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## An investigation of human immune response to perennial ryegrass (*Lolium perenne*) pollen cytochrome c (*Lol p X*)\*

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*Cytochrome c (Lol p X) was purified from the pollen of perennial ryegrass (Lolium perenne). It was used in a sensitive double-antibody radioimmunoassay to determine specific IgG and IgE antibody (Ab) levels in the sera of grass-allergic human subjects and the relationship between immune responsiveness and HLA type. A total of 139 subjects were studied, including 63 subjects treated by grass immunotherapy and 76 untreated subjects. The sera of only four subjects were found to contain anticytochrome c IgG Ab; there was no evidence of cytochrome c-specific IgE Ab in any of these patients. Based on these data, we conclude that the prevalence of IgE and IgG Ab responses to this protein is low. Because of the small number of responders, it was not possible to demonstrate a significant association between any HLA-D specificity and immune responsiveness to ryegrass cytochrome c. (J ALLERGY CLIN IMMUNOL 1987;80:229-35.)*

The cytochromes c represent a group of proteins that appear to be an excellent choice for studying the molecular and genetic basis of immune response because (1) the proteins are easy to isolate because they can be identified by their unique spectral properties,

### Abbreviations used

|           |                                                           |
|-----------|-----------------------------------------------------------|
| Ab:       | Antibody                                                  |
| DARIA:    | Double-antibody radioimmunoassay                          |
| SDS-PAGE: | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| DEAE:     | Diethylaminoethyl                                         |

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\**Lol p X* is the designation given under the new International Union of Immunological Societies Allergen Nomenclature System.<sup>1</sup>

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(2) they are present in all eukaryotes, and (3) amino acid sequences of more than 100 cytochromes c have been determined and have been found to exhibit a high degree of homology extending across the entire plant and animal kingdoms.<sup>2-4</sup> Recent immunogenetic studies in animals have served to emphasize the usefulness of cytochromes c as model antigens.<sup>3-6</sup> Finally, cytochromes c from ragweed and grass pollens have been implicated in causing IgE-mediated sensitivity

in some allergic patients.<sup>7-10</sup> In view of the potential importance of the use of these cytochromes c in human immunogenetic studies, we have examined the prevalence of Ab response to *L. perenne* cytochrome c in grass-allergic subjects and the relationship between responsiveness and HLA type.

## MATERIAL AND METHODS

### Cytochrome c

*Lolium perenne* pollen (Greer Laboratories, Lenoir, N. C.) was defatted with petroleum ether and extracted in 0.125 mol/L of ammonium bicarbonate, pH 8.0, for 18 hours at 4° C.<sup>11</sup> High-molecular-weight proteins were removed from the extract by passing it through an Amicon (Amicon Corp., Danvers, Mass.) hollow-fiber cartridge, HIP5, with a nominal cutoff of 5000 daltons. We have observed that this cartridge allows the passage of cytochrome c. The ultrafiltrate containing cytochrome c was concentrated with an Amicon YM-2 membrane, dialyzed against deionized water, and lyophilized. After reconstituting in 0.2 mol/L of ammonium bicarbonate, the ultrafiltrate was chromatographed successively on Sephadex G-75 superfine, CM-Sephadex C-25, DEAE-Sephadex A-25, and Sephadex G-50 superfine. Experimental details are presented in the legends to the individual chromatograms.

### Antisera

Five different antisera raised against grass-pollen antigens were used to investigate the purity of the cytochrome c preparation. Goat anticrude rye-pollen antiserum No. A-617-601-557 (National Institutes of Health Research Resources reagent) was a pool of high-titer sera from three goats that were each hyperimmunized with repeated, multiple-site, intramuscular injections of 2 to 10 mg of dialyzed rye-pollen extract emulsified with Freund's complete adjuvant containing 10 mg/ml of *Mycobacterium butyricum*. The next two reagents were two purified rabbit IgG fractions, antiorchard grass and antifive grass mix (rye, orchard, fescue, timothy, and velvet), kindly supplied by Dr. Henning Løwenstein. Each was prepared in three rabbits hyperimmunized with the respective antigens emulsified in Freund's complete adjuvant (see reference 23). High-titer sera were pooled, and the IgG fractions were each isolated and concentrated fivefold (see reference 23). The fourth serum was a pool obtained by combining two exceptionally high-titer rabbit antirye and rabbit antifescue sera prepared in our laboratory by a similar procedure. The fifth serum was a rabbit anti-*Lol p II* serum prepared in our laboratory by hyperimmunizing a single rabbit with *Lol p II* antigen emulsified in Freund's complete adjuvant.

