

DETERMINANTS OF RYEGRASS POLLEN CYTOCHROME c RECOGNIZED BY HUMAN IgE AND MURINE MONOCLONAL ANTIBODIES*

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Abstract—In attempts to produce fragments of an allergenic molecule which would retain allergenic and/or antigenic determinant(s), the cytochrome c of ryegrass (RG) pollen, which had been shown to be an allergenic constituent of this pollen, was digested with trypsin and chymotrypsin and the resulting fragments were separated by high performance liquid chromatography. Several of these fragments were shown, with the aid of the radioallergosorbent test and solid phase radioimmunoassays, to bind IgE antibodies present in a pool of six sera from grass-sensitive patients and three murine monoclonal antibodies, designated as Mab 41, Mab 42 and Mab 43, which had been originally produced against the crossreacting cytochrome c of Kentucky bluegrass (KBG). In summary, (i) fragments C-67 and C-74 reacted with all antibodies, (ii) fragments T-45, T-46 and C-69 bound to human IgE antibodies as well as to Mab 41 and Mab 42, but not to Mab 43, (iii) fragment T-44 reacted only with Mab 41 and Mab 42, and (iv) fragment C-83 bound only Mab 42 and Mab 43. On the basis of these results, it is concluded that (i) immunochemically active fragments of the RG cytochrome c can be readily produced by enzymatic degradation, (ii) there is significant crossreaction between the antigenic determinants of RG and KBG cytochromes c, (iii) whereas all fragments possessed at least two of the original antigenic determinants, fragments C-83 and T-44 were devoid of allergenic determinants, (iv) the antigenic determinants recognized by Mab 41 and Mab 42 were different from those reacting with human IgE antibodies and Mab 43, (v) each of the three monoclonal antibodies recognized a distinct antigenic determinant, (vi) fragments C-67 and C-74 possessed all determinants recognized by the human IgE and mouse antibodies used.

INTRODUCTION

In relation to the regulation of antibody formation, it is now generally accepted that (i) collaboration (Loor and Roelants, 1977) between T helper (T_H) and B cells is a prerequisite for the induction of the IgG and IgE antibody responses to most protein antigens in mice (Katz *et al.*, 1970; Michael and Berstein, 1973) and rats (Tada and Okimura, 1971); (ii) the population of T cells contains not only T_H cells, but also suppressor (T_S) cells (Möller, 1975) and amplifier (T_A) cells and that T_A cells may enhance the effects of T_H and T_S cells (Möller, 1977, 1978), and (iii) the homeostatic balance among these different subpopulations of T cells is responsible for controlling the magnitude and duration of antibody responses (Katz, 1977; Mitchison *et al.*, 1970; Moller, 1975, 1977, 1978).

For therapy of allergic conditions mediated by IgE antibodies, it would be desirable to stimulate the activation of T_S cells which would lead to the suppression of IgE antibodies to the allergen in question. In support of this concept, one may cite the results of Sercarz *et al.* (Sercarz *et al.*, 1978) who have demonstrated the presence of a single suppressive

determinant on the lysozyme molecule which promoted the activation of T_S cells capable of abrogating the formation of antibodies directed against the antigenic determinant(s) of the lysozyme molecule as a whole. Another therapeutic modality would involve inhibiting the cross-linking of cell-fixed IgE antibodies by natural multivalent allergens by the administration of univalent haptens of the appropriate allergenic molecules or of fragments of these molecules containing only univalent determinants. The feasibility of this approach gains support from the successful regimen developed by de Weck (de Weck and Girard, 1972) for the treatment of penicillin allergy by the injection of large doses of the univalent penicillin derivative, benzyl penicilloyl-formyl lysine, prior to administration of penicillin to sensitive patients.

An increasing body of experimental evidence also supports the view that administration of haptens or univalent fragments in the form of conjugates with appropriate non-immunogenic carriers would lead to the induction of tolerance of B cells and/or to the activation of T_S cells. Thus, abrogation of the ongoing reaginic responses in experimental animals was achieved by the injection of conjugates of the appropriate hapten (e.g. benzyl penicilloyl, 2,4 dinitrophenyl, trimellityl, 3-nitro-4-hydroxy-5-iodo-phenyl acetyl groups) with non-immunogenic carriers, such

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as isologous λ -globulins (Lee and Schon, 1975), the synthetic copolymer of D-glutamic and D-lysine (Katz *et al.*, 1973; Tse *et al.*, 1978), polyvinyl alcohol (Lee and Schon, 1979; Wei *et al.*, in press) or polyvinyl pyrrolidone (Lee *et al.*, 1981).

