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Grass Pollen Allergens: Detection on Pollen Grain Surface Using Membrane Print Technique

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Abstract. A microtechnique is described to visualize pollen allergens by making prints from the surface of mature grains on nitrocellulose membrane. The allergen *Lol p I* is detected by specific monoclonal antibodies or specific IgE from sera of grass-pollen-allergic patients, visualized by the immunoperoxidase method. This technique offers the potential to study the distribution of individual allergens in aerobiology samples by bright-field microscopy, and to determine the sensitivity spectra of allergic individuals to known and unknown pollen sources.

Introduction

The presence of allergenic proteins and glycoproteins in wind-borne pollen types such as grass pollen led to the first immunocytochemical studies of flowering plant antigens being carried out on pollen [1, 2]. Immunofluorescent studies of ragweed pollen, freeze-sectioned within the anther to minimize diffusion of antigens, show that the major ragweed allergen, Antigen E, is concentrated in the pollen walls and surface [3], and similar techniques were used to determine the surface location of grass pollen allergens using polyclonal antibodies [4].

Biochemical analyses of pollen allergens require large quantities; for example, more than 100 µg of pollen protein needs to be extracted. It is possible to extract and visualize microquantities of protein from pollen walls by a 'pollen print' technique [5]. The method involves applying dry pollen grains attached to a piece of adhesive tape to an agarose film on a microscope slide. The pollen protein components are released into the moist agarose, pollen grains removed with the tape, and the agarose film dried and stained. Immunofluorescence studies of the agarose films with polyclonal antibodies showed the progressive release of Antigen E from the surface and wall pores of ragweed pollen [5].

In this paper, we describe a modification of this method in which moist nitrocellulose or cellulose ni-

trate paper is used rather than agarose as a substrate to capture pollen surface proteins. Nitrocellulose membrane is used extensively for electroblotting of protein from polyacrylamide gels [6] and is also used for dot-blotting of antigens for immunoassay studies [7, 8] since proteins bind electrostatically to the membrane. Immunofluorescence microscopy of nitrocellulose membranes has also been recently used to identify cross-reactivity between various grass pollens and polyclonal antibodies to Bermuda grass pollen [9].

In a preliminary account [10], we established optimal times for exposing pollen to the moist membrane. In this paper, we show that the immunodominant allergen of ryegrass pollen, *Lol p I*, is present at the surface of mature viable pollen, using monoclonal antibodies or specific IgE from sera of allergic patients and immunoperoxidase methods for light microscopy.

Materials and Methods

Pollen-Printing Procedure

Fresh mature pollen of perennial ryegrass, *Lolium perenne* L., was used for pollen printing. A monolayer of fresh pollen was applied to a defined circle of 0.5 cm in diameter on a clean microscope slide. Strips of nitrocellulose membrane, 3 × 1 cm² (Schleicher & Schuell, Dassel, FRG; Membranfilter 50, pore size 0.1 µm) were soaked in 0.05 M Tris HCl buffer, pH 7, containing 0.15 M NaCl.