### Agarose electrophoresis and immunoelectrophoresis

Agarose electrophoresis was performed at 10° C in 1% agarose (SeaKem ME agarose, FMC Corp., Rockland, Maine) with Tris-tricine buffer, pH 8.6, 10.1, according to

the method of Roebber et al.<sup>12</sup> Proteins were run for 45 minutes at 10 V/cm and then stained with "Boston" stain (50 ml of methanol, 124 ml of water, 20 gm of trichloroacetic acid, 6 gm of sulfosalicylic acid, and 0.18 gm of Coomassie brilliant blue R250). Immunoelectrophoresis was performed essentially as described.<sup>12</sup>

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed in slab gels according to the method of Laemmli<sup>13</sup> with 10% to 20% gradient in polyacrylamide.

### High-performance liquid chromatography

Analytical high-performance liquid chromatography was performed with Waters 501 pumps (Waters Associates, Milford, Mass.), U6K injector and model 441 absorbance detector.

### Radioiodination of cytochrome c

Purified ryegrass pollen cytochrome c was labeled with <sup>125</sup>I with the chloramine-T method<sup>14</sup> to a specific activity of about  $1.1 \times 10^5$  cpm/ng.

### Double antibody radioimmunoassay

DARIAs were used to study the binding of human IgG and IgE Abs to cytochrome c. For IgG Ab assays, the human serum was diluted 1:10 in borate-buffered saline. For IgE Ab assays, the human serum was diluted 1:2 in IgE myeloma serum PS "carrier" (1/200). In each case, 100 µl of the diluted human serum was incubated for 4 hours at 23° C with 100 µl of radiolabeled cytochrome c (0.5 ng) containing 5% bovine serum albumin. Then, 100 µl of goat antihuman IgG (γ-chain specific) or goat antihuman IgE (ε-chain specific) was added, and immune precipitates were allowed to form overnight at 4° C. The precipitates were washed four times with borate-buffered saline and counted in a gamma counter. Ab content was expressed in terms of units, where 1 unit of Ab was equivalent to the highest dilution of a standard serum giving counts per minute value that was consistently significantly higher than the control counts per minute value.

### Study groups

Two groups of unrelated subjects, described in earlier articles,<sup>15, 16</sup> were studied. The first group consisted of 63 individuals (41 male and 22 female) who were participating in various clinical studies in the Division of Clinical Immunology at the Johns Hopkins University Medical School. All these individuals were highly allergic to ryegrass and had been receiving high-dose immunotherapy with mixed grass-pollen antigens for a minimum of 3 years.

The second group comprising 76 individuals (68 male and eight female; mean age  $36.2 \pm 8.9$  years; range 20 to 56 years) were employees of the Westinghouse Electric Corporation, Hunt Valley, Baltimore County, Md. All these selected individuals were also highly allergic to ryegrass