Clearly, the implementation of these different treatment modalities with respect to common allergens will be predicated on the availability of well characterized allergenic determinant(s) of allergens, which could then be synthesized in sufficient amounts. With these objectives in mind, the present investigation was undertaken to explore the possibility of enzymatically cleaving an allergenic molecule to obtain fragments that would possess determinants detectable with human IgE antibodies as well as with murine monoclonal antibodies. For this study, ryegrass (RG)* cytochrome c was selected as the allergen of choice since RG cytochrome c was available for carrying out fragmentation studies and that there was allergenic crossreactivity between Kentucky bluegrass (KBG) cytochrome c and ryegrass (RG) pollen cytochrome c (Ekramoddoullah *et al.*, 1982). In this report we describe: (i) the production of murine monoclonal antibodies to KBG cytochrome c, (ii) the results of enzymatic cleavage of RG cytochromes c, (iii) the separation of the resulting fragments by high performance liquid chromatography (HPLC), (iv) the capacity of various fragments to combine with the human IgE antibodies and/or the murine monoclonal antibodies to KBG cytochrome c and (v) the elucidation of the specificities of the various antibodies relative to each other.

MATERIALS AND METHODS

Isolation of cytochromes c from KBG and RG pollens

The respective cytochromes c were isolated from extracts of KBG and RG pollen as described previously (Ekramoddoullah *et al.*, 1981). Briefly, the aqueous extract of a given defatted pollen (Hollister-Stier Laboratory, Mississauga, Ontario) was dialysed through spectrapor membrane tubing (mol. wt cut off = 6000–8000 daltons; Spectrum Medical Industries Inc., Los Angeles, CA); and the nondialysable components were fractionated on Sephadex G-50 (Pharmacia AB, Uppsala, Sweden). The fraction containing cytochrome c was separated by preparative isoelectrofocusing using ampholyte buffer, pH range 3.5–10 (LKB Products, Stockholm-Bromal, Sweden). The extreme cathodal zone containing cytochrome c was subjected further to gel chro-

matography on Ultrogel AcA 44 (LKB 2204-440, Industrie Biologique Française, Genevilliers, France) and the fractions containing cytochrome c were pooled and rechromatographed on Ultrogel AcA 44.

Production of monoclonal antibodies to KBG-cytochrome c

(i) *Immunization.* BALB/c female mice (Timco Breeding Laboratories, Houston, Texas) were given on days 1, 28 and 70, a total of 50 μ g KBG-cytochrome c emulsified with FCA (Difco Laboratories, Detroit, MI, U.S.A.) in multiple s.c. injections into sites of the ventral region. After day 70 the sera of the mice contained antibodies to KBG cytochrome c as demonstrated by a SPRIA. On day 98, each mouse received an additional i.p. injection of 50 μ g of KBG cytochrome c in saline and three days thereafter the spleens of these animals were removed for cell fusion.

(ii) *Cell fusion and production of monoclonal cell lines.* A single cell suspension was prepared from four spleens and washed three times in RPMI complete [RPMI-1640 (Grand Island Biological Co. (GIBCO), Grand Island, NY) tissue culture medium supplemented with 15% v/v fetal calf serum (GIBCO), 1% w/v glutamine (GIBCO) and 1% w/v penicillin-streptomycin (GIBCO)].

