

## PRODUCTION OF A MONOCLONAL ANTIBODY AGAINST A MAJOR ALLERGEN OF *DACTYLIS GLOMERATA* POLLEN (Dg1)

by S. Mécheri, G. Peltre, A. Weyer and B. David

*Service d'Immuno-Allergie, Institut Pasteur, 75724 Paris Cedex 15*

### SUMMARY

A mouse monoclonal antibody was produced against the most frequent allergen Ag Dg1<sub>ief</sub>, purified from the *Dactylis glomerata* grass pollen by isoelectric focusing. Using the immunoprint technique, this monoclonal antibody detected Ag Dg1<sub>ief</sub> in *D. glomerata* pollen and some cross-reacting molecules in four other grass pollens. This hybridoma was grown in ascitic fluid. An immunosorbent was made from it and, by affinity chromatography, was used to purify monoclonal Ag Dg1<sub>m</sub> from the pollen extract. Ag Dg1<sub>m</sub> (6,000 to 8,000 daltons) appeared to be 4 to 5 times smaller than Ag Dg1<sub>ief</sub>. It appeared as a weak allergen in passive cutaneous anaphylaxis in rats, but was more potent in triggering histamine release on human basophils from grass-pollen sensitive patients.

These results suggest that Ag Dg1<sub>m</sub> could be a part of the Ag Dg1<sub>ief</sub> used to induce production of this monoclonal antibody.

**KEY-WORDS:** Pollen, Allergen, Monoclonal antibody; *Dactylis glomerata*, Immunochemistry.

---

### INTRODUCTION

The isolation of highly purified allergens is an essential condition for the knowledge of allergen immunochemistry and for determining the genetic basis of allergy in man. The genes control IgE as well as IgG responses to low doses of antigens injected in mice [20]. The association between H-2 type and IgE- and IgG-antibody responses to low doses of purified allergens such as rye grass group I and Asc-I allergens was previously established [2, 16]. For the AgE molecule of ragweed pollen [9], no association with any HLA type was found. This is probably due to the

high immunogenicity of AgE and the cross-reactivity with at least two other allergens, AgK and BPA-R. This allergen is considered by these authors to be a poor choice for studies of genetic control of IgE responsiveness in man. However, the IgE, and not the IgG, response to the ragweed pollen RA5 antigen has been linked to HLA-Dw2 in allergic patients [10, 11].

Another field of interest for purified antigens is the standardization of allergens and their nomenclature. Recently, the use of mouse monoclonal antibody technology was proposed as a mean of standardization applied to allergens extracted from *Dermatophagoides pteronyssinus* [1]. Monoclonal mouse antibodies against allergenic components of rye grass pollen (*Lolium perenne*) have been developed [19].

We had previously isolated a highly purified major allergen, which we now propose to name Dg1, from *Dactylis glomerata* pollen using several steps of preparative isoelectric focusing (IEF) on granulated gels. We followed its purification by analytical IEF and by the nitrocellulose immunoprint technique [13, 15] giving some of its physicochemical and biological properties. Dg1 was defined as an allergen in man and mouse showing an isoelectric point (pI) of 5.9 and one major band of 33,000 daltons in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

In this study, we report the production of a mouse monoclonal antibody (McAb P<sub>3</sub>B<sub>2</sub>) with anti-Dg1 specificity. With this monoclonal antibody, we made an immunosorbent used to purify the antigen Dg1. We obtained a highly purified component with allergenic activity, as measured by passive cutaneous anaphylaxis (PCA) in rats and *in vitro* histamine release from human basophils.

## MATERIALS AND METHODS

### *Allergen extracts.*

*D. glomerata* pollen was obtained from our laboratory and soluble extracts (SE) in distilled water were made [15]: 10% (weight/volume) extraction for 1 h at room temperature.

Antigen Dg1 was prepared as follows: SE was separated by preparative IEF in granulated gel in a wide pH range: 3-10 [17]. After focusing, the gel bands (0.5 cm wide) were cut off and collected in different tubes. The samples corresponding to each band of preparative gel were analysed by analytical IEF. The gel fractions having pH 5.9 were focused again in a preparative way in a narrow pH range. Fractions were collected and analysed by analytical IEF agarose gel. By zone electrophoresis at pH 5.5 in acetic-acid acetate 0.05 M buffer, we obtained a single protein band stained by Coomassie blue with pI 5.9.

---

Ag = antigen.  
ELISA = enzyme-linked immunosorbent assay.  
IEF = isoelectric focusing.  
IP = intraperitoneal(ly).  
McAb = monoclonal antibody.

---

PCA = passive cutaneous anaphylaxis.  
pI = isoelectric point.  
SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis.  
SE = soluble extract.

### *Immunization.*

Female BALB/c mice 7-weeks old were primed intraperitoneally (IP) with 10 µg Dg1 in AlPO<sub>4</sub>. Three weeks later, the mice were challenged IP with the same dose. A second booster injection with 10 µg Dg1 was made 2 weeks after. Three days later, as the sera from these mice showed a high PCA titre (1/810) and a positive IgG response tested by ELISA technique, their spleens were removed for cell fusion.

