

ALLERGENIC CROSS-REACTIVITY OF CYTOCHROMES *c*
OF KENTUCKY BLUEGRASS AND PERENNIAL
RYEGRASS POLLENS*

A. K. M. EKRAMODDOULLAH, F. T. KISIL and A. H. SEHON

MRC Group for Allergy Research, Department of Immunology, Faculty of Medicine,
University of Manitoba, Basic Medical Sciences Building, 730 William Avenue,
Winnipeg, Manitoba, Canada R3E 0W3

(First received 19 February 1982; accepted in revised form 22 April 1982)

Abstract—The allergenic cross-reactivity of cytochromes *c* isolated from Kentucky bluegrass (KBG) and ryegrass (RG) pollens was demonstrated by the finding that both cytochromes elicited PCA reactions of comparable titers in rats sensitized with murine reaginic antiserum to either KBG or RG cytochrome *c*. However, allergenic differences were revealed at limiting doses of the cytochromes *c*; under these conditions RG cytochrome *c* elicited PCA reactions only with the homologous reaginic serum, whereas KBG cytochrome *c* elicited PCA reactions with both murine reaginic sera. Moreover, in experiments involving inhibition of PCA, RG cytochrome *c* failed to neutralize some of the IgE antibodies of the antiserum to KBG cytochrome *c*, whereas KBG cytochrome *c* neutralized all IgE antibodies of both murine reaginic antisera. From these results it may be concluded that: (1) the two cytochromes *c* share common allergenic determinants, and (2) the KBG cytochrome *c* possesses additional allergenic determinant(s) not present on RG cytochrome *c*. The radioallergosorbent test, utilizing KBG cytochrome *c* discs and a pool of sera of individuals allergic to KBG, was inhibited to the extent of 87 or 41%, by the addition of 10 µg of KBG or RG cytochrome *c*, respectively. By contrast both cytochromes *c* at 10 µg were equally effective in the inhibition (92%) of the binding of the IgE antibodies in this serum pool to RG cytochrome *c* allergosorbent discs. These experiments confirmed that the two cytochromes share common allergenic determinants.

INTRODUCTION

The identification of site(s) on a protein molecule that could constitute or contribute to its antigenic specificity has been greatly facilitated by the availability of a family of related proteins such as the cytochromes *c*. Thus, in studies involving several mammalian cytochromes *c* (Corradin & Chiller, 1979; Jemmer-son & Margoliash, 1977; Reichlin & Eng, 1977; Reichlin *et al.*, 1979; Schwartz *et al.*, 1977; Solinger *et al.*, 1979; Urbanski & Margoliash, 1977), the extent of the antigenic cross-reactivity of the different cytochrome *c* molecules was successfully correlated with differences in their amino acid sequences, which also led to the delineation of the sequence(s) responsible for their antigenic specificity.

Recently, pollen cytochromes *c* isolated from ragweed, Kentucky bluegrass (KBG†) (*Poa*

pratensis) and Bermuda grass pollens were demonstrated to be allergenic in humans with allergies to the respective pollens (Goodfriend *et al.*, 1979; Ekramoddoullah *et al.*, 1981; Schumacher *et al.*, 1982). The frequency of an allergic response to pollen cytochromes *c* has not yet been determined. Nevertheless, the observations suggest that other pollen may also contain cytochromes *c* with allergenic activities.

Cytochromes *c* are considered to represent an excellent group of proteins for elucidating the molecular and immunogenetic basis of allergenic specificity(ies). One of the advantages of using cytochrome *c* as a model allergenic protein is that the primary structures of more than 85 cytochromes have been elucidated (Dayhoff *et al.*, 1972). This vast repertoire of data can be expected to facilitate studies to evaluate the effects of differences in amino acid sequences on the determinants recognized by the IgE antibodies. With this goal in mind, the present study describes the isolation of an allergenically active cytochrome *c* from RG (*Lolium perenne*) pollen and data demonstrating allergenic cross-reactivity between KBG and RG cytochromes *c* are presented.