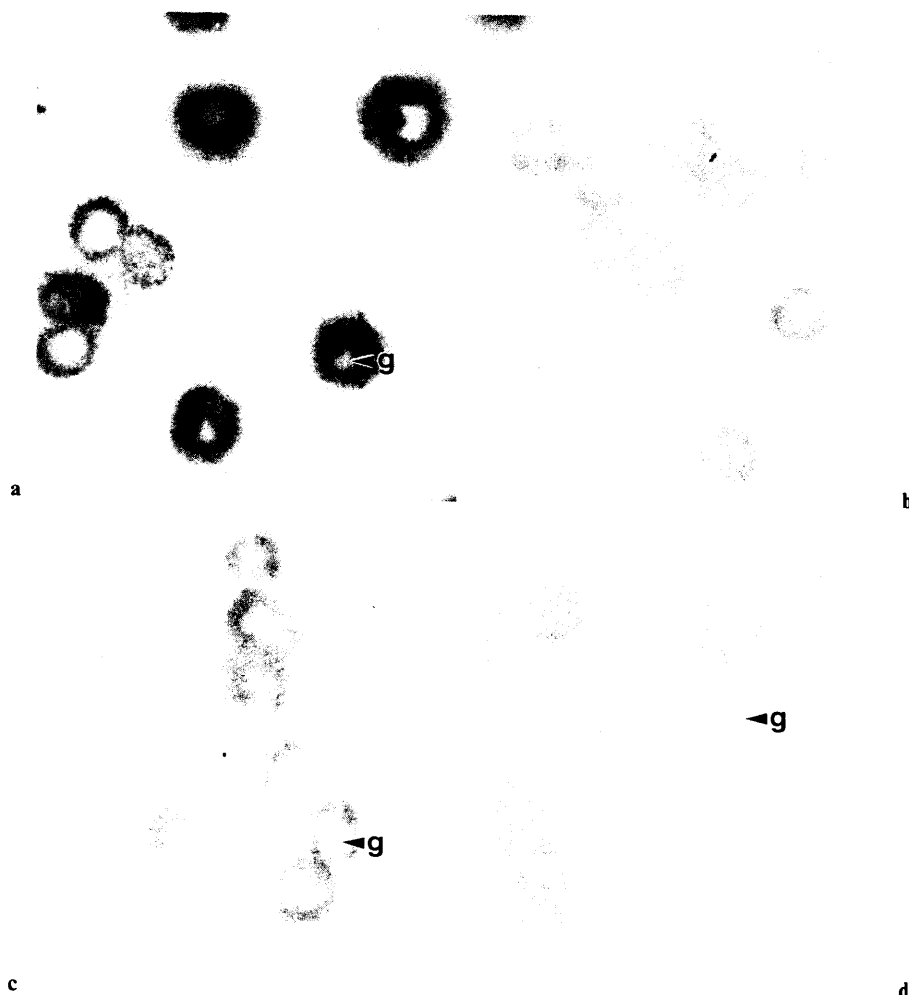


Fig. 1. Pollen protein prints of rye grass, *L. perenne*, exposed to membrane for 2-min periods. g = Germinal aperture. **a** Anti-*Lol p I* monoclonal antibody binding using the peroxidase indirect labeling method. FMC A1 antibody. $\times 250$. **b** As **a** but with FMC A2 antibody. $\times 250$. **c** Con A-peroxidase technique for glycoproteins. $\times 250$. **d** Binding of human serum IgE from a patient with clinical reactivity to rye-grass pollen using anti-IgE and indirect labeling with peroxidase. $\times 200$.

Each strip of paper was blotted to remove excess buffer, secured to the slide at one end with a piece of adhesive tape and gently laid over the pollen. Another clean microscope slide bearing a 100-gram weight was placed over the paper at right angles to the first to apply light pressure and ensure pollen-paper contact for the required printing time.

After this time, the weight and top slide were removed, the paper sliced from the adhesive tape with a razor blade and carefully lifted from the slide. Adhering pollen grains, if present, were then removed from the paper gently with a camel hair brush. The paper-strips were then allowed to air-dry after which they were either processed immediately or stored desiccated at -20°C until required. Throughout the procedure, papers were handled with fine forceps.

Printed pollen was tested for viability using the FCR test described by Heslop-Harrison and Heslop-Harrison [11]. A solution of 0.2% (w/v) fluorescein diacetate (Sigma, St. Louis, Mo., USA) is made up in 50% (v/v) acetone in water. Two or three drops of this

solution were added to 2 ml of a 20% (w/v) sucrose in water solution. Pollen grains were then added to a drop of this mixture on a microscope slide, left to incubate for 5 min and then observed under fluorescence microscopy.