### *Cell fusion, cloning and production of monoclonal antibody (McAb).*

Spleen cells from the immunized mice were fused with non-secreting SP2-O myeloma cells at a ratio of 10/1 in the presence of polyethylene glycol 42% (W/v), MW 4,000 according to Köhler and Milstein [5].

Fused cells were resuspended in HAT (hypoxanthine, aminopterin and thymidine) medium containing 10% heat-inactivated foetal calf serum and seeded at a density of 10<sup>5</sup> to 10<sup>6</sup> cells per well into 24-well tissue culture plates. Supernatants of wells in which vigorous growth was observed were tested 3 weeks after cell fusion for antibody production using an antigen-binding enzyme-linked immunosorbent assay (ELISA). Positive hybridomas were cloned by limiting dilution with mouse thymocytes as a feeder layer. Single colonies were then retested for antibody production.

The hybrid clones were also propagated *in vivo* in adult BALB/c mice after IP injection of 10<sup>6</sup> hybrid cells. Large quantities of McAb were produced.

### *Antigen-binding ELISA.*

The wells of microtitre plates were coated with 200 µl Dg1 allergen at 20 µg/ml in carbonate-bicarbonate buffer pH 9.6, 0.05 M for 2 h at 37° C. The wells were washed extensively with PBS-Tween 0.1%, and 200 µl of each culture supernatant were added in duplicate. After 2-h incubation at 37° C, the wells were again washed with PBS-Tween 0.1%. Then the specific McAb were detected by addition of 200 µl of sheep anti-mouse IgG (H+L) (Institut Pasteur Production, Marnes) labelled with horseradish peroxidase (diluted 500 times). These labelled antibodies were incubated 2 h at 37° C and, after several washings, 200 µl of *ortho*-phenylene diamine (4 mg/ml) in citrate buffer pH 5, 0.05 M containing H<sub>2</sub>O<sub>2</sub> were added. The reaction was stopped by addition of 50 µl of HCl 3N.

### *Nitrocellulose immunoprint technique.*

Aliquots (10 µl) of the positive culture supernatants, ascitic fluids or allergen extracts were subjected to electrophoresis in 0.4-mm thick agarose gels in a pH 3-10 range. IEF were performed at 4° C at a constant power of 4 W for 90 min [15]. After focusing, the separated components were transferred onto a nitrocellulose sheet by blotting and pressing. This sheet was saturated in a 3% bovine serum albumine solution for 1 h at 45° C. For the McAb detection, the nitrocellulose print was incubated with sheep anti-mouse IgG (H+L) labelled with horseradish peroxidase. After 6 washings with PBS-Tween-20 (0.1%), the nitrocellulose print was incubated with the diamino-benzidine stain (0.5 mg/ml in Tris buffer 0.1 M, pH 7.4).

To analyse the specificity of the McAb the nitrocellulose print of IEF-separated pollen extracts were incubated with positive culture supernatants or ascitic fluids and then incubated as previously with purified peroxidase labelled Ab to mouse immunoglobulins.

### *Purification of Dg1 by affinity chromatography using immobilized McAb.*

The ascitic fluid containing the specific monoclonal antibody P3B2 (McAb P<sub>3</sub>B<sub>2</sub>) was subjected to an 18% Na<sub>2</sub>SO<sub>4</sub> precipitation. The supernatant was discar-

ded and the pellet was dissolved in saline and dialysed against large volumes of phosphate buffer pH 6.2 (0.0175 M).

The purification of IgG antibodies was carried out by ion exchange liquid chromatography [7]. The proteins were loaded onto a DEAE-cellulose column (DE-52 Whatman) previously equilibrated with 0.0175 M, pH 6.3 phosphate buffer. The protein concentration of the purified McAb P<sub>3</sub>B<sub>2</sub> was determined by the Lowry assay [8].

To prepare the immunosorbent, 3 g of AH-sepharose 4B (Pharmacia Fine Chemical) were activated with glutaraldehyde and then 100 mg of McAb P<sub>3</sub>B<sub>2</sub> were coupled by means of the free aldehyde groups. After 3-h incubation at room temperature, the beads were washed with 0.05 M sodium carbonate until the eluate was protein-free. The gel was then saturated with the lysine solution by overnight incubation at room temperature.

The Ag Dg1<sub>ief</sub> (prepared by preparative IEF) dialysed against 0.05 M sodium carbonate buffer pH 8.5 was loaded onto the immunosorbent column. The elution was carried out with the same buffer until the effluent was protein-free. The bound material was then eluted with 0.1 M HCl-glycine buffer pH 2.5 and the pH was immediately neutralized with K<sub>2</sub>HPO<sub>4</sub> 1 M. The eluted component was named Dg1<sub>m</sub>.

#### *Histamine-release triggering.*

Leukocytes were isolated from 30 ml of venous blood from patients sensitive to *D. glomerata* pollen [12, 3]. Washed cells were suspended in Tris-ACM buffer containing 0.025 M Tris, 0.12 M NaCl, 0.005 M KCl,  $6 \times 10^{-4}$  M CaCl<sub>2</sub>,  $10^{-3}$  M MgCl<sub>2</sub> and 0.05% human serum albumin pH 7.4. The cells were challenged with SE of *D. glomerata* pollen or with Dg1<sub>ief</sub> or with Dg1<sub>m</sub>. After incubation for 40 min at 37° C, the content of histamine in the cell-free supernatant was measured by an automated technique [18, 6].