* This work was supported by grants from the Medical Research Council of Canada.

† Abbreviations: KBG, Kentucky bluegrass; RG, ryegrass; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RAST, radioallergosorbent test; PCA, passive cutaneous anaphylaxis; i.v., intravenous; i.p., intraperitoneal; i.d., intradermal; cpm, counts per minute.

MATERIALS AND METHODS

Preparation of cytochromes c from KBG and RG pollens

Cytochromes *c* were isolated as recently described in detail by Ekramoddoullah *et al.* (1981). Briefly, the aqueous extract of a given pollen (Hollister-Stier Laboratory, Mississauga, Ontario) was dialysed through spectrapor membrane tubing (mol. wt cut off = 6000–8000; Spectrum Medical Industries Inc., Los Angeles, California) and the nondialysable components were fractionated on Sephadex G-50 (Pharmacia AB, Uppsala, Sweden). The fraction containing cytochrome *c* (detectable by its characteristic absorption at 410 nm) was separated by preparative isoelectrofocusing using ampholyte buffer, pH range 3.5–10 (LKB Products AB, Stockholm-Bromal, Sweden). The extreme cathodal fraction which contained cytochrome *c* was further separated by gel filtration on Ultrogel AcA 44 (LKB 2204-440, Industrie Biologique Francaise, Gennevilliers, France) and the fractions containing cytochrome *c* were pooled and rechromatographed on Ultrogel AcA 44.

Protein measurement

Unless stated otherwise, the amounts and concentrations of various fractions are expressed in terms of their protein content which was determined by the method of Lowry *et al.* (1951).

Absorption spectrum

The absorption of the fraction containing purified cytochrome *c*, dissolved in water, was measured at different wavelengths at intervals of 5 nm using a Zeiss spectrophotometer (Zeiss, Oberkochen, Federal Republic of Germany).

Amino acid analysis

Samples containing RG cytochrome *c* were hydrolysed with HCl at 110–120 °C for 24, 48 and 72 hr and the hydrolysates were analysed with the Beckman automatic amino acid analyser, Model 120-139 (Beckman, Palo Alto, California). The concentrations of serine, threonine and tyrosine were calculated by extrapolation to zero-time hydrolysis and those of valine and isoleucine were calculated from the analysis of the 72-hr hydrolysate. Cysteine was determined as cysteic acid following oxidation of the samples with performic acid (Hirs, 1956) prior to acid hydrolysis and the tryptophan

content was measured spectrophotometrically (Edelhock, 1967). The concentrations of other amino acids were calculated from the analysis of the 48-hr hydrolysate.

Polyacrylamide gel electrophoresis

Disc electrophoresis in a polyacrylamide gel was performed at pH 4.3 using the method described by Reisfeld *et al.* (1962). For mol. wt determination, SDS-PAGE was used as described by Laemmli (1970). The gel was stained according to the method of Fairbank *et al.* (1971) and the mobility of the bands was measured as described by Weber & Osborn (1969).

Skin tests

The stock solution of cytochrome *c* in saline (0.1 mg/ml) was passed through a sterile Millipore membrane (0.45-µm pore size). A volume of 20 µl of the stock solution, diluted 100-fold, was injected i.d. into patients sensitive to grass pollen(s); saline was injected into control sites. Fifteen minutes later, the size of the wheal was measured.

Immunization

In accordance with an established procedure for the production of IgE antibody to KBG pollen allergens (Ekramoddoullah *et al.*, 1977), for the induction of IgE antibodies to cytochromes *c*, the KBG or RG cytochrome *c* (dose range 10–100 µg) admixed with freshly prepared Al(OH)₃ (5 mg) was injected i.p. into C3H/HeJ mice. The sera obtained from the blood of eight mice immunized in an identical manner were pooled; each pool is referred to as the corresponding murine reagenic serum.

Measurement of antibodies

The levels of murine IgE antibodies were established by PCA in hooded rats obtained from the University of Manitoba vivarium (Guntton, Manitoba) (Ekramoddoullah *et al.*, 1977).

