

Purification and Characterization of a Major Allergen from *Dactylis glomerata* Pollen: The Ag Dg1

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Abstract. We have isolated an allergen (Ag Dg1) from *Dactylis glomerata* pollen which is recognized by the serum of 95% of human patients sensitive to *D. glomerata* pollen, as has been shown by the nitrocellulose immunoprint technique. After two successive purifications by preparative isoelectric focusing (IEF), Dg1 was characterized as a single band in analytical agarose IEF with a pI of 5.9 and was found to display 3 bands by sodium dodecyl sulfate polyacrylamide gel with the respective molecular weights: 21,000, 31,000 and 33,000 daltons. The high recognition frequency by IgE antibodies of Dg1 in the sera of allergic patients and its ability to trigger histamine release from sensitized human basophils allow to consider that Ag Dg1 is the main major allergen extracted from *D. glomerata* pollen.

Introduction

It is well-established that pollens are involved in human allergies causing hay fever. All the early studies had suggested that pollens were highly complex mixtures of allergenic, antigenic proteins and carbohydrates together with nonallergenic components [1, 2].

Attempts were made by many workers to identify allergenic components and particularly major allergens in pollen. Thus, Ag E, the major allergen, was purified from ragweed pollen [3] and Ag B is considered as the major allergen of timothy grass pollen [4].

Concerning *Dactylis glomerata* pollen (orchard grass or cocksfoot) few studies have been undertaken.

In 1961, Augustin and Hayward [14] have isolated a highly purified preparation of A antigen which has been found to be highly immunogenic in rabbits. With regard to allergenicity in human patients, it appeared as a minor allergen to which only a part of the allergenic population is sensitive. Nevertheless, up to now, the determination of the major allergen of *D. glomerata* pollen remained unsuccessful.

In France and other European countries *D. glomerata* pollen is as widespread and abundant as *Phleum pratense* and *Lolium perenne*.

In this paper, we report the purification of a major allergen (Ag Dg1) from *D. glomerata* pollen. By the

nitrocellulose immunoprint technique [6] we found that the Ag Dg1 was recognized by the serum of more than 90% of the patients studied who were sensitive to *D. glomerata* pollen. We then isolated and purified this Ag Dg1 by two successive preparative isoelectric focusings (IEF). Dg1 has an isoelectric point of 5.9. By sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) electrophoresis one major band was found corresponding to a molecular weight of 33,000 daltons.

Materials and Methods

Antigen Extract from the Pollen

A 10% sample (w/v) of pollen was suspended in distilled water and was gently agitated for 1 h at room temperature. The pollen suspension was centrifuged for 5 min at 10,000 g, and the supernatant, named soluble extract (SE), was used as source of allergens [2].

Purification of Ag Dg1 by IEF

Preparative IEF. The Radola technique (1969-1973) using a flat bed of granulated gel was used, adapted for the Pharmacia FBE 3000 electrophoretic apparatus. (a) Preparation of the first preparative IEF gel: the granulated gel (14 g of Sephadex IEF, Pharmacia) was swollen in 250 ml of degassed distilled water containing 12.5 ml Servalyt T (4-9, Serva) and 2.5 ml of Pharmalyte (4-6.5, Pharmacia). This gel was cast in the chamber formed by the FBE 3000 cooling plate and a homemade plastic frame surrounding the cooling plate forming an inner chamber of 25 × 25 × 0.5 cm. The

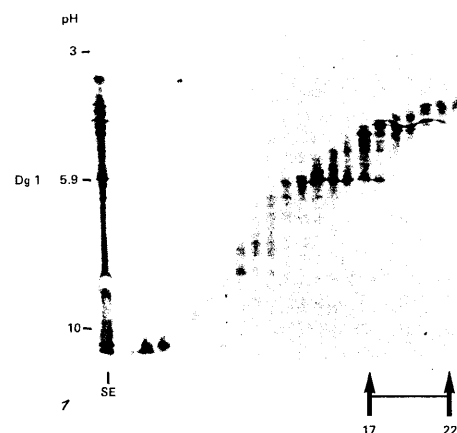


Fig. 1. Analytical IEF in a wide pH range of the fractions obtained from the first preparative IEF of *D. glomerata* SE. Fractions 17–22 were submitted, without elution from the gel, to a second preparative IEF in a narrower pH range.