## RESULTS

#### *Selection and characterization of McAb.*

From one hybridization, a total of 22 hybrids were screened for antibody production by ELISA. Among these, only 6 were positive in Ag-binding ELISA. The monoclonality of these 22 hybridomas was analyzed by the nitrocellulose immunoprint technique (fig. 1). The same 6 supernatants appeared to contain McAb.

---

FIG. 1. — Nitrocellulose immunoprint analysis of monoclonality of 22 hybridomas.

Twenty-two culture supernatants from growing hybridoma cells were focused on agarose gel and a nitrocellulose immunoprint was made. Only six supernatants were revealed to be positive, among them the McAb P<sub>3</sub>B<sub>2</sub> (sample n° 9).

FIG. 2. — Nitrocellulose immunoprint analysis of ascitic fluid monoclonality.

Cells (10<sup>6</sup>) of six secreting hybridoma were propagated *in vivo* in adult BALB/c mice. One week later, ascitic fluids were harvested and analysed for their monoclonality by immunoprint technique. The *in vivo* secretory activity remained only with the P<sub>3</sub>B<sub>2</sub> hybridoma.

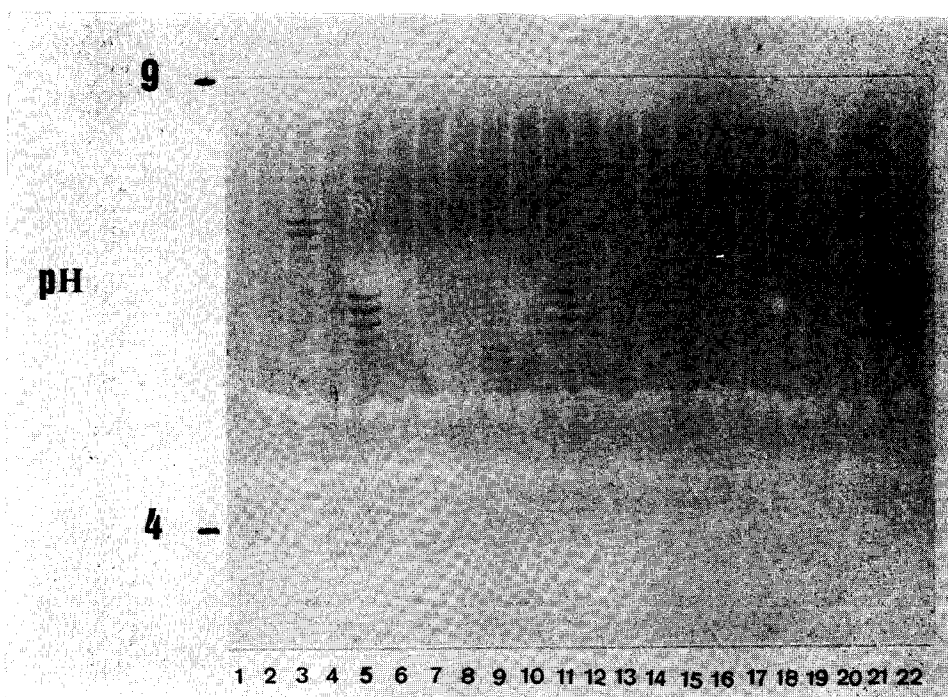


FIG. 1

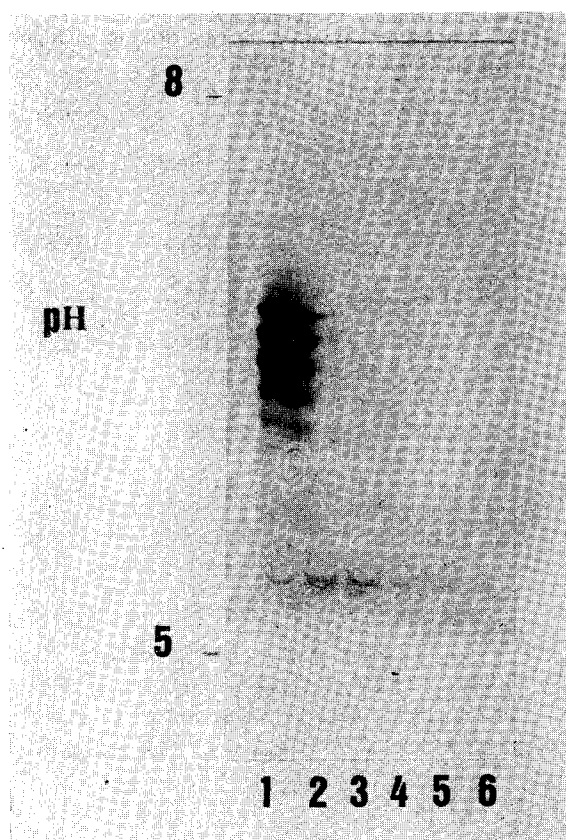


FIG. 2

FIG. 3. — *Specificity of McAb P<sub>3</sub>B<sub>2</sub> by the nitrocellulose immunoprint technique.*

The McAb P<sub>3</sub>B<sub>2</sub> reacted with components from only five pollen extracts: *A. sativa* (2), *D. glomerata* (4), *F. pratensis* (5), *A. odoratum* (6) and *L. perenne* (8). For each pollen extract, a low pI heterogeneity of the antigen recognized was observed.