Relationship of allergenic specificity between KBG and RG cytochromes c

Inhibition of PCA. To evaluate the allergenic cross-reactivity of the two cytochromes *c* in terms of specificity(ies) recognized by the murine reagenic sera to KBG and RG cytochromes *c*, the procedure of inhibition of PCA was employed (Ekramoddoullah *et al.*, 1980). For this purpose, different amounts of the inhibitor

(i.e. a given cytochrome *c*) were mixed with a fixed volume of a murine antiserum to the same or to the alternative cytochrome *c* and the mixtures were maintained for 1 hr at room temperature. Thereafter, 50 µl of each mixture was injected i.d. into hooded rats and 24 hr later the sensitized rats were challenged by i.v. administration of a solution containing either of the two cytochromes *c* (0.2 mg/rat) and Evan's blue dye (1% in saline). For establishing reproducibility of these determinations, two rats were sensitized with identical mixtures.

The PCA titer of either of the two reagenic sera was of the order of 640. However, since the purified cytochromes were available in limiting amounts and in order to minimize the amount of inhibitor required to neutralize all the reagenic antibodies the sera were used at a dilution of 160; even at this dilution the neat sera gave a strong PCA reaction, i.e. size of wheal was 18 × 22 mm. As would be expected, the mixtures containing increasing amounts of an inhibitor gave PCA reactions of decreasing intensity. Accordingly, the absence of a PCA reaction was taken to indicate that the corresponding mixture contained the equivalent amount, or an excess of the inhibitor necessary to neutralize all the reagenic antibodies present in the diluted serum in the mixture. By contrast, a positive PCA reaction indicated that not all of the murine anti-cytochrome *c* IgE antibodies had been neutralized by the inhibitor.

Inhibition of RAST. For the evaluation of the allergenic cross-reactivity of the two cytochromes *c* in man, the procedure of inhibition of RAST was employed with the aid of a pool of six sera from patients allergic to KBG pollen (Gleich & Jones, 1975). Briefly, individual allergosorbent discs were prepared by coupling each cytochrome *c* to cyanogen bromide activated paper discs (10 µg of cytochrome/disc). A volume of 20 µl of the serum pool was mixed with different quantities of a putative inhibitor and the mixture, after 3 hr at room temperature, was added to an allergosorbent disc. The amount of IgE antibodies bound to the allergosorbent disc was determined by the addition of ¹²⁵I-labeled rabbit anti-human IgE antibodies (Pharmacia) and with the aid of a Beckman gamma counter. The reduction in the uptake of the ¹²⁵I-label by the allergosorbent disc, which had been exposed to mixtures of the serum pool and the inhibitor, was expressed in terms of the per cent inhibition of the maximum

uptake of the label by the allergosorbent disc in the absence of inhibitor using the formula:

$$\% \text{ inhibition} = (A - B)/A \times 100,$$

where *A* = cpm in the absence of inhibitor and *B* = cpm in the presence of inhibitor.

RESULTS

Physico-chemical properties of cytochrome c isolated from RG pollen

The protocol developed for isolating KBG cytochrome *c* was found to be applicable also



Fig. 1. Polyacrylamide gel electrophoresis (pH 4.3) of RG cytochrome *c*; the sample applied on the gel disc was 25 µg.

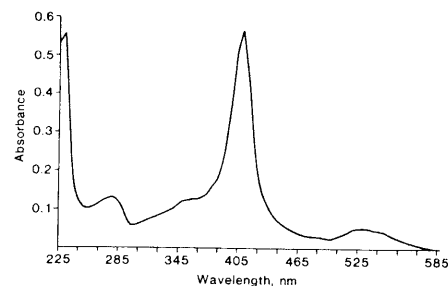


Fig. 2. Absorption spectrum of RG cytochrome *c* (0.1 mg/ml in H₂O).

for the isolation of cytochrome *c* from RG pollen. Thus, as may be seen in Fig. 1, RG cytochrome *c* migrated as a single component on PAGE and its mol. wt estimated by SDS-PAGE was of the order of 12,000.