For each fusion, 100 $\times$ 10⁶ spleen cells were pelleted at 350 g for 5 min with 25 $\times$ 10⁶ P3 $\times$ 20 myeloma cells (generously donated in 1978 by Dr Myron A. Leon, Detroit, MI). The cell pellet was gently resuspended for 60 sec in 2 ml of a polyethylene glycol (PEG) solution prepared by dissolving 20 g PEG-1540 (J. T. Baker Chemical Company, Phillipsburg, NJ) in 28 ml RPMI-1640 supplemented with 15% w/v dimethylsulfoxide (Fisher Scientific Company, Fairlawn, NJ). Thereafter, 5 ml RPMI complete was added dropwise over a period of 90 sec to the cell suspension, followed by an additional volume of 5 ml RPMI complete; the cell suspension was allowed to stand for 2.5 min and was then centrifuged at 350 g for 5 min. Finally, the cell pellet was resuspended in 5 ml RPMI complete and 50 μ l aliquots were placed into each well of 4 Costar 24-well plates also containing 1 $\times$ 10⁶ normal BALB/c spleen cells in 1 ml HAT medium [RPMI complete containing 0.1 mM hypoxanthine (Sigma Chemical Co., St. Louis, MO), 0.4 μ M aminopterin (Sigma), 16 μ M thymidine and 20 μ M 2-mercaptoethanol (Sigma)]. The cultures were incubated at 37 $^{\circ}$ C in a humidified atmosphere containing 5% CO₂ and were maintained by alternate addition or replacement of HAT medium every few days starting on the fifth day following fusion. After 14 days HAT was replaced by HT medium (HAT medium without aminopterin) and after 28 days with RPMI complete. Five fusions were performed, generating a total of 480 separate primary cultures. Eight days following fusion, aliquots of the culture supernatants were assayed for antibody activity as described below.

Selected cultures were then expanded and retested for antibody production prior to cloning. To produce monoclonal cell lines, cultures were diluted with RPMI complete to a concentration of 10 viable cells/ml. Aliquots (50 μ l) of this cell suspension were placed into wells of 96-well microtiter plates, which in addition contained 100 μ l HT medium containing 1 $\times$ 10⁵ normal BALB/c spleen cells. At least two plates were prepared for each selected polyclonal culture (i.e. 196 wells).

Typically, one-third to one-half of the wells contained expanding clones of cells. Samples of supernatants were tested 10 days after cloning for antibody activity. Selected monoclonal cultures were subsequently expanded in 24-well plates before being maintained in flasks for mass culture and ascitic fluid preparation.

(iii) *Ascitic fluid.* Three monoclonal cell lines were propagated in the peritoneum of C2B6F1/J (BALB/cJ $\times$ C57B1/6J) female mice (Jackson). For this purpose, each mouse was injected i.p. with 0.5 ml of pristane (2,6,10,14-tetramethyl pentadecane, Aldrich Chemical Co., Milwaukee, WI) and two weeks later with a suspension of 4–5 $\times$ 10⁶ viable hybrid cells in RPMI complete (the viability of the cells was examined by trypan blue exclusion) were injected i.p. into each animal. The ascitic fluid of the mice was harvested 14–21 days later.

Solid phase radioimmunoassay (SPRIA)

The SPRIA was performed in disposable flexible polyvinyl chloride microtiter plates (12.7 $\times$ 9.5 cm, 96 round "U" wells, Dynatech Laboratories, Inc., 900 Slaters Lane, Alexandria, VA, U.S.A.). Ten 10 μ g of antigen in 100 μ l coating buffer (1.59 g of Na₂CO₃, 2.93 g of NaHCO₃ and 0.2 g of NaN₃ made up to 1 liter with distilled water, pH 9.6) was placed into each well and the plates were kept at 4 $^{\circ}$ C in a moist chamber for 18 hr. The solution of antigen was then withdrawn and the plates were washed with phosphate-buffered saline, PBS (0.54 g KH₂PO₄, 2.14 g Na₂HPO₄, 7.75 g NaCl, 0.5 g NaN₃, H₂O to make 1 liter, pH 7.2). The capacity of the wells to further adsorb proteins was blocked by adding a solution of BSA (1% in PBS) according to the conditions described for the coating with antigen. Finally, the plates were washed three times with PBS (200 μ l/well). Volumes of 100 μ l of cell culture supernatant or ascitic fluid, serially diluted with assay buffer (AB) consisting of PBS containing 0.5% BSA, 5% Tween 20 (Fisher), was added to the wells and kept overnight at room temperature; the fluids were then withdrawn and the wells washed three times with AB. ¹²⁵I-labelled affinity purified sheep anti-murine Fab antibodies, SAMA (100 μ l containing 15–20 $\times$ 10⁵ cpm, the preparation of SAMA was described by Kelly *et al.*, 1980), in AB was added and incubated overnight at room temperature. The wells were washed three times with PBS containing 1% Tween 20 and cut out from the plates. The levels of

radioactivity were measured in a Beckman gamma counter. For screening of clones secreting anti-KBG cytochrome c antibodies, the time for incubation of antigen coated plates with the culture supernatant and the labelled SAMA was 2 hr at room temperature.