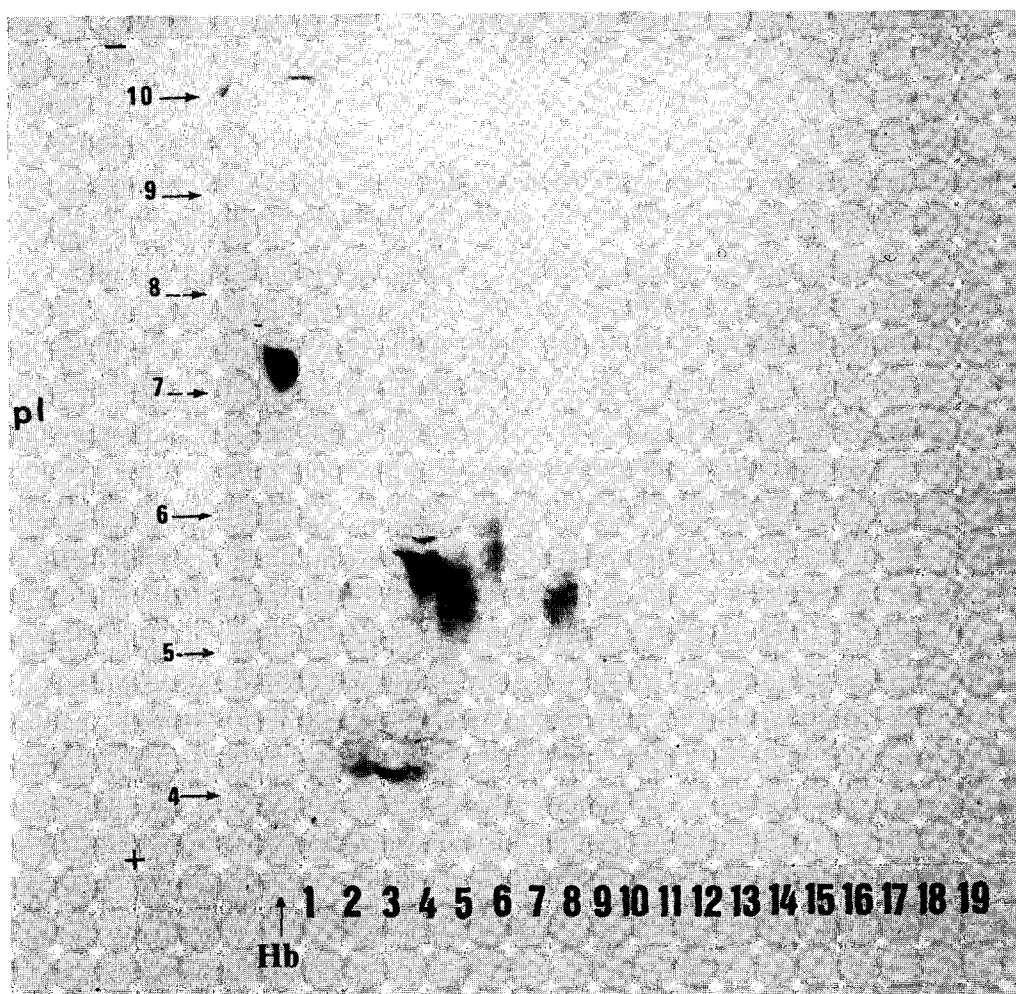


FIG. 3



The P<sub>3</sub>B<sub>2</sub> clone producing an IgG isotype was propagated *in vivo* in adult BALB/c mice after IP inoculation of 10<sup>6</sup> hybrid cells. The ascitic fluid was harvested 1 week later and analysed by the immunoprint technique. Figure 2 displays the appearance of the McAb P<sub>3</sub>B<sub>2</sub> in the ascitic fluid. The other 5 hybridomas did not secrete any antibody in the ascitic fluids.

#### *Specificity of the McAb.*

Only the McAb P<sub>3</sub>B<sub>2</sub> was subjected to this experiment. As mentioned above, this hybridoma gave a positive antigen-binding ELISA, the immobilized antigen being the *D. glomerata* SE.

By the immunoprint technique, when *D. glomerata* SE is separated by IEF, the McAb P<sub>3</sub>B<sub>2</sub> recognizes the Dg1 band at pI 5.9 and a group of weaker bands close to it. This can be seen in figure 3, where *D. glomerata* SE (n° 4) was studied in analytical IEF side by side with 10% W/V SE of 18 different pollens, 14 of which were grass pollens (n° 1 to 14), 2 were tree pollens (15 and 18) and 3 were weed pollens (17 to 19). An immunoprint of the whole focusing plate was made and incubated with 1 ml of ascitic fluid containing the McAb P<sub>3</sub>B<sub>2</sub>, finally revealed by peroxidase-labelled anti-mouse immunoglobulins. One can see that this McAb, besides the Dg1 from *D. glomerata*, recognized some antigenic bands in *Festuca pratensis* (n° 5), *Anthoxanthum odoratum* (n° 6), *Lolium perenne* (n° 8) and *Avena sativa* (n° 2) and very weakly in *Agrostis alba* (n° 1). No epitope was detectable in the other grass pollen extracts: *Bromus arvensis* (n° 3), *Holcus lanatus* (n° 7), *Panicum miliaceum* (n° 9), *Hordeum sativum* (n° 10), *Poa pratensis* (n° 11), *Phleum pratense* (n° 12), *Secale cereale* (n° 13) and *Alopecurus pratensis* (n° 14). No detectable cross-reaction was observed with the tree pollens from *Betula alba* (n° 15) and *Platanus acerifolia* (n° 16) or the weed pollens from *Ambrosia elatior* (n° 17), *Amaranthus retroflexus* (n° 18) and *Brassica campestris* (n° 19).