From the absorption spectrum of RG cytochrome *c* (Fig. 2), it may be concluded that the absorption maximum at 280 nm is due to a protein, the other maxima at 350, 410, 520 and 550 nm being characteristic of a hemochrome (Margoliash & Schejter, 1966). The results listed in Table 1 indicate that RG cytochrome *c* contained all the naturally occurring amino acids and that one of its lysine molecules was trimethylated.

Immunogenic properties of KBG and RG cytochromes *c*

From the dose-response profile for IgE anti-

Table 1. Amino acid composition of rye cytochrome *c*

Amino acid	Residues/molecule
Alanine	13
Arginine	3
Aspartic acid	8
Cysteine	2
Glutamic acid	9
Glycine	12
Histidine	2
Isoleucine	4
Leucine	8
Lysine	10
Trimethylated lysine	1
Methionine	2
Phenylalanine	4
Proline	8
Serine	7
Threonine	7
Tryptophan	2
Tyrosine	5
Valine	5

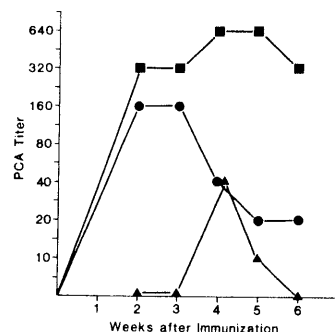


Fig. 3. IgE antibody responses following single injections of three different doses of KBG cytochrome *c* in C3H/HeJ mice expressed in terms of PCA titers in hooded rats: ● = 100 µg, ■ = 50 µg, ▲ = 10 µg.

body responses following a single immunization with KBG cytochrome *c* shown in Fig. 3, it is obvious that KBG cytochrome *c* was capable of inducing IgE antibody response in C3H/HeJ mice. The level of IgE antibodies in mice immunized at a dose of 50 µg was higher than that in mice immunized at doses of 10 or 100 µg. Therefore, in subsequent experiments a dose of 50 µg of cytochrome was used to produce murine anti-cytochrome reagenic sera. A similar IgE antibody response with RG cytochrome *c* was obtained (data not shown). It was also found that an anamnestic IgE antibody response to either cytochrome *c* could be obtained by reimmunization of the mice (data not shown).

Attempts to produce precipitating rabbit antibodies detectable by immunodiffusion were unsuccessful. Thus, seven injections of each of a group of three rabbits over a period of 6 months with mixtures consisting of 0.2 mg of a given cytochrome *c* and Freund's complete adjuvant (i.e. each rabbit received 1.4 mg of the antigen) did not result in the production of detectable precipitating antibodies.

Allergenic properties

Evidence for the allergenicity of RG cytochrome *c* was obtained by skin testing of three allergic patients who had been shown to be sensitive to the extract of KBG pollen. The i.d. injection of RG cytochrome *c* (at a concentration of 1 µg/ml) elicited immediate skin reactions in the three patients, the size of the wheals being 9 × 9, 5 × 5 and 5 × 5 mm. However, no skin reactions were observed on injection of saline into control sites.

Table 2. Comparison of allergenic activities of KBG and RG cytochromes *c*

Cytochromes <i>c</i> used for challenge	Challenging dose per rat (mg)	PCA titer	
		Anti-KBG cytochrome <i>c</i> ^a	Anti-RG cytochrome <i>c</i> ^a
KBG cytochrome <i>c</i>	0.2	640	640
	0.02	320	320
	0.002	20	80
Rye cytochrome <i>c</i>	0.2	320	640
	0.02	20	640
	0.002	0	80

^aDifferent skin sites of a rat were sensitized i.d. with 50 µl of the two murine reagenic sera at serial dilution. The rats received 24 hr later i.v. injections of either of the two cytochromes *c* at the dose indicated above.