The radioallergosorbent test (RAST)

The RAST was performed in microtiter plates as described above with the exception that the incubation buffer, IB (PBS containing 0.3% HSA), was employed for the blocking stage. A pool of six allergic sera obtained from grass-sensitive patients was diluted five-fold with IB before adding to the allergen-coated wells. A solution of ¹²⁵I-labelled rabbit anti-human IgE antibodies (100 μ l containing 3–5 $\times$ 10⁴ cpm, Pharmacia AB, Uppsala, Sweden) in IB, was added to each well for determination of the IgE antibody bound.

Determination of the isotypicity of each monoclonal antibody by enzyme-linked immunoassay (ELISA)

The ELISA was performed with EIA plates (Cat. # 3590, Costar, 205 Broadway, Cambridge, MA 02139, U.S.A.), which had been coated with KBG cytochrome c (10 μ g/well) overnight, washed and incubated for 1 hr with ascitic fluid diluted 1000-fold with AB. After washing the plates, rabbit antibodies specific for one of the various immunoglobulins and their subclasses (Mono Ab-ID EIA Kit, Zymed Laboratories, Inc., South San Francisco, CA, U.S.A.) were added to the wells for a period of 1 hr the wells were then washed and incubated with the conjugate of goat anti-rabbit immunoglobulin and alkaline phosphatase (Zymed Laboratories Inc.) for 1 hr. Following washings, substrate was added and the color reaction was allowed to develop for 40 min and then stopped by the addition of a solution of NaOH (3 M) in a volume of 50 μ l. The optical density was measured at 405 nm using the ELISA automatic reader (Model MR 580, Dynatech).

Enzymatic digestion of rye cytochrome c

To a solution of 2 mg of RG cytochrome c in 0.96 ml of 0.05 M NH₄HCO₃, pH 7.75, was added 0.04 ml of a solution of enzyme, trypsin TPCK or TLCK treated α -chymotrypsin (1 mg/ml in 0.05 M NH₄HCO₃, pH 7.75; the enzymes were purchased from Worthington Biochemical Corporation, Freehold, NJ, U.S.A. and Sigma Chemical Co., St. Louis, MO, U.S.A., respectively). The mixture was maintained at room temperature for 16 hr with continuous shaking and the reaction was stopped by the addition of 3 drops of 2 N acetic acid. The enzymatic digests were filtered through millex-HA membranes (Millipore Corporation, Bedford, MA, U.S.A.) and lyophilized.

High-performance liquid chromatography (HPLC)

The enzymatic digests of RG cytochrome c were fractionated by HPLC using the reversed-phase chro-

*Abbreviations: RG, ryegrass; KBG, Kentucky bluegrass; HPLC, high-performance liquid chromatography; s.c., subcutaneous; i.p., intraperitoneal; FCA, Freund's complete adjuvant; PEG, polyethylene glycol; SPRIA, solid phase radioimmunoassay; RAST, radioallergosorbent; cpm, counts per minute; ELISA, enzyme linked immunoassay; TFA, trifluoroacetic acid; Mab, monoclonal antibody; SAMA, sheep anti-murine Fab antibodies; AB, assay buffer; IB, incubation buffer.

matography. The HPLC system (Waters Associates, Inc., Milford, MA, U.S.A.) consisted of a μ Bondapak C-18 column (10 μ particle size), a guard column packed with C-18 packing material, a U6K injector, the solvent delivery system consisting of 6000A and M-45 pumps, 720 system control, model 441 absorbance detector set to 214 nm, connected to a two-channel recorder (LKB model 2210). A multi-trac (LKB model 2111) was used for collection of the peptide fractions.

Buffers were prepared using distilled water which had been further purified by a Millipore MILLI-Q system. A solution of 0.1% (w/v) TFA (99%, Aldrich Chemical Co. Inc., Wisconsin, U.S.A.) was prepared by diluting an appropriate volume of TFA in distilled water and the pH was adjusted to 3 with 1 N NaOH. The TFA solution was then passed through a column packed with the C-18 matrix. Acetonitrile and methanol, HPLC grade (Fisher Scientific Limited), were used without further purification. All solvents were filtered through Millipore filters (HVLP04700) and degassed under suction in an all glass filtering apparatus (Millipore).