It can be seen that antigens bearing common epitopes recognized by McAb P<sub>3</sub>B<sub>2</sub> among these different pollens did not focus at exactly the same pI.

#### *Purification of Ag Dg1<sub>m</sub> and some of its biological and physicochemical properties.*

The purification of Ag Dg1<sub>m</sub> was carried out using McAb P<sub>3</sub>B<sub>2</sub> immobilized as an immunosorbent. The column was loaded with a solution of Ag Dg1<sub>ief</sub> purified by preparative IEF. The Ag Dg1<sub>m</sub> was eluted at pH 2.5, instantaneously neutralized and its absorbence spectrum was made. The peak absorbence of Ag Dg1<sub>ief</sub> was 280 nm, compared with 270 nm for Ag Dg1<sub>m</sub> (fig. 4). No Dg1 was detectable by human IgE antibodies in the fraction not absorbed by the McAb P<sub>3</sub>B<sub>2</sub> column.

In an attempt to determine the molecular size of Ag Dg1<sub>m</sub>, an analytical electrophoresis in polyacrylamide gel was performed in presence of SDS (SDS-PAGE). Ag Dg1<sub>m</sub> was not stainable with the common protein stain

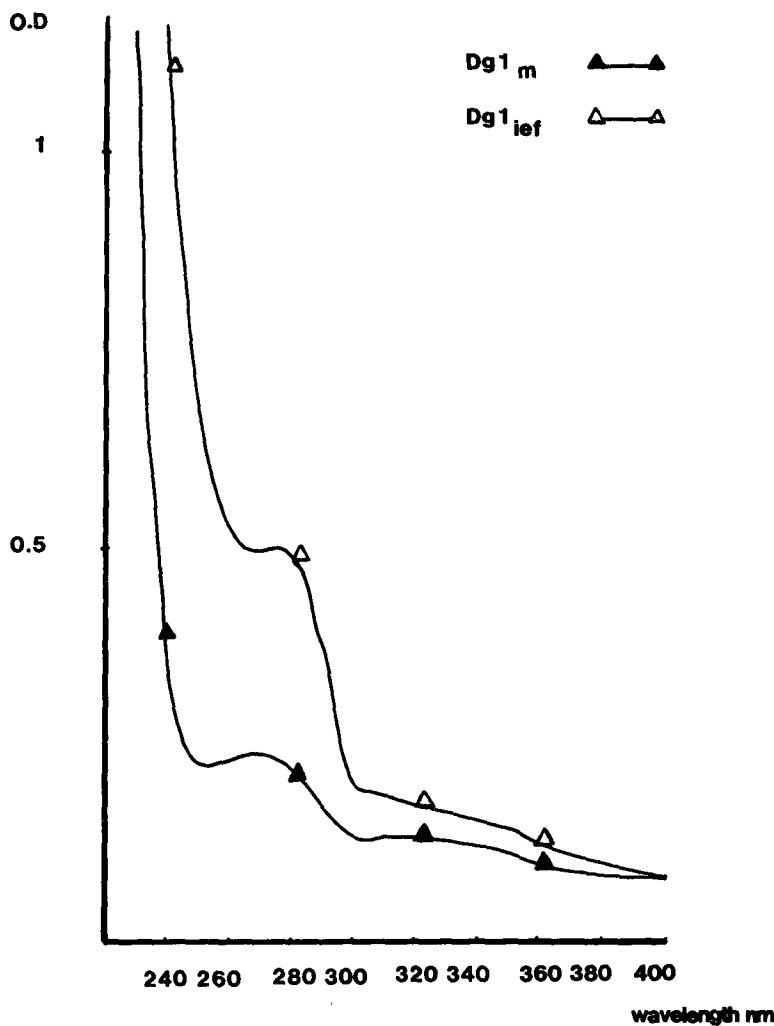


FIG. 4. — Absorbance spectra of Ag Dg1<sub>m</sub> (▲) and Ag Dg1<sub>ief</sub> (△) between 240 and 400 nm.

Coomassie blue. It was stained neither with silver nitrate, usually more sensitive than Coomassie blue, nor with ethyidium bromide, the common nucleic acid stain. It should be noted that Ag Dg1<sub>ief</sub> was also not stained with ethyidium bromide, but was strongly stained with silver nitrate. Finally, the molecular size of Ag Dg1<sub>m</sub> was estimated, by gel filtration liquid chromatography, to be between 6,000 and 8,000 daltons.