Partial allergenic cross-reactivity of the two cytochromes *c* demonstrated

By PCA. The allergenic activities of KBG and RG cytochromes *c* were compared in terms of their ability to elicit PCA reactions in rats which had been passively sensitized with the two murine reagenic sera produced to each of the cytochromes. The results listed in Table 2 demonstrate that KBG cytochrome *c* at challenging doses ranging from 0.02 to 0.2 mg and RG cytochrome *c* at the dose of 0.2 mg elicited PCA reactions of comparable titers with either of the murine reagenic sera. However, RG cytochrome *c* elicited significantly greater PCA titers with the homologous reagenic antiserum than with the antiserum to KBG cytochrome *c*; in fact at the lowest dose of 0.002 mg RG cytochrome *c* did not give any detectable PCA reaction in sites sensitized with the antiserum to KBG cytochrome *c*. By contrast, KBG cytochrome *c* at the lowest dose of 0.002 mg elicited positive PCA reactions in skin sites sensitized with either serum and it is to be noted that, even at this lowest dose, it was as effective as RG cytochrome *c* in eliciting PCA reactions in sites sensitized with the antiserum to RG cytochrome *c*. It is also worth pointing out that reactions elicited by KBG cytochrome *c* at this limiting dose with the heterologous serum were larger than with the homologous serum. Hence, it may be concluded that: (1) the two cytochromes are allergenically cross-reactive, and (2) RG cytochrome *c* may lack an allergenic determinant(s) which is (are) present on the KBG cytochrome *c* molecule.

In an attempt to evaluate more precisely the extent of the allergenic cross-reactivity of the two cytochromes, the procedure of inhibition of PCA was resorted to. In Table 3(A) are

listed the PCA reactions elicited in rats which had been passively sensitized with mixtures, containing a constant volume of murine reagenic serum to KBG cytochrome *c* (which had been diluted 160-fold) and varying concentrations of either of the two cytochromes *c*, and which were subsequently challenged with KBG or RG cytochrome *c*. The skin sites sensitized with the reagenic serum alone (i.e. in the absence of inhibitor) served as reference control sites. From results (a) and (b) it may be concluded that KBG cytochrome *c* (when used as the inhibitor with the homologous serum) neutralized all the anti-KBG cytochrome *c* IgE antibodies (reactive to KBG cytochrome *c*) even at the highest concentration of 500 µg/ml. However, RG cytochrome *c* did inhibit that portion of the antibody population present in anti-KBG cytochrome *c* reagenic serum that was reactive with RG cytochrome *c* as demonstrated by the result in (c). These results confirm the earlier conclusion that KBG cytochrome *c* contained allergenic determinants in excess of those present in RG cytochrome *c*. Further confirmation for this conclusion is provided by the findings listed in Table 3(B), which demonstrate that KBG cytochrome *c* was just as effective, if not even more effective, than RG cytochrome *c* in neutralizing IgE antibodies of the reagenic serum to RG. [See experiments (d) and (e).]

By RAST. The extent of the allergenic cross-reactivity between the two cytochromes was also evaluated by the inhibition of RAST resulting from the addition of a given cytochrome *c* to the reference serum obtained from patients allergic to KBG pollen, prior to inter-

Table 3. Inhibition of PCA

(A) PCA reactions elicited by challenge with KBG and rye cytochromes *c* in rats passively sensitized with mixtures consisting of 50 μ l of 160-fold diluted murine reaginic serum* to KBG cytochrome *c* and varying concentrations of either of the two cytochromes *c*

Inhibitor	Cytochromes <i>c</i> used for challenge	PCA reactions ^b				
		Concentration of inhibitor (μ g/ml)				
		500	50	5	0.5	0
(a) KBG cytochrome <i>c</i>	KBG cytochrome <i>c</i>	—	—	—	+	+
		—	—	—	+	+
(b) RG cytochrome <i>c</i>	KBG cytochrome <i>c</i>	+	+	+	+	+
		+	+	+	+	+
(c) RG cytochrome <i>c</i>	RG cytochrome <i>c</i>	—	—	+	+	+
		—	—	—	+	+