A sample (2 mg) of an enzymatic digest of RG cytochrome c was dissolved in a volume of 0.2 ml of a solution of TFA (0.1%, pH 3) and then injected into the C-18 column equilibrated with the solution of TFA (solvent A). A linear gradient was established with solvent B (70% acetonitrile, 0.1% TFA, pH 3) at

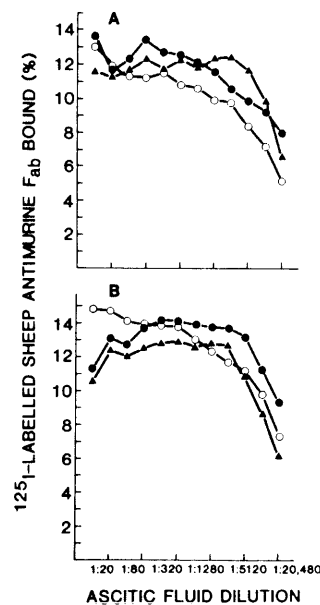


Fig. 1. Solid phase radioimmunoassays of murine monoclonal antibodies present in ascitic fluids (●—● = Mab 41, ○—○ = Mab 42, ▲—▲ = Mab 43). A = assays performed with KBG cytochrome c on the solid phase; B = assays performed with RG cytochrome c on the solid phase.

a combined flow rate of 1 ml/min over a period of 2 hr. Various peptide fractions were collected in disposable glass tubes which were previously coated with solvent B (to minimize the loss of peptides due to adsorption). The fractions (usually in volumes of 0.5 ml) were lyophilized and redissolved in coating buffer for analysis by SPRIA using the ascitic fluids containing the monoclonal antibody diluted 640-fold (cf. Fig. 1) or RAST using the pool of human allergic sera diluted 5-fold. The activity of the individual peptide fractions in SPRIA or RAST was expressed as a percentage of that bound by the intact rye cytochrome c.

RESULTS

Production and characterization of monoclonal antibodies

The initial screening by SPRIA of 480 supernatants of hybridoma culture fluids revealed that 84 had antibody activity. Single clones of the cells secreting antibody were expanded by *in vitro* culture. Three stable cell lines were injected into mice and the corresponding ascitic fluids were designated as Mab 41, Mab 42 and Mab 43. The first two monoclonal antibodies had isotypic determinants of IgM and Mab 43 was IgG1. The light chains of the three monoclonal antibodies were of the kappa type.

Antigenic crossreactivity of KBG and RG cytochromes c

From the results shown in Fig. 1, it is evident that all three monoclonal antibodies resembled closely one another in their affinity for KBG and RG cytochromes c. These observations clearly indicated that the two cytochromes c possessed some common antigenic determinant(s). To examine the binding ability of these monoclonal antibodies towards the various antigenic determinant(s), enzymatically derived peptides of RG cytochrome c were prepared with the anticipation that the cytochrome molecule would be cleaved into fragments which would retain at least some of their antigenic properties capable of being recognized by one or more of the Mabs.

Separation of peptides by HPLC on a C-18 column

The elution profiles of the tryptic or chymotryptic peptides resolved by HPLC on C-18 columns are illustrated in Figs 2 and 3.

Allergenic and antigenic activity of peptide fractions

The capacity of the peptide fractions to bind to human IgE antibodies and to murine monoclonal antibodies was evaluated by RAST and SPRIA, respectively, with reference to the capacity of these relative assay values are referred to as immunoreactivity levels.

From the immunoreactivity profiles of the peptides illustrated in Fig. 4 it is evident that (i) different peptides had different characteristics depending on

Table 1. Demonstration of differences between binding of human IgE antibodies and murine monoclonal antibodies to various peptide fractions

Peptide fraction ^a	% Antibody bound by a given peptide fraction relative to the intact RG cytochrome c			
	Human IgE	Mab 41	Mab 42	Mab 43
T-44	0	42	79	0
T-45	61	76	75	9
T-46	90	72	78	0
C-67	42	73	97	20
C-69	206	53	74	7
C-74	65	98	87	16
C-83	0	5	40	106

^aThe letters T and C refer to the peptide fractions obtained by digestion of RG cytochrome c with trypsin and chymotrypsin, respectively, and which were subsequently separated by HPLC (cf. Figs 2 and 3).

the enzyme used for the digestion of the cytochrome and antibody used for the assays, (ii) some peptide fragments retained the allergenic and/or antigenic activity of the intact rye cytochrome c molecule, (iii) peptide fragments 69 and 70 had substantially higher immunoreactivity levels than the intact cytochrome c molecule with reference to the human IgE antibodies.