upper surface of the gel was leveled by a ruler gliding on the plastic frame edges. The excess of liquid was absorbed by adding dry Sephadex IEF beads sprinkled on the wet parts (as suggested by Pharmacia). The gel was ready for use after a final leveling of the surface. Its viscosity should be such that it can withstand a 45° inclination without sliding. Under the electrodes the sponge wicks were soaked in 0.5 M acetic acid at the anode and in 1 M NaOH at the cathode. The temperature of the cooling plate was set at 4°C. (b) Sample preparation for the first preparative IEF gel: *D. glomerata* pollen from our own production was kept desiccated at 4°C. The crude extract was obtained by incubating 10 g of pollen grains with 100 ml of distilled water for 1 h at room temperature. This suspension was filtered through a buchner funnel under vacuum, the pollen being washed with 100 ml of distilled water. The filtrate was lyophilized and the dry powder resolubilized in 20 ml distilled water, centrifuged at 10,000 g for 10 min at 4°C. To the supernatant, Sephadex IEF was added to obtain a viscous liquid that could be poured in a trough cut out in the Sephadex IEF gel ready for the focusing. (c) First preparative IEF in the wide pH range: a constant power of 30 W was applied to start and gradually increased to a final 55 W by increments of 5 W every 15 min. This gradual power increase was needed to avoid overheating and excess evaporation due to the high conductivity of such a concentrated sample and so the salts, present in great amount in the sample, were electrophoretically removed prior to the establishment of the pH gradient. After a run of 5 h, a final voltage of 1,150 V, a Vh value of 3,500, samples of the gel were collected with a spatula. The presence of colored bands helped to select the fractions. The Dg1 zone was clearly visible as a 3-mm wide white-yellowish band. Another confirmation of its location could be done by a rapid pH evaluation either with pH paper laid on the gel, or by pH measurements using a miniaturized combined pH electrode dipped into the gel (Heito, Paris, France). By analytical IEF on a flat bed agarose gel, 10- μ l samples of each of 28 fractions were analyzed in a wide pH gradient together with the starting pollen SE (fig. 1). (d) Second preparative IEF in the narrow pH range: the fractions of the first preparative IEF to be refocused in a narrower pH range were chosen after the IEF patterns were ob-

tained by the analytical IEF (fractions 17–22 of fig. 1). This second preparative IEF was performed in a new separation chamber. This chamber was made, as the one used in the first preparative IEF by a frame of plastic laid on the cooling plate of the Pharmacia FBE 3000 apparatus to form an inner volume of 25 $\times$ 5 $\times$ 0.5 cm, the electrodes being perpendicular to the long axis of this chamber. The more acidic fraction to be refocused (fraction 22) was cast with the help of a spatula near the anode, the other fractions being added to it following their increasing pH. As much care as possible was taken to avoid air bubbles in the gel. The surface of the gel was flattened and leveled with the flat part of a spatula. No new ampholytes were added to the ones already present in the gel from the previous preparative IEF. Under the electrodes the Pharmacia sponge wicks were soaked in 0.5 M acetic acid for the anode and 1 M NaOH for the cathode. The temperature of the cooling plate was set at 4°C. The focusing was performed overnight for 12 h at a constant power of 5 W and a total Vh value of 11,000. The fractions were collected with a spatula in 1-ml tubes. The width of the collected fractions was arbitrarily decided, taking into account that some fractions were pigmented. The heterogeneity of each fraction was tested by a second analytical IEF on a flat bed agarose in a wide pH range (Pharmalyte) (fig. 2).

Analytical IEF. It was performed on Agarose-IEF (Pharmacia) gels: 1% agarose, containing 12% sorbitol and 2% ampholytes (as most commercial ampholyte solutions are 40% solutions, 300 μ l of ampholytes were used for a 6-ml gel). The gels were cast in a 12 $\times$ 12 $\times$ 0.4 cm chamber made of 2 glass plates separated by a 0.4-cm thick U-shaped plastic spacer on a Gelbond film (Marine colloids). To ease the casting of the gel, the upper opening of this chamber was widened by two paper clips as suggested by Görg et al. [7]. The boiled agarose gel containing the sorbitol was kept in surfusion in a 60°C water bath. The ampholytes were added, carefully mixed avoiding air bubbles and the gel was cast in the preheated chamber (10 min at 60°C in a dry oven).