(B) PCA reactions elicited by challenge with RG cytochrome *c* in rats passively sensitized with mixtures consisting of 50 μ l of 160-fold diluted murine reaginic serum to RG cytochrome *c* and varying concentrations of RG or KBG cytochromes *c*

Inhibitor	Cytochromes <i>c</i> used for challenge	PCA reactions				
		Concentration of inhibitor (μ g/ml)				
		500	50	5	0.5	0
(d) RG cytochrome <i>c</i>	RG cytochrome <i>c</i>	—	—	+	+	+
		—	—	—	+	+
(e) KBG cytochrome <i>c</i>	RG cytochrome <i>c</i>	—	—	—	+	+
		—	—	—	—	+

*The PCA titers of both reaginic sera used were 640 prior to dilution.

^bThe dose of allergen used for challenge was 0.2 mg per rat. Positive and negative PCA reactions observed after i.v. challenge with the appropriate cytochromes are indicated by (—) and (+) respectively. Each test was performed in duplicate utilizing two rats and the reactions denoted in a given row were observed in one rat.

action of this serum with the allergosorbent discs prepared with KBG cytochrome *c*. (This reference serum consisted of a pool of six sera

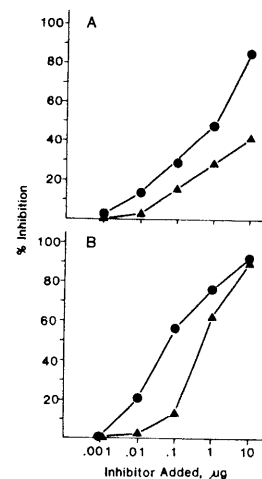


Fig. 4. Inhibition of RAST: (A) allergosorbent disc prepared with KBG cytochrome *c*. (B) Allergosorbent disc prepared with RG cytochrome *c*. ● = inhibition by KBG cytochrome *c*. ▲ = inhibition by RG cytochrome *c*. Each point represents an average value of three determinations.

collected during 1976 and used in this laboratory for the long-term studies on the isolation and characterization of the allergenic constituents of KBG pollen.)

It is evident from Fig. 4 that the addition of either of the two cytochromes in increasing amounts, over the range of 0.01–10 μ g, resulted in increasing degrees of inhibition of the binding of human IgE antibodies to the KBG cytochrome *c* allergosorbent and that the inhibition by KBG cytochrome *c* was more effective. Thus, whereas the addition of 10 μ g of KBG cytochrome *c* resulted in 87% inhibition of the binding of the human IgE to the KBG cytochrome *c* disc, the inhibition due to the same amount of RG cytochrome *c* was only 41% (Fig. 4A). It was not clear from this experiment if the value of 41% represented the maximum inhibition achievable with the RG cytochrome. Thus, this observation could only be interpreted to mean that KBG cytochrome *c* was more effective than RG cytochrome *c* in inhibiting the binding of the human IgE antibodies to the KBG cytochrome *c* allergosorbent discs. The results illustrated in Fig. 4B, of experiments utilizing allergosorbent discs prepared with RG cytochrome *c* and the two cyto-

chromes *c* over the range of 0.001–10 μ g, revealed that the inhibition of binding of IgE antibodies of the KBG reference serum caused by KBG cytochrome *c* was more effective than that by RG cytochrome *c*, except at 10 μ g where both cytochromes were equally effective, leading to 92% inhibition of binding. The findings of the RAST inhibition experiments provide further support for the conclusion that both cytochromes share some allergenic specificities and that KBG cytochrome *c* is more effective than RG cytochrome *c* in combining with and inhibiting the IgE antibodies.

DISCUSSION

The isolation of cytochrome *c* from KBG pollen by a series of fractionation steps was recently described and the cytochrome *c* was shown to be allergenic in man (Ekramoddoullah *et al.*, 1981). By the application of similar procedures, the isolation of cytochrome *c* from RG pollen was successfully accomplished in the present study and it was shown, by means of direct skin tests in patients allergic to grass(es) and by RAST, that it was an allergenic constituent of RG pollen. RG cytochrome *c* had an absorption spectrum characteristic of a heme protein and the amino acid composition was comparable to that of KBG cytochrome *c* (Ekramoddoullah *et al.*, 1981) and other plant cytochromes *c* (Dayhoff & Eck, 1976).