Differences in the specificity of antibodies

The observation (Fig. 4) that several tryptic peptide fractions were capable of binding the human IgE antibodies and murine Mab 41 and 42 but not Mab 43 was considered to indicate that Mab 43 recognized

a determinant on the RG cytochrome molecule that was no longer present after fragmentation. Similarly, some chymotryptic peptide fragments (within the region encompassed by HPLC fractions 50-60) which bound human IgE antibodies did not possess antigenic determinants which could be recognized by any of the three monoclonal antibodies.

A comparison was made of the ability of various peptide fragments to bind either the human IgE antibodies and/or one or more of the murine monoclonal antibodies. The results listed in Table 1 indicate that T-44 strongly bound mab 42 and to a lesser extent Mab 41, but did not bind either human IgE

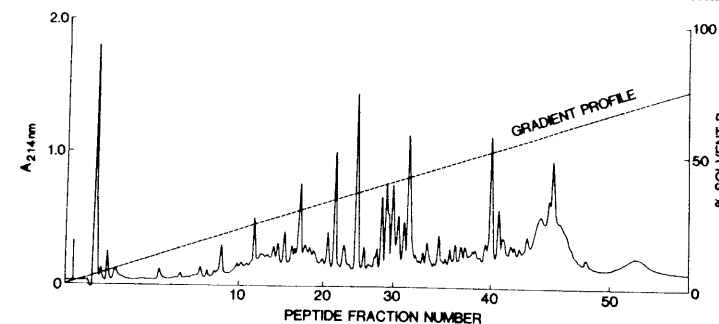


Fig. 2. Separation of tryptic digest of RG cytochrome c (2 mg) by HPLC on reversed phase C-18 column equilibrated with solvent A (0.1% TFA, pH 3.0); a linear gradient of solvent B (70% acetonitrile, 0.1% TFA, pH 3.0) was applied over a period of 2 hr.

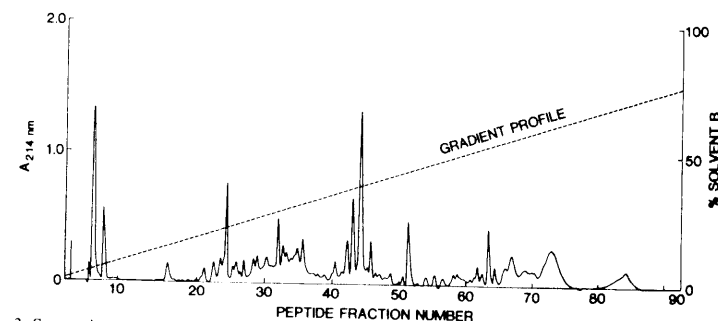


Fig. 3. Separation of chymotryptic digest of RG cytochrome c (2 mg) by HPLC on reversed phase C-18 column equilibrated with solvent A (0.1% TFA, pH 3.0). A linear gradient of solvent B (70% acetonitrile, 0.1% TFA, pH 3.0) was applied over a period of 2 hr.

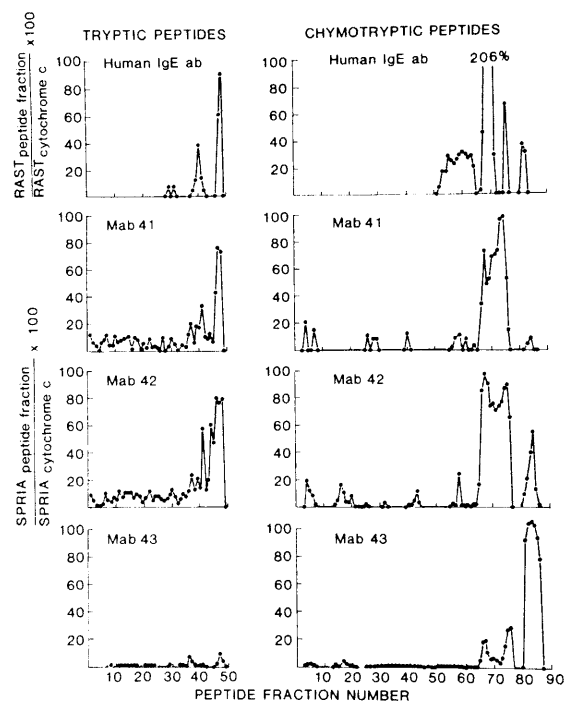


Fig. 4. Immunoreactivity profiles of enzymatically derived peptides of RG cytochrome c separated by HPLC.