The wide pH gradient was obtained by mixing ampholytes from the three major commercial manufacturers (LKB, Pharmacia and Serva) who have different recipes to produce ampholytes. This was

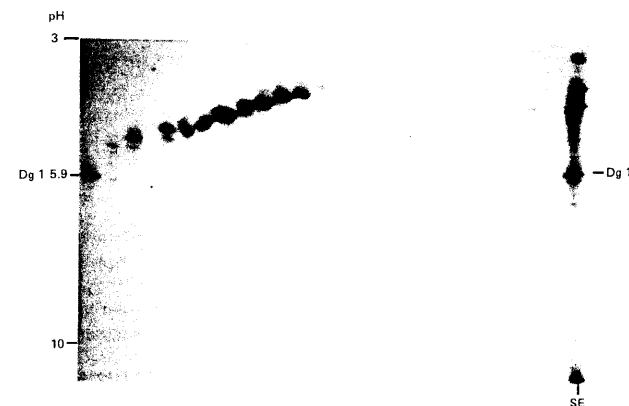


Fig. 2. Analytical IEF in a wide pH range of the fractions obtained from the second preparative IEF.

done to increase the heterogeneity of the ampholytes and to have a better resolution than with only one ampholyte source. To the wide pH ranges 3.5–10 (LKB), 3–10 (Pharmacia), 4–9 (Serva, T grade), 120 μ l each, were added a group of acid (2–4, Serva, 30 μ l; 4–5, Serva, 30 μ l and 4–6.5, Pharmacia, 60 μ l) and basic ampholytes (7–9, LKB, 60 μ l and 9–11, Serva, T grade, 60 μ l). The total volume of 600 μ l was used to prepare two plates requiring each 6 ml of gel. The gelification was allowed to occur for at least 30 min at 4°C. The focusing was performed on the same apparatus used for the preparative runs. The temperature of the cooling plate was set at 4°C. The samples were added onto the surface of the uncasted gel soaked on filter papers. Under the platinum electrodes, at two opposite edges of the agarose plate Whatman glass fiber (GF/B) paper strips were laid, soaked in 1 M NaOH for the cathode and in 0.5 M acetic acid for the anode. A constant voltage of 300 V was applied for 15 min, then a constant power of 4 W was kept up to the end of the run, usually 75–90 min. The protein detection on the gel was done by Coomassie staining.

Reaginic Sera

For mice reaginic serum 7-week-old Balb/C mice (INRA, Grignon, France) were immunized by intraperitoneal injection of Ag Dg1 (10–0.01 μ g) included in AlPO₄ gel (2 mg). 21 days later the animals received a booster injection and 7 days later they were bled.

The reaginic Ab titer of the pooled antisera was evaluated by the 2-hour passive cutaneous anaphylaxis (PCA) reaction in rats.

PCA Reaction

The measurement of circulating IgE antibodies of mice was carried out with Lewis strain rats. 2 h after sensitization of the rat skin with 200 μ l of serial twofold dilution of reaginic mice serum, animals received an intravenous injection of 1 ml 1% Evans blue including either 2 mg SE or 500 μ g Ag Dg1 [8]. The PCA titer was expressed by the maximum dilution giving a positive reaction.

SDS-PAGE Electrophoresis

Samples of Ag Dg1 and molecular weight markers were subject-

ed to electrophoresis in 1.5-mm thick slabs of PAGE (15% acrylamide) according to the method of Laemmli [9].

Spectrofluorometric Determination of Histamine

Leukocytes of blood samples from patients sensitive to *D. glomerata* pollen were isolated with a technique described by May et al. [10]. Serial dilutions were made on allergens: SE of *D. glomerata* pollen and Ag Dg1. 0.1 ml of each dilution was mixed with 0.9 ml cell suspension containing 1–10⁶ cells/ml. The mixture was incubated with constant agitation at 37°C for 40 min. All tubes were centrifuged at 300 g for the total histamine determination. Samples were deproteinized with perchloric acid and the histamine content was determined by the automated fluorometric method of Shore et al. [11]. All values presented are corrected for the spontaneous release.

Results

Immune Response after Sensitization with Ag Dg1 in Balb/C Mice

The results of IgE response to Ag Dg1 are shown in figure 3a. The animals were sensitized with different doses of Ag Dg1: 10, 1, 0.1 μ g adsorbed on 2 mg AlPO₄ as adjuvant. In all cases, the primary IgE response was not observed except with the 10- μ g dose for which the response occurred relatively late at the third week. Concerning the booster injection, the secondary IgE response differed in persistence and intensity according to the dose of Ag Dg1: no response was obtained with 0.01 μ g; 0.1 μ g induced a weak and transient IgE response, but only after a booster injection at week 5; 10 and 1 μ g were followed by a long-lasting IgE response.

