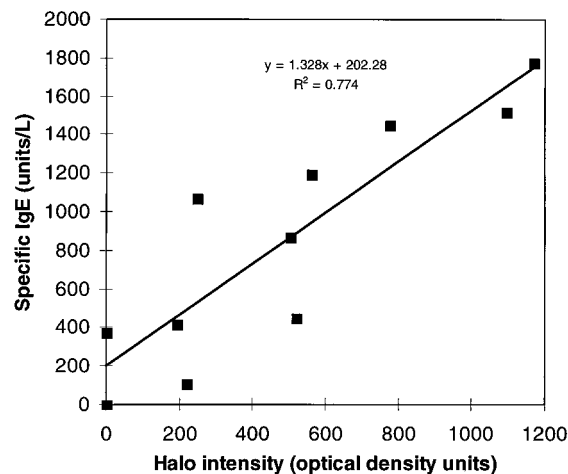


FIG 5. Relationship between level of *Lolium*-specific IgE in serum of patients and intensity of immunostained halos surrounding *L. perenne* pollen grains.

TABLE I. Size range of pollen and nonpollen particles and their halos

Probe	Size range of intact pollen grains (μm)	Size range of halos around pollen grains (μm)	Size range of nonpollen particles (μm)	Size range of halos around nonpollen particles (μm)
Polyclonal	10-55	17.5-67.5	1.25-30	10-90
mAb	37.5-50	55-225	5-30	25-62.5
Human IgE	15-30	35-75	1.25-60	15-130

Size was determined by the longest length of particle and halo, respectively.

and the immunostaining method can also be used to explore and validate specificity and antigenic relationships, for example, by the use of mAbs in immunoblotting assays with whole pollen grains from a range of species.^{17,19}

Immunostaining with a patient's IgE and viewing the source of allergens allows for determination of unknown or unsuspected allergens and identification of cross-reactive allergens that may be present in a patient's environment. The strong relationship found between the density of immunostained halos surrounding pollen grains and the level of specific IgE in the patient's sera suggest that this method may be used to assess the level of immune response of a patient to allergen-carrying particles in the environment. This assessment of an individual's allergen load when combined with personal sampling methods, such as personal air samplers^{18,20} or nasal air samplers,^{18,21} may provide insight into the relationship between allergen exposure and symptoms of allergic disease. In addition to studies on individuals, this method may have specific application in community studies where the range of possible allergen sources is not widely known or requires confirmation. The width of the Burkard tapes (20 mm) allows these to be split into at least 10 strips, which can then be incubated with individual sera from symptomatic individuals.

This technique is more sensitive than conventional ELISA immunoassays used for aeroallergen quantification because the allergen is concentrated in a halo around the particle and is not diluted in elution buffer.^{21,22} The method is as sensitive for a single particle as it is for

thousands of particles. High-volume samplers are not required for this technique; thus the integrity of collected particles is not compromised.²² The technique also allows particles that are aggregated or overlapping to be differentiated from single particles, and primary allergen sources can be distinguished from secondary allergen sources such as dust particles, which were problems with previous immunoblotting techniques.^{4,10,23,24} The amount of allergen in each individual halo was not determined; however, with our current image analysis systems this should be possible provided suitable standards can be found. Other studies have also considered the use of image processing for counting of the number of particles carrying allergens and quantifying the total amount of allergen.^{4,5,11,18}

The size of allergen halos produced by intact pollen grains was about 2.3 times the size of the pollen grain, which is similar to the results reported by Takahashi et al (1993)¹⁰ for Japanese cedar. Paucimicronic particles carrying rye grass allergen were also detected and ranged in size from 5 to 15 μm , confirming previous reports.^{2,4,25} The Burkard 7-day volumetric spore trap used in these studies is thought to be relatively inefficient at collecting particles less than 4 μm in diameter²⁴; thus the amount of allergen carried on smaller particles may be an underestimate of the true level of such particles in air. It was recently shown that the use of acrylic adhesive tapes greatly improves the apparent collection efficiency of Hirst-type spore traps for small particles (1-20 μm in diameter) compared with the standard adhesive.¹⁵ In

addition, smaller particles may be collected more efficiently with low-volume filter samplers, which have traditionally collected too little allergen for conventional analysis but can also be immunostained with our assay.¹⁸ With low-volume filter samplers the particles are collected onto a porous membrane, followed by lamination with the adhesive tape.¹⁸

The technique described here allows visualization of allergen released from a single particle such as a pollen grain or fungal spore or from smaller particles that also carry pollen or other allergens. The ability to visualize both the released allergen and its source is a powerful analytic tool that can be used to answer many questions about allergen exposure, including the size and nature of particles carrying allergen in the air and the amount of allergen carried by each particle. A range of probes can be used that allows identification of unknown and cross-reactive allergens. Factors that influence the amount of allergen released from pollen grains,²⁶ mold spores, and other allergen sources can also be examined with use of this technique.

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