antibodies or Mab 43 and hence it may be suggested that the specificities of the human IgE antibodies and Mab 43 differed from those of Mab 41 and 42. On the other hand, T-46, which bound strongly human IgE antibodies and Mab 41 and Mab 42, did not bind Mab 43. Although the pattern of immunoreactivity of fragment T-45 was similar to that of T-46, its extent of the binding to Mab 43 was of a low order (9%). Taken together, these results were interpreted to mean that the specificity of the antigenic determinant(s) reacting with human IgE antibodies, Mab 41 and Mab 42, was different from Mab 43. Furthermore, the results of the comparison of immunoreactivity of selected tryptic peptide fractions indicated that the human IgE antibodies had a specificity that was different from those of Mab 41, 42 or Mab 43. A unique specificity of Mab 41, which was different from Mab 42, was revealed by differences in their reactivities towards the chymotryptic peptide, C-83. In summary, the human IgE antibodies, Mab 41, 42 and 43, each had a different antibody specificity.

DISCUSSION

Because of the complex composition of the constituents of a given allergenic pollen, attempts to isolate the individual allergens in a pure state have been fraught with great difficulties and the number of

chemically characterized allergens is limited only to a few. The protocols which have been used for isolating allergens have involved time-consuming combinations of several physico-chemical methods and, in general, the yields of the isolated allergens have been too low for further studies which may have been directed toward the elucidation of their structural features and toward the preparation of tolerogenic derivatives as may be achieved with antigens such as albumin (Sehon, 1982). Clearly, the new hybridoma technology opens new avenues for the use of monoclonal antibodies for (i) for the isolation of individual allergenic constituents in a pure state in a single step by the use of reverse immunosorbents, and (ii) the standardization of allergenic preparations used for immunotherapy (Kahn and Marsh, 1983; Agarwal *et al.*, 1983). Moreover, the availability of a whole spectrum of the allergenic constituents of different pollens and other environmental allergens in a pure state offers also the incomparable advantage of being able to include in the preparations used for the immunotherapy of different patients only the constituents to which each of the patients is allergic rather than injecting, as is the current practise, crude extracts containing also constituents to which the individual may not be allergic and to which they may actually become sensitized in the course of treatment (Richter *et al.*, 1957; Marsh *et al.*, 1972).

To achieve these objectives, we have recently produced monoclonal antibodies to the heterogeneous extract of KBG pollen (Kisil *et al.*, 1980) and are in the process of selecting appropriate monoclonal antibodies which are suitable for isolating the individual allergens. In the present study we have exemplified the procedure by limiting ourselves to the generation of monoclonal antibodies to cytochrome c of KBG and these monoclonal antibodies were utilized also for establishing the antigenic crossreactivity between the cytochromes c of KBG and RG.

In a recent study (Ekramoddoullah *et al.*, 1982), we reported that KBG and RG cytochromes c possess common allergenic specificities, which were manifested as determinant(s) recognized by antibodies of the IgE class. Consistent with this finding, the present study revealed also that these cytochromes c possess crossreactive antigenic specificities which are considered to be represented by determinant(s) recognized by the same monoclonal antibodies of an isotype(s) other than IgE. To further our understanding of the antigenic composition of an allergenic molecule an attempt was made to isolate as many monoclonal antibodies as could be conveniently handled which possessed a variety of specificities representative of the different epitopes on the cytochrome c. The reason for selecting RG cytochrome c for enzymatic fragmentation was based simply on its availability in a highly purified form.

The examination of the peptide fragments for their capacity to bind the human IgE antibodies served to identify those fragments that were relevant to the human allergies to the pollen cytochrome c. The IgE antibodies present in the pool of sera of grass allergic patients are obviously polyspecific and, in principle, should be capable of recognizing all the different allergenic specificities present on the KBG cytochrome c molecule. Therefore, the human IgE antibodies, as employed in this study, would not be able to discern differences in allergenic specificities, if at all present, in the various peptide fractions. Nevertheless, it was found that the collective specificity of the IgE antibodies was different from any of the monoclonal antibodies. On the other hand, the examination of the peptide fragments for their capacity to bind the monoclonal antibodies provided an opportunity to establish whether or not there were differences in antigenic composition between various fragments and, as a corollary, to establish the specificities of these antibodies relative to one another. These monoclonal antibodies proved invaluable as specific molecular probes for detecting their homologous antigenic determinants and resolving various peptide fragments on the basis of their antigenic composition.