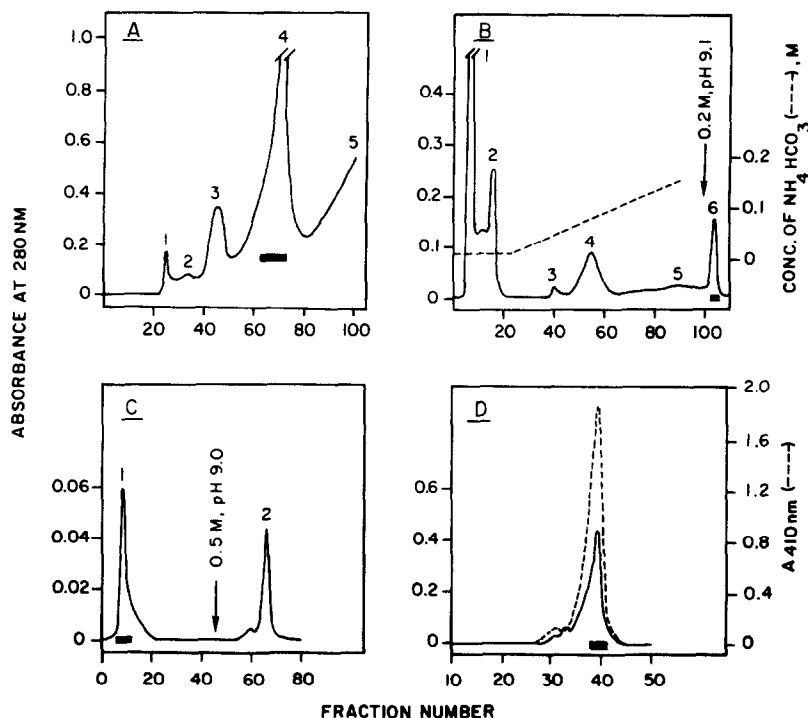


**FIG. 1.** A, Sephadex G-75 superfine chromatography of ryegrass pollen extract. Column: 5 by 95 cm; sample: 15 ml containing 1.28 gm of lyophilized material; eluent: 0.2 mol/L of ammonium bicarbonate, pH 8.0, containing 0.02% sodium azide; flow rate: 20 ml/hr; fraction size: 10 ml. B, CM-Sephadex C-25 chromatography of cytochrome c-containing fraction (peak 4, A). Column: 2.6 by 40 cm; sample: approximately 100 ml of the pool (peak 4, A) was concentrated to 25 ml through Amicon YM-2 membrane and applied on this column; elution: initially with 0.01 mol/L of ammonium bicarbonate, pH 8.0, then linear gradient with increasing ammonium bicarbonate concentration (to 0.1 mol/L) at constant pH of 8.0, and finally with 0.2 mol/L of ammonium bicarbonate plus ammonium hydroxide, pH 9.1; flow rate: 40 ml/hr; fraction size: 10 ml. C, DEAE-Sephadex A-25 chromatography of peak 6, A. Column 2.5 by 50 cm; sample: 7 ml; elution: initially with 0.01 mol/L of ammonium bicarbonate plus ammonium hydroxide, pH 9.0, and finally with 0.5 mol/L of the same buffer; flow rate: 40 ml/hr; fraction size: 10 ml. D, Sephadex G-50 superfine chromatography of peak 1, C. Column: 1.4 by 160 cm; sample: 1.5 ml; eluent: 0.2 mol/L of ammonium bicarbonate, pH 8.0; flow rate: 15 ml/hr; fraction size: 3.5 ml.

but, unlike the participants of the other group, were not receiving immunotherapy with grass-pollen extract.

### HLA typings

HLA-A, B, C, DR, and DQ typings were performed on 43 of the treated subjects and 66 of the Westinghouse subjects in the immunogenetics laboratory of Dr. Wilma B. Bias, Johns Hopkins University School of Medicine, with standard lymphocytotoxicity procedures.

### Amino acid analysis

The protein sample was hydrolyzed for 21 hours at 110° C with 6 N of HCl. Analysis was performed with the PICO TAG method with Waters' high-performance liquid chromatography system. Cysteine and tryptophan contents were not determined.

## RESULTS AND DISCUSSION

### Purification of cytochrome c

Purification of cytochrome c involved four successive chromatographic steps depicted in Fig. 1. The starting material was a "medium molecular weight fraction" of the pollen extract, obtained by removing high-molecular-weight and low-molecular-weight substances, first by passing the pollen extract through an Amicon hollow-fiber H1P5 cartridge and discarding the retentate and then by passing the ultrafiltrate through an Amicon YM-2 membrane followed by dialysis and lyophilization of the YM-2 retentate. Although the nominal cutoff of H1P5 hollow-fiber cartridge is 5000 daltons, we have observed that it allows the passage of cytochrome c and of some larger