Concerning its biological properties, Ag Dg1<sub>m</sub> appeared to be an allergenic component either by the PCA technique or by histamine release triggering experiments. Table I shows the results of PCA reactions in Lewis rats challenged intravenously with SE, Dg1<sub>ief</sub> or Dg1<sub>m</sub> after 2-h skin

TABLE I. — Comparison between PCA titres induced by Dg1<sub>m</sub>, Dg1<sub>ief</sub> and SE in rats sensitized with an anti-Dg1<sub>ief</sub> serum.

| Rat skin sensitization        | Challenge with     |        | PCA titre |
|-------------------------------|--------------------|--------|-----------|
| Anti-Dg1 <sub>ief</sub> serum | Dg1 <sub>m</sub>   | 2 mg   | 30        |
|                               | Dg1 <sub>ief</sub> | 0.5 mg | 270       |
|                               | SE                 | 2 mg   | 270       |

Dry weights of the allergen amounts used per rat were estimated after dialysis and lyophilization.

sensitization with an anti-Dg1<sub>ief</sub> reagenic serum (PCA titre 270). Ag Dg1<sub>m</sub> appeared to be weakly allergenic compared to Dg1<sub>ief</sub> and SE (PCA titre 270), since its PCA titre reached only 30. The allergenicity of Ag Dg1<sub>m</sub> was also investigated by histamine release triggering of basophils from two *D. glomerata*-sensitive patients. The initial amount of total IgE of the two patients was different: 55 IU/ml in patient A and 4,000 IU/ml in patient B. Figure 5

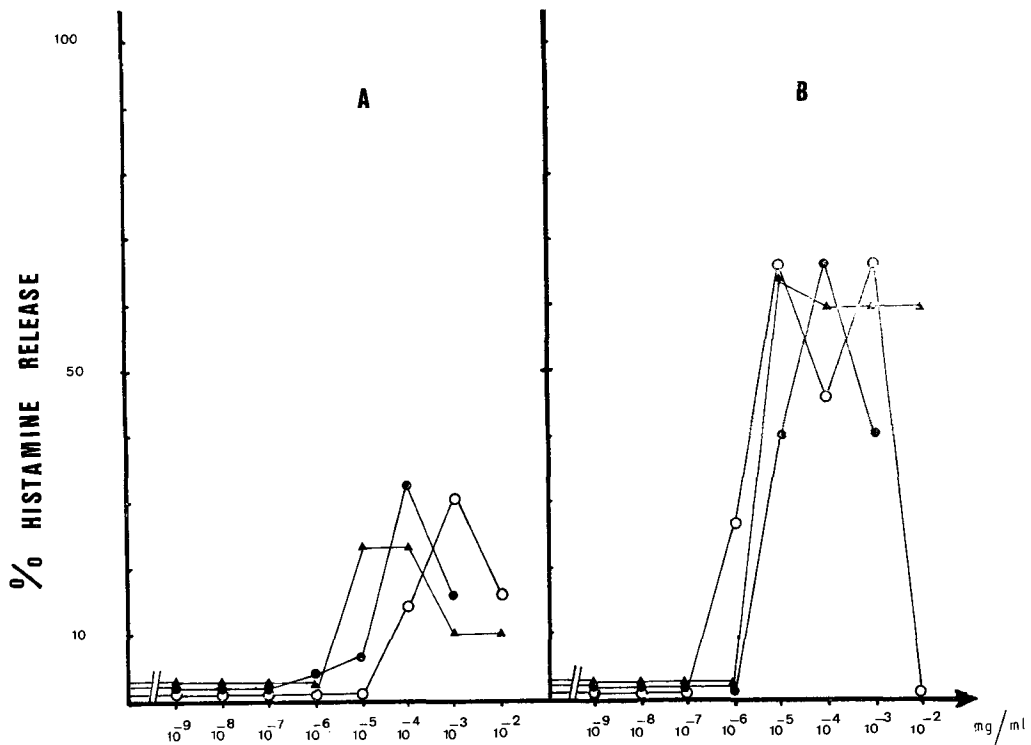


FIG. 5. — Histamine release from human blood leukocytes of two *D. glomerata* sensitive patients.

Blood leukocytes from 2 patients, A and B, were challenged with varying doses of SE of *D. glomerata* pollen (○), with Dg1<sub>m</sub> (●) or with Dg1<sub>ief</sub> (▲). The allergen concentrations in abscissa are expressed in mg of dry weight (after dialysis and lyophilization) per ml final volume.

shows the amounts of histamine released with Ag Dg1<sub>m</sub> compared to Ag Dg1<sub>ief</sub> and SE. In the two cases, Dg1<sub>m</sub> was able to trigger histamine release. Compared to Ag Dg1<sub>ief</sub>, the maximum histamine release corresponded to the concentration of 10<sup>-4</sup> mg/ml, whereas the maximum was reached at 10<sup>-5</sup> mg/ml concentration for the Ag Dg1<sub>ief</sub> in the two patients.

## DISCUSSION

In this work, we have reported the development of a McAb which reacted with components of *D. glomerata* pollen extract. The antigenic determinants recognized by the P<sub>3</sub>B<sub>2</sub> monoclonal antibody using the immunoprint technique, are carried by molecules of a pI around 5.9, which is the pI of the immunizing pollen component which we named Ag Dg1<sub>ief</sub>. As far as we know, this allergen has not been described by other authors.