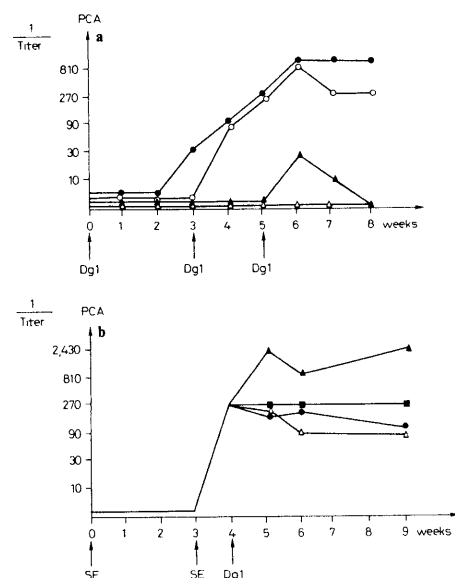


Fig. 3. **a** IgE murine response of 4 groups of BALB/c mice measured by 2 h PCA in rats: the mice were primed intraperitoneally with a sensitizing injection of varying doses of Dg1: 10 µg (●), 1 µg (○), 0.1 µg (▲) and 0.01 µg (△) together with 2 mg Al (OH)₃ as adjuvant. A booster injection at the same doses of Dg1 was administered 3 weeks later. **b** PCA reaction in BALB/c mice injected once intraperitoneally with 10 µg SE with 2 mg Al (OH)₃. Mice were boosted with a second 10-µg SE injection 3 weeks after. 1 week later, the mice were divided into 4 groups and each group was treated with a different dose of Dg1: 1 µg (▲), 0.1 µg (△) and 0.01 µg (●); ■ no Dg1 at week 4.

Figure 3b shows the effect of Ag Dg1 on the IgE response to SE. The mice were sensitized twice with 10 µg SE in 2 mg AlPO₄. At week 4, the PCA reaction reached 1/270. The same week, the mice were treated with 1, 0.1 or 0.01 µg Ag Dg1 and 2 mg AlPO₄. An important booster IgE response was obtained only with the 1 µg dose of Ag Dg1 (PCA: 1/2,430) compared to the 0.1 and 0.01 µg doses and the control.

Heterogeneity of the Allergens Recognized by Serum IgE Ab from Patients Sensitive to Grass Pollen

Sera from 17 *D. glomerata*-sensitive patients were

investigated for their ability to recognize the *Dactylis glomerata* allergen components by the immunoprint technique. For this the crude pollen SE was focused and, as shown in figure 4a, revealed at least 60 bands (with pIs ranging from 3.8 to 10.5) visualized by Coomassie blue staining.

The print was made on a nitrocellulose sheet which was cut into 17 strips and each strip was incubated with an individual patient serum. The serum IgE antibodies were revealed by a further incubation of the strips with an anti-IgE labeled with ¹²⁵I. A marked difference was observed from one patient to the other (fig. 4a). The first evidence was the number of allergens interacting with each patient serum IgE. The sera of patients No. 2, 3, 10, 11 and 14 displayed a high heterogeneity between 14 and 30 bands, whereas a lower heterogeneity of allergens was detected in patients No. 4, 7 and 15 (between 4 and 6 bands). Patients No. 1, 6, 9, 13 and 17 had a low number of allergens (one or two) reacting with their IgE antibodies.

Another observation is the relative high frequency of some allergens recognized by patient sera. The band with a pI of 5.9 and called Ag Dg1 was detected in 14 patients among the 17 patient sera analyzed (82% frequency). Another allergen with a pI of 10 was recognized by the patient sera with the same frequency (82%) as Ag Dg1.

To confirm that our purified Ag Dg1 was really the major allergen, this Ag Dg1 was focused again and analyzed by the immunoprint technique. As shown in figure 4b, Ag Dg1 focused again at pH 5.9 and was recognized by the IgE antibodies from 10 different patients sensitive to *D. glomerata*. As shown in figure 4a, the binding of the IgE antibodies to Ag Dg1 varies in intensity from one patient to another. This reflects the heterogeneity of the specific IgE antibody affinity and their different concentrations among the patient sera studied.

Evaluation of Molecular Weight and pI

The determination of the molecular weight of Ag Dg1 was performed by SDS-PAGE (15%) electrophoresis. An aliquot of Ag Dg1 (50 µg) was applied on the gel. Figure 5 shows that Ag Dg1 was separated into 3 distinct bands with a molecular weight of 21,000, 31,000 and 33,000 daltons as compared to molecular weight calibration kit. The major band, stained with Coomassie blue, has a molecular weight of 33,000 daltons.