According to earlier reports (Urbanski & Margoliash, 1977) cytochromes *c* were shown to be poorly immunogenic unless used in a polymerized form. However, in those studies, no mention was made of pollen cytochromes and of any attempts to induce the formation of anti-cytochrome *c* antibodies of the IgE class. Since plants and mammals are phylogenetically distant, it is surprising that repeated injections of KBG or RG cytochromes *c* into rabbits in this study have failed to produce antisera that would contain antibodies detectable by immunodiffusion in agar gel. Hence, the possibility of eliciting the production of precipitating anti-cytochrome *c* antibodies by immunization of rabbits with a heterogeneous extract of a grass pollen, of which cytochrome *c* is only a minor constituent, would seem even more remote and, therefore, it may be suggested that a cytochrome *c* present in a grass pollen may escape detection by a method such as radiocrossed-immunoelectrophoresis (Lowenstein *et al.*, 1976; Chakrabarty *et al.*, 1981) which is based

on the availability of a precipitating antibody for the corresponding component(s). By contrast, KBG and RG pollen cytochromes *c* were shown in this study to be capable of inducing the formation of antibodies of the IgE class in C3H/HeJ mice which were readily demonstrable by PCA reactions in hooded rats.

KBG and RG cytochromes *c* were shown to be cross-reactive in terms of their allergenic specificities. This allergenic relationship held true regardless of whether human or murine reaginic sera were employed for the allergenic assessment by the inhibition of RAST or PCA procedures, respectively. However, at this stage of our studies it is not known if the allergenic determinant(s) of the cytochromes *c* that are recognized by the human IgE antibodies are identical to those recognized by the murine IgE antibodies.

The observation that KBG cytochrome *c* possessed an allergenic determinant not present on the RG cytochrome *c* is analogous to that of another study (Urbanski & Margoliash, 1977). Thus, a rabbit antiserum to human cytochrome *c* contained antibodies that recognized an antigenic specificity on the human cytochrome *c* which was not present on monkey cytochrome *c*. In addition, the rabbit antiserum contained antibodies that recognized determinants common to both cytochromes *c*. The unique antigenic specificity of the human cytochrome *c* was related to a single difference between the amino acid sequences of human and monkey cytochromes *c*. Accordingly, it may be inferred that the allergenic specificity unique to KBG cytochrome *c* is related to a limited difference between the amino acid sequences of KBG and RG cytochrome *c*. A comparison of allergenic cross-reactivity between pollen cytochromes *c* of known amino acid sequences could lead to the identification of the amino acid site(s) relevant to a particular immunochemical specificity.

Acknowledgements—The skilful technical assistance of Mr Tom Cook is acknowledged. The amino acid analysis was kindly performed by Dr F. Stevens of the Department of Biochemistry, University of Manitoba, and the assistance of Dr R. Warrington of our Department in performing the direct skin tests in allergic individuals is gratefully acknowledged.

REFERENCES

- Chakrabarty S., Lowenstein H., Ekramoddoullah A. K. M., Kisil F. T., Sehon A. H. (1981) Detection of cross-reactive allergens in Kentucky bluegrass and six other grasses by crossed radioimmuno-electrophoresis. *Int. Archs Allergy appl. Immun.* **66**, 142–157.