We had reported earlier that Ag Dg1<sub>ief</sub> was the most frequent allergen recognized in humans [15]. It was detected as an allergen and was able to induce an IgE response in mice [13].

This antigen is distinct from the A antigen described by O'Sullivan [14], which is a basic component and fails to induce IgE formation. Figure 3 shows cross-reactivity between Ag Dg1 of *D. glomerata* and other components belonging to four other pollens: *A. sativa*, *F. pratensis*, *A. odoratum* and *L. perenne*. The component of *L. perenne* which reacted with McAb P<sub>3</sub>B<sub>2</sub> is very similar in pI (5.25) with an antigenic determinant of the group-I allergens described by Johnson and Marsh [4].

To determine whether the *D. glomerata* epitope recognized by McAb P<sub>3</sub>B<sub>2</sub> was carried by an allergen, the purification of this molecule was performed using an immunosorbent with immobilized P<sub>3</sub>B<sub>2</sub> antibodies. A purified component named Ag Dg1<sub>m</sub> was obtained. The Ag Dg1<sub>m</sub> was shown to be allergenic, since it reacts with mouse IgE antibodies (PCA reaction in rats) and human IgE antibodies, as shown by histamine release triggering from human patient basophils. In a recent paper, Smart *et al.* [19] described the production of monoclonal antibodies specific for allergenic components from rye grass (*L. perenne*) pollen. They induced their McAb by immunizing mice with a rye grass crude extract, as opposed to our approach, which consisted of immunizing mice with a purified pollen allergen, Ag Dg1<sub>ief</sub>.

They developed two elaborate ELISA techniques in order to screen hybridomas specific to antigens and allergens. Their technique for studying the immune specificity of their McAb differs from our immunoprint technique. They separate the pollen extract by SDS-PAGE, which may denature some of the labile pollen components.

The fact that Ag Dg1<sub>m</sub> differs from the immunizing Ag Dg1<sub>ief</sub> may be due to the Ag Dg1<sub>m</sub> column elution step, namely acidic exposure. Such a treatment may have split the Ag Dg1<sub>ief</sub> molecule into a 4-to-5-times smaller component, Dg1<sub>m</sub> and another component of bigger size (25,000 daltons). Preliminary experiments showed that this component was not recognized

by McAb P<sub>3</sub>B<sub>2</sub>. This may explain the modified absorbance spectrum, the difference in chemical reactivity responsible for the ability of Dg1<sub>m</sub> to be stained either by Coomassie blue or by silver nitrate. Due to its small size, Ag Dg1<sub>m</sub> may have not been immobilized after electrophoretic separations and obviously not visualized by the stains we used. Pretreatment of Ag Dg1<sub>ief</sub> by 0.1 M glycine-HCl buffer pH 2.5 leaves intact the recognition of Ag Dg1<sub>m</sub> by McAb P<sub>3</sub>B<sub>2</sub>.

Such a smaller molecule may be a potent allergen in histamine release triggering. For patient A, basophil sensitivity was greater with Ag Dg1<sub>m</sub> than with crude extract. In both patients A and B, the maximum percentages of histamine release obtained with Ag Dg1<sub>ief</sub>, Ag Dg1<sub>m</sub> or SE were comparable, but not at the same allergen concentrations.

It is evident that more physico-chemical data are needed to better characterize the Ag Dg1<sub>m</sub> we purified.

This monoclonal antibody may be very useful in several fields: (1) in fundamental allergy, in order to study the structures responsible for IgE induction and target cell (mast cell and basophil) degranulation, (2) in allergen standardization, to quantitate the amount of Ag Dg1 in different *Dactylis* extracts or cross-reacting extracts, (3) in genetics, where highly purified allergens are needed to study the genetic basis of their recognition in man, and (4) in botany to establish the phylogenetic order of the different grasses through their pollen components.

## RÉSUMÉ

### PRODUCTION D'ANTICORPS MONOCLONAUX CONTRE UN ALLERGÈNE MAJEUR DU POLLEN DE « DACTYLIS GLOMERATA » (Dg1)

Nous avons préparé un anticorps monoclonal de souris dirigé contre l'antigène (Ag) Dg1<sub>ief</sub> purifié par isoélectrofocalisation préparative qui est l'allergène le plus fréquent du pollen de la graminée *Dactylis glomerata*. Par la technique d'immuno-empreinte sur nitrate de cellulose, cet anticorps monoclonal reconnaît l'Ag Dg1 dans le pollen de dactyle ainsi que des constituants de quatre autres pollens de graminées. Cet hybridome développé en ascite nous a permis de faire un immunoabsorbant et de purifier, par chromatographie d'affinité, un Ag Dg1<sub>m</sub> (de 6 000 à 8 000 daltons), 4 à 5 fois plus petit que l'Ag Dg1<sub>ief</sub>. Ce constituant Ag Dg1<sub>m</sub> est un faible allergène chez le rat (réaction d'anaphylaxie cutanée passive). *In vitro*, lorsque l'Ag Dg1<sub>m</sub> est mis en présence de leucocytes de patients allergiques au pollen de dactyle, on observe une libération d'histamine par les basophiles du sang.