By analytical IEF in thin agarose gels of the pollen

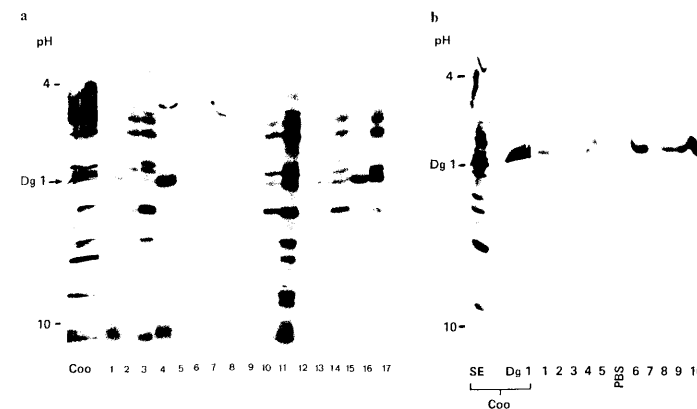


Fig. 4. **a** Heterogeneity of the *D. glomerata* pollen allergens recognized by IgE antibodies from 17 *D. glomerata*-sensitive patients. The band on the left shows the heterogeneity of *D. glomerata* stained by Coomassie blue (COO) with pIs ranging from 3.8 to 10.5. **b** Binding activity of isolated Ag Dg1 with IgE antibodies from patient sera. The purified Ag Dg1 was focused together with SE in pH ranges from 3.8 to 10.5. The band on the left shows the heterogeneity of SE and Ag Dg1 stained with Coomassie blue (COO). On the right, the print was made on a nitrocellulose sheet which was cut into strips and each strip was incubated with an individual patient serum. The negative control was made by incubating a strip with phosphate-buffered saline PBS.

SE with pI markers, the pI of Dg1 was found to be 5.9 after Coomassie blue staining (fig. 1, 2).

Allergenic Properties of Ag Dg1

PCA Reaction. To investigate how Ag Dg1 reacts with mouse IgE antibodies, PCA experiments were performed. Skins of rats were sensitized with reaginic sera raised against SE of Ag Dg1. The results (table I) showed that in both cases positive PCA reactions were obtained when the rats were challenged with Ag Dg1. In the reciprocal manner, the intravenous challenge of the rat with SE induced positive PCA reaction with both sera. The challenge doses of SE and Ag Dg1 needed to reach the optimal PCA titer were different: 2 mg for SE and only 0.5 mg for Ag Dg1.

Histamine Release Triggering. In order to know if Ag Dg1 is able to interact with IgE-bearing basophils, histamine release experiments were performed. Figure 6 shows the dose-response curve of histamine release for 1 hay fever-suffering patient sensitive to *D. glomerata* pollen. In the 10⁻¹⁰-10⁻⁴ g/ml protein concentration range of the Ag Dg1 the peak of histamine release was observed at 10⁻⁵ g/ml protein followed by a plateau. The 10⁻³ g/ml concentration of Ag Dg1 was not assayed because at this concentration the allergen was too fluorescent itself.

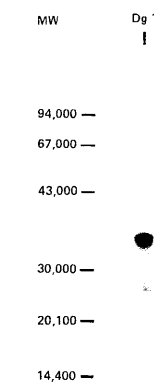


Fig. 5. Evaluation of the molecular weight of Dg1 by SDS-PAGE electrophoresis (15%).

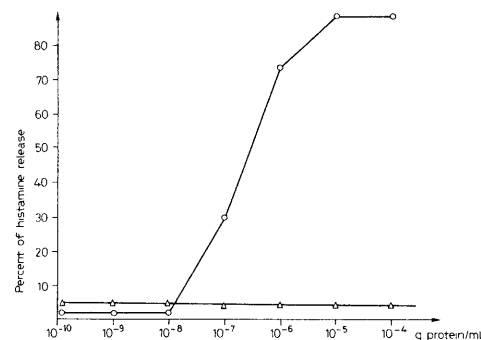


Fig. 6. Histamine release from human basophils. Basophils from *D. glomerata*-sensitive patients (O—O) were challenged with varying doses of Ag Dg1. A nonallergic control (Δ—Δ) failed to release histamine.