The enzymatically derived peptides of RG cytochrome c were well resolved by high-performance liquid chromatography. In general, the highly immunoreactive peptides were eluted from the C-18 column with the solvent mixture composition favouring

the less polar solvent. Peptides which were eluted at the beginning of the solvent gradient were mainly devoid of immunoreactivity. The lack of activity of these peptides may be explained by one or more of the following reasons: (i) these peptide fragments did not constitute that portion of the molecule which encompassed an antigenic determinant(s); (ii) these peptides may be part of a determinant(s) which is conformational in nature and, therefore, immunoreactive only in native cytochrome c; (iii) they may be fragments of determinant(s) that were susceptible to the enzymatic digestion; (iv) these peptides did not bind to the plates used in SPRIA or RAST; (v) the specificities of the monoclonal murine antibodies did not correspond *in toto* to those reacting with human IgE antibodies.

The immunoreactivity of chymotryptic peptide fractions C69 (Table 1) and C70 (cf. Fig. 4) with human IgE antibodies was higher than that of the intact cytochrome c molecule. Again, considering the limitations of the assay procedures described above and specifically recognizing that the native cytochrome c molecule and the different peptide fractions may not bind to an equal extent in the preparation of the micro-titer assay plates, it may be interpreted as indicating that (i) that these two peptide fractions possess a major allergenic determinant(s) of cytochrome c and (ii) this (these) determinant(s) are not as readily accessible on the intact molecule for reaction with human IgE antibodies. An alternate explanation to account for the highest immunoreactivity levels being obtained with these peptide fractions is to suggest that they bound to the assay plate to a significantly higher extent as compared to the intact allergen. Therefore, one would expect that the immunoreactivity levels established with the monoclonal antibodies would also be high. However, this was not the case, i.e. the immunoreactivity levels of the peptide fractions did not exceed those of the intact cytochrome c molecule (cf. Table 1). This observation further supports the conclusion that the epitopes recognized by the monoclonal antibodies are different from those which are detected by the human IgE antibodies.

At this stage of our investigations, it is not known if the murine model used for the production of monoclonal antibodies was intrinsically capable of recognizing the determinant(s) to which the IgE antibodies present in the sera of allergic humans were directed. Notwithstanding this finding, it is conceivable that additional monoclonal antibodies with specificities different from those presently available may still be found among the remaining 80 clones with antibody activity to KBG cytochrome c, which have been temporarily stored in liquid nitrogen.

The fact that several tryptic and chymotryptic fractions (cf. Figs 1 and 2; Table 1) were immunoreactive with both Mab 41 and 42 suggested that the determinants recognized by these two monoclonal antibodies were closely linked along a linear segment

of the cytochrome c molecule. The alternate possibility that these two determinants are identical is considered to be most improbable since peptide fractions C-83 (Table 1) reacted with Mab42 but not with Mab41.

Because of the availability of the different fragments in limiting amounts only, no attempt was made in this study to evaluate the ability of the various peptide fractions to inhibit the binding of intact RG cytochrome c to human IgE antibodies or to monoclonal antibodies. Such studies would have provided the information as to the proportion of determinants of the intact cytochrome c that are represented by the various peptide fractions. Attempts are in progress to use the monoclonal antibodies in the form of reverse immunosorbents for the isolation of these fragments in larger quantities and more expeditiously. Nevertheless, it may be concluded that this study has indicated that immunoreactive fragments can be obtained from an allergen by appropriate enzymatic cleavage and by the use of the techniques described it is reasonable to suggest that an allergenic determinant could be isolated in the near future in high yields. Moreover, because of the relatively low molecular weight of these determinants it is also reasonable to anticipate that their amino acid sequence would be readily established and that, consequently, they could be synthesized with relative ease by the application of modern procedures of peptide synthesis. It is therefore not unreasonable to suggest that in the not too distant future it would be possible to establish a library of peptides representing the determinants of the major allergens responsible for common hypersensitivities of the immediate type in man and in turn these peptides could be used for the synthesis of tolerogenic conjugates which would be employed for the effective immunotherapy of allergic patients.

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