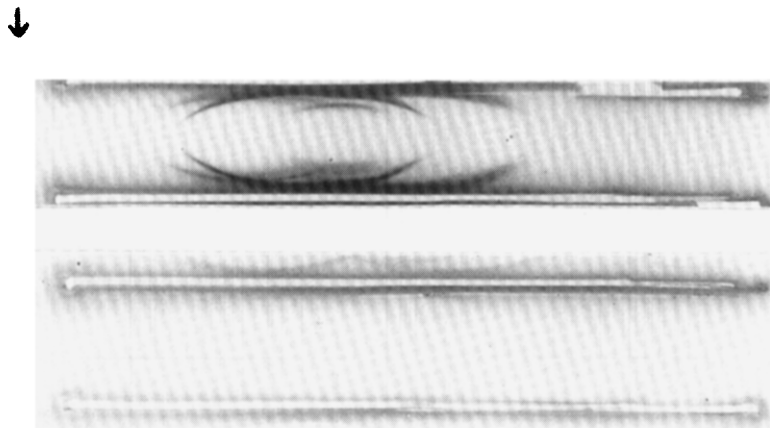


**FIG. 2.** Immunoelectrophoresis of ryegrass cytochrome c before (*upper panel*) and after (*lower panel*) purification with a highly potent antiserum pool from three goats hyperimmunized with crude rye-pollen extract. Samples were run at 10° C for 45 minutes at 10 V/cm in 1% agarose in Tris-tricine buffer (pH = 8.6; I = 0.1), and antiserum was added to troughs cut in the gels, which were then incubated overnight at 23° C. After washing and drying, the gels were stained with 0.1% Coomassie blue R250 in 45% methanol and 10% acetic acid.

molecules as well. On Sephadex G-75 column (Fig. 1, A), the "medium molecular weight fraction" was resolved into four protein peaks and a fifth, broad band, largely containing low-molecular-weight pigments. (The fifth "peak" is not depicted in Fig. 1, A because it emerged from the column as a smear in a large volume, probably resulting from the interaction of the pigments with the gel.)

The fourth peak from the Sephadex G-75 column was tentatively identified by its pink color to contain cytochrome c. The pink fractions were pooled, concentrated through an Amicon YM-2 membrane, diluted with water to 25 ml, and chromatographed on a CM-Sephadex column (Fig. 1, B). Acidic proteins were eluted in two peaks in the initial buffer (0.01 mol/L of ammonium bicarbonate, pH 8.0), followed by other proteins when a salt gradient was applied. By their color, peaks 5 and 6 (Fig. 1, B) also appeared to contain cytochrome c. Peak 6 was used for further purification because it contained significantly larger amounts of the protein.

The pooled pink fraction under peak 6 (Fig. 1, B) was concentrated through a YM-2 membrane and diluted with water to decrease the salt concentration in the protein to <0.01 mol/L. It was then chromatographed on a DEAE-Sephadex A-25 column (Fig. 1, C). Two peaks were obtained; peak 1 was identified by its spectral properties to be cytochrome c. These spectral properties included absorption maxima at 280, 350, 410, 520, and 550 nm.<sup>17</sup>

The cytochrome c pool was lyophilized, and after

reconstitution in 1.5 ml of buffer, it was chromatographed on Sephadex G-50 superfine (Fig. 1, D). The column was monitored both at 280 nm and 410 nm. One major peak and a minor peak of higher molecular weight were obtained. Since the minor, higher molecular weight peak also absorbs at 410 nm, we suspect that it might represent a polymerized form of cytochrome c. The protein fraction under the major peak indicated in Fig. 1, D was used in the present studies.

#### Characterization of the purified cytochrome c

Immunoelectrophoresis was initially performed with the pool of antisera from three goats hyperimmunized with crude rye-pollen extract (National Institutes of Health Research Resources reagent, No. A-617-601-557). This serum elicited at least 15 precipitin lines against the crude extract<sup>18</sup> and at least four lines (Fig. 2, *upper lane*) with partially purified extract (the cytochrome c-containing fraction under peak 4 in Fig. 1, A). Purified cytochrome c, however, elicited no precipitin line against the same antiserum (Fig. 2, *lower lane*), demonstrating not only that the antiserum contains little or no Ab against cytochrome c but also suggesting that the cytochrome c preparation is devoid of any antigenic impurity originally present in the partially purified preparation. No evidence of impurity in the cytochrome c preparation was found even after repeating immunoelectrophoresis with four additional hyperimmune antiserum pools raised against crude and purified extracts of rye and

other grass-pollen extracts (see Material and Methods). Three of these sera were able to detect 30 to 40 immunoprecipitates against crude rye extract; the fourth serum was essentially monospecific for *Lol p* II. Taken together, these data provide evidence that antigenic impurities are undetectable in our cytochrome c preparation. On agarose electrophoresis, the purified *L. perenne* cytochrome c elicited a major band and a smear, probably corresponding to oxidized and reduced forms.<sup>7</sup> SDS-PAGE of the purified cytochrome c elicited one band corresponding to a molecular weight close to 12,000 daltons (Fig. 3). The absorption spectrum of the protein demonstrated major peaks at 280 and 410 nm, and minor peaks at 350, 520, and 550 nm (Fig. 4). The absorption maxima at 280 nm is due to protein; the maxima at 350, 410, 520, and 550 nm are the characteristics of the heme spectrum of cytochrome c.<sup>17</sup> The amino acid composition (Table I) of this cytochrome c was found to be similar to that previously reported for ryegrass cytochrome c.<sup>10</sup>

### Immune response to cytochrome c

A total of 139 grass-allergic subjects were studied, including 63 subjects treated by grass immunotherapy and 76 untreated subjects. The sera of four subjects who had received high-dose grass immunotherapy were found by DARIA to contain anticytochrome c IgG Ab; there was no evidence of cytochrome c-specific IgE Ab in any of these patients. With the possible exception of one patient (C. St.) the pretreatment blood samples of these four patients had no detectable Ab against cytochrome c, and the specific IgG Ab was produced in response to immunotherapy with whole grass-pollen extracts. None of the other subjects studied demonstrated any detectable anticytochrome c IgE or IgG Abs. With such a low proportion of responders, it was not possible to demonstrate a significant association between an HLA-A, B, C, or DR specificity and immune responsiveness to cytochrome c. It should, however, be noted that all three responder subjects for whom HLA-D typings were available possessed DQ1. The corresponding proportion of DQ1 in D-typed treated nonresponders was 31/43 (72%).

The low prevalence of IgG Ab response to ryegrass cytochrome c (even after high-dose immunotherapy) and absence of detectable IgE Ab in all 139 patients studied is in contrast to an earlier study suggesting that ryegrass cytochrome c is allergenically active.<sup>10</sup> With a high concentration (1 µg/ml) of ryegrass cytochrome c (*Lol p* X) in intradermal tests, three ryegrass-allergic individuals were found to demonstrate positive skin tests.<sup>10</sup> Cytochromes c from

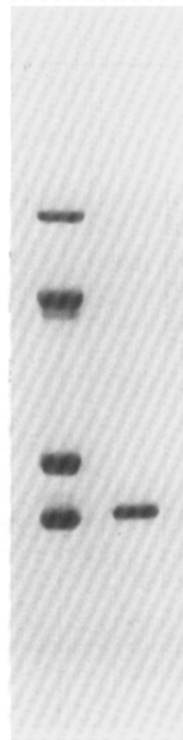


**FIG. 3.** SDS-PAGE analysis of purified *Lol p* cytochrome c in a gradient gel. The sample and molecular weight standards were denatured and reduced by boiling in 62 mmol/L of Tris HCl, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol and were then electrophoresed for 4 hours at 30 mA in the gel (1.5 mm thick, 12 cm long, and 14 cm wide). The stacking gel (4% concentration) was prepared in 125 mmol/L of Tris HCl, pH 6.8, containing 0.1% SDS. The separating gel consisted of a gradient of 10% to 20% gel in 375 mmol/L of Tris HCl, pH 8.8, with 0.1% SDS. The tank buffer was 25 mmol/L of Tris-192 mmol/L of glycine, pH 