Cytochemical Detection Methods

Proteins. Dried paper strips were stained with Amido black (Sigma), made up as a 0.1% (w/v) solution in 45% methanol and 7% acetic acid, for 5 min, then placed in 70% methanol and 7% acetic acid for 15 min, to remove excess stain. When papers were destained, they were air-dried and attached to microscope slides.

Glycoproteins. Pollen prints were probed for glycoproteins utilizing the concanavalin A (Con A)-peroxidase method [12]. Control prints were treated identically, with the omission of the Con A incubation step, and stained for peroxidase.

Allergens. Pollen of ryegrass was printed for 10 s, 30 s, 1 min and 2 min. These prints were incubated for 2 h with one of the fol-

lowing monoclonal antibodies: FMC A1, A2 and A7 [13]. Antigenic sites were then indirectly labeled with immunoperoxidase (Cappel Labs, Downington, Pa., USA) for 1 h [7]. Some prints were incubated in human serum from a grass-pollen-allergic patient overnight, then IgE binding visualized using anti-IgE labeled with peroxidase (CSL, Melbourne, Australia) for 3 h [14]. Pollen prints were observed by bright-field microscopy using either transmitted or epi-illumination and photographed with Kodak Tech Pan film.

Results and Discussion

Pollen Prints Using Fresh Viable Pollen

Printing times between 10 s and 2 min result in sufficient pollen protein binding to the nitrocellulose membrane to be detected by immunoperoxidase techniques. The viability of the printed pollen samples remained high (>95%), up to a printing time of 2 min. The protein released in this time period represents only the wall fraction of the protein, as shown by the positive FCR test for membrane integrity, when pollen is sampled after exposure.

Pollen Prints Using Monoclonal Antibodies and Lectins as Probes

After 2 min printing, different degrees of reactivity to each monoclonal antibody FMC A1 and A2 can be distinguished (fig. 1a, b), indicating that allergens may be present in different concentrations at the pollen surface.

Some of the ryegrass pollen allergens are glycoproteins, and Con A-peroxidase staining reveals the glycoprotein component of the pollen surface (fig. 1c). The germinal pores in the pollen wall show dense binding in some prints. Control prints which are treated only with peroxidase show no endogenous peroxidase staining (not illustrated). This confirms that peroxidase labeling is suitable for use as an indirect staining method in immunocytological analyses of pollen prints.

Pollen Prints Using Specific IgE as Probe

Specific IgE in serum from a ryegrass-sensitive patient gave a positive reaction with the ryegrass pollen prints (fig. 1d). Control prints, exposed to normal human serum, gave no positive prints.

Advantages of the Pollen Print Technique

The nitrocellulose membrane print technique is a unique method for the resolution and observation of very small quantities of surface protein such as the

pollen allergens. The protein is immobilized on the nitrocellulose membrane immediately after its release from the pollen grain and is firmly bound to the paper by air-drying. This procedure of protein capture avoids denaturing the protein that, for example, freezing or chemical fixation may cause. Pollen protein which has been blotted, dried and stored on nitrocellulose membrane at -20°C has been shown in this study to retain its antigenicity for at least 6 months.

Recently, a whole pollen protein blotting technique has been used to detect aeroantigens on nitrocellulose membranes using polyclonal antibodies and fluorescein isothiocyanate labeling [9]. The method has enabled the detection of Bermuda and closely related grass antigens in both pollen grains and fragments present in the atmosphere. No distinction between surface and internal pollen antigens could be made.

The pollen print method of protein capture presented in this paper allows the antigenic activity at the surface of individual pollen grain walls to be detected using a library of monoclonal antibodies against allergens. The utilization of nitrocellulose membrane as a solid phase to observe the distribution of specific pollen allergens provides a new micromethod that can be visualized by bright-field microscopy without the need for fluorescence microscopy, a feature that is useful for field studies.

The use of this micromethod with specific IgE from allergic individuals will enable the spectrum of pollen species to which an individual is allergic to be rapidly probed with the minimum amount of pollen.

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