- Corradin G. & Chiller J. M. (1979) Lymphocyte specificity to protein antigens. II. Fine specificity of T cell activation with cytochrome *c* and derived peptides as antigenic proteins. *J. exp. Med.* **149**, 436-447.
- Dayhoff M. O. & Eck R. V. (1976) *Atlas of Protein Sequence and Structure*. National Biomedical Research Foundation, Washington, D.C.
- Dayhoff M. O., Hunt L. T., McLaughlin P. J. & Baker W. C. (1972) In *Atlas of Protein Sequence and Structure* (Edited by Dayhoff M. O.), Vol. 5. National Biochemical Research Foundation, Washington, D.C. See also Suppls 1 (1973), 2 (1976) and 3 (1978).
- Edelhock H. (1967) Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* **6**, 1948-1954.
- Ekraddoullah A. K. M., Chakrabarty S., Kisil F. T. & Sehon A. H. (1980) Isolation of a purified allergen from Kentucky bluegrass pollen. *Int. Archs Allergy appl. Immun.* **63**, 220-235.
- Ekraddoullah A. K. M., Kisil F. T. & Sehon A. H. (1977) Suppression of the IgE antibody response in mice to Kentucky bluegrass pollen allergens. *Int. Archs Allergy appl. Immun.* **55**, 247-254.
- Ekraddoullah A. K. M., Kisil F. T. & Sehon A. H. (1981) Isolation of allergenically active cytochrome *c* from Kentucky bluegrass pollen. *Int. Archs Allergy appl. Immun.* **65**, 367-376.
- Fairbanks G., Steck T. L. & Wallach D. F. H. (1971) Electrophoretic analysis of the major polypeptides of human erythrocyte membrane. *Biochemistry* **10**, 2606-2616.
- Gleich G. J. & Jones R. T. (1975) Measurement of IgE antibodies by the radioallergosorbent test. II. Analysis by quantitative relationships in the test. *J. Allergy* **55**, 346-357.
- Goodfriend L., Choudhury A. M., Carpio J. D. & King T. P. (1979) Cytochrome *c*: new ragweed pollen allergens. *Fedn Proc. Fedn Am. Soc. exp. Biol.* **38**, 1415.
- Hirs C. H. S. (1956) The oxidation of ribonuclease with performic acid. *J. biol. Chem.* **219**, 611-621.
- Jemmerson R. & Margoliash E. (1977) Analysis of a complex antigenic site on horse cytochrome *c*. *Adv. exp. Med. Biol.* **98**, 119-129.
- Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature Lond.* **227**, 680-685.
- Lowenstein H., Markussen B. & Weake B. (1976) Identification of allergens in extracts of horse hair and dandruff by means of crossed radioimmuno-electrophoresis. *Int. Archs Allergy appl. Immun.* **51**, 38-47.
- Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265-275.
- Margoliash E. & Schejter A. (1966) Cytochrome *c*. *Adv. Protein Chem.* **21**, 114-287.
- Margoliash E. & Schwarz R. H. (1979) T lymphocyte response to cytochrome *c*. I. Demonstration of a T cell heteroclitic proliferative response and identification of a topographic antigenic determinant on pigeon cytochrome *c* whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes. *J. exp. Med.* **150**, 830-847.
- Reichlin M. & Eng J. (1977) Identifying antigenic determinants on cytochrome *c* for B and T cells. *Adv. exp. Med. Biol.* **98**, 5-18.
- Reichlin M., Parnis R. A. & Wang K. M. (1979) Molecular complexity and the induction of immune responses. *Immun. Lett.* **1**, 121-124.
- Reisfeld R. A., Lewis U. J. & Williams D. E. (1962) Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature, Lond.* **195**, 281-283.
- Schumacher M. J., Wagner C. M. & Cusanovich M. A. (1982) Cytochrome *c* as a possible allergen in Bermuda grass pollen extract. *J. Allergy Clin. Immun.* **69**, No. 1, Part 2, 145 (abstract No. 209).
- Schwartz R. H., Solinger A. M., Ultee M. & Margoliash E. (1977) Genetic control of the T lymphocyte proliferative response to cytochrome *c*. *Adv. exp. Med. Biol.* **98**, 371-386.
- Urbanski G. J. & Margoliash E. (1977) The antigenicity of cytochrome *c*: in *Immunochemistry of Enzymes and Their Antibodies* (Edited by Salton, R. H.) pp. 203-225. John Wiley, New York.
- Weber K. & Osborn M. (1969) The reliability of molecular weight determinations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. biol. Chem.* **244**, 4406-4412.