Table 1. Allergenic properties of Ag Dg1

Rat skin sensitizer	Challenge	PCA titers
IgE anti SE	2 mg SE	810
	0.5 mg SE	90
	2 mg Dg1	810
	0.5 mg Dg1	810
IgE anti Dg1	2 mg Dg1	270
	0.5 mg Dg1	270
	2 mg SE	270
	0.5 mg SE	30

Rat skin was passively sensitized with reaginic serum against SE or against Dg1. 2h later, the rats were challenged intravenously with different doses of either SE or Ag Dg1. The PCA titers were expressed as the mean of 2 values.

Absorbance Spectrum of Ag Dg1

Figure 7 shows the absorbance spectrum of Ag Dg1 from 400–230 nm. It clearly appears that one peak corresponds to a wavelength of 270 nm followed by a shoulder corresponding to a wavelength of 290 nm.

Discussion

Among the investigations undertaken by many authors, the major allergen from *D. glomerata* pollen has never been clearly determined. O'Sullivan [5] and

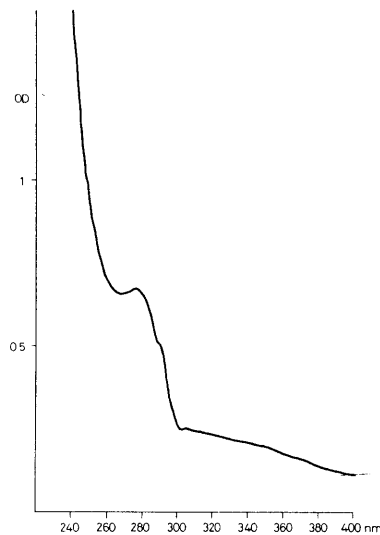


Fig. 7. Absorbance spectrum of Ag Dg1. The diagram shows a peak at 270 nm. OD = Optical density.

Augustin [12] have isolated a highly purified antigenic complex: Ag A with an undefined molecular weight between 56,000 and 88,000 daltons. This Ag A was highly antigenic in rabbits but of low skin reactivity in human patients: it was 10–10,000 times less active than whole extract and therefore considered as a minor allergen.

In the studies performed by Topping et al. [13] using a fraction obtained from an IEF separation of the cocksfoot pollen and assayed by prick test in humans, PCA reaction in monkeys and RAST were not able to identify a major allergen.

The immunoprint technique used in our laboratory, consisting in the separation of *D. glomerata* pollen components allows to distinguish for each constituent between its allergenic (reacting with IgE antibodies) or antigenic (reacting with IgG, IgM, IgA antibodies) properties [6].

Thus, we have isolated the Ag Dg1 which is a major allergen since it was recognized by 95% of allergic patients sera having RAST classes 3 and 4. The Ag Dg1 was characterized as a single band in analytical agarose IEF with a pI of 5.9 and was found to display

3 bands by SDS-PAGE with the respective molecular weights of 21,000, 31,000 and 33,000 daltons. According to the physicochemical properties and the allergenicity in man and mouse, the Ag Dg1 appears to be clearly different from the Ag A earlier described by Augustin et al. [14].

As proposed by Marsh [15] the high recognition frequency by IgE antibodies in sensitized patients allows to consider that Ag Dg1 is a major allergen extracted from *D. glomerata* pollen. Together with Ag Dg1 most of the patients also recognize a basic allergen (pI 10) which we are presently studying. For these reasons, we suggest that Ag Dg1 should be considered as a standard in the international nomenclature of purified pollen allergens.

Dr. David Marsh provided us with his monoclonal anti-Rye I (clone 21.3). By the immunoprint technique this antibody recognized also our Ag Dg1 showing the presence of a common epitope on these two major grass pollen allergens.

SDS-PAGE revealed that Ag Dg1 is dissociated in denaturing conditions (reducing agent, detergent after 2 min boiling) into three distinct components. We do not yet know the actual structure of the native molecule and further studies remain to be done to find out which fragments are the carriers of the allergenic epitopes.

In addition to its high allergenicity as shown by the immunoprint technique with allergic patient sera, by PCA in rats, and by the triggering of histamine release from human basophils, Ag Dg1 is also one of the very first allergens to be released by the pollen grain in water (data not shown). Such a rapid releasability of Ag Dg1 could play a determinant role in the hay fever pathogenesis. The fact that by the immunoprint technique some patients have IgE antibodies directed only to Ag Dg1 suggests an immunotherapy performed only with this allergen. This could be an approach to a personified immunotherapy sometimes proposed to avoid the exposure of patients to potential allergens they have not yet recognized and to whom a 'desensitization' treatment with a crude or even 'semi-purified' allergen extract may sensitize.

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