Ces résultats nous suggèrent que l'Ag Dg1<sub>m</sub> est un allergène qui paraît être une fraction de l'Ag Dg1<sub>ief</sub> utilisé pour l'obtention de l'anticorps monoclonal.

MOTS-CLÉS : Pollen, Allergène, Anticorps monoclonal ; *Dactylis glomerata*, Immunochimie.

## ACKNOWLEDGEMENTS

We gratefully acknowledge Dr J. C. Mazié and S. Rouyre (Hybridolab, Pasteur Institute) for their collaboration, J. Lapeyre, J. Rabillon and R. Peronet for their skillful technical assistance, J. Weyer for helpful discussion and G. Allain for typing.

## REFERENCES

- [1] BALDO, B. A., KRILIS, S. & BASTEN, A., Selective approaches to the isolation and standardization of allergens, in « Contemporary topics in molecular immunology » (Imman, Mandy), vol. 8 (p. 41-52). Plenum Press, New York, 1981.
- [2] CHANG, E. B. & MARSH, D. G., Immune responsiveness of inbred mice to pollen allergens. *J. Allergy clin. Immunol.*, 1974, **53**, 65-66.
- [3] ISHIZAKA, T., ISHIZAKA, K., JOHANSSON, S. G. O. & BENNICHT, H., Histamine release from human leukocytes by anti- $\gamma$ E antibodies. *J. Immunol.*, 1969, **102**, 884-892.
- [4] JOHNSON, P. & MARSH, D. G., « Isoallergens » from rye grass pollen. *Nature* (Lond.), 1965, **206**, 935-936.
- [5] KOHLER, G. & MILSTEIN, C., Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* (Lond.), 1975, **256**, 495-497.
- [6] LEBEL, B., A high-sampling-rate automated continuous-flow fluorometric technique for the analysis of nanogram levels of histamine in biological samples. *Analyt. Biochem.*, 1983, **133**, 16-29.
- [7] LEVY, H. B. & SOBER, H. A., A simple chromatographic method for preparation of gamma-globulin. *Proc. Soc. exp. Biol.* (N. Y.), 1960, **103**, 250-252.
- [8] LOWRY, O., ROSEBROUGH, N., FARR, A. & RANDALL, R., Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, 1951, **193**, 265-275.
- [9] MARSH, D. G., CHEESE, G. A. & BRIES, W. B., An immune response gene for ragweed Ra3: most probable location within the HLA-2,12 haplotype. *Fed. Proc.*, 1975, **34**, 980.
- [10] MARSH, D. G., HSU, S. H., ROEBBER, M., EHRLICH-KAUTZKY, E., FREIDHOFF, L. R., MEYERS, D. A., POLLARD, M. K. & BIAS, W. B., HLA-DW2: a genetic marker for human immune response to short ragweed pollen allergen. — I. Response resulting primarily from natural antigenic exposure. *J. exp. Med.*, 1982, **155**, 1439-1451.
- [11] MARSH, D. G., MEYERS, D. A., FREIDHOFF, R., EHRLICH-KAUTZKY, E., ROEBBER, M., NORMAN, P. S., HSU, S. H. & BIAS, W. B., HLA-DW2: a genetic marker for human immune response to short ragweed pollen allergen. — II. Response after ragweed immunotherapy. *J. exp. Med.*, 1982, **155**, 1452-1463.
- [12] MAY, C. D., LYMAN, M., ALBERTO, R. & CHENG, J., Procedures for immunochemical study of histamine release from leukocytes with small volume of blood. *J. Allergy*, 1970, **46**, 12-20.
- [13] MÉCHERI, S., Régulation de la réponse IgE par des constituants de pollen *Dactylis glomerata*. Thèse de Doctorat de 3<sup>e</sup> cycle. Université Paris VII, 1983.
- [14] O'SULLIVAN, S., PhD thesis, University of Liverpool, 1968.
- [15] PELTRE, G., LAPEYRE, J. & DAVID, B., Heterogeneity of grass pollen allergens (*Dactylis glomerata*) recognized by IgE antibodies in human patients sera by a new immunoprint technique. *Immunol. Letters*, 1982, **5**, 127-131.
- [16] PETRUCCO, R. & MARSH, D. G., Immune response to ascaris antigens in inbred mice. *J. Allergy clin. Immunol.*, 1974, **53**, 65.

- [17] RADOLA, B. J., Isoelectric focusing in layers of granulated gels. — II. Preparative IEF. *Biochim. biophys. Acta* (Amst.), 1974, **386**, 181-195.
- [18] SIRAGANIAN, R. P., An automated continuous flow system for the extraction and fluorometric analysis of histamine. *Analyt. Biochem.*, 1974, **57**, 383-394.
- [19] SMART, I. J., HEDDLE, R. J., ZOLA, H. & BRADLEY, J., Development of monoclonal mouse antibodies specific for allergenic components in rye grass pollen. *Int. Arch. Allergy*, 1983, **72**, 243-248.
- [20] VAZ, N. M. & LEVINE, B. B., Immune response of inbred mice to repeated low doses of antigens. Relationship to histocompatibility (H-2) type. *Science*, 1970, **168**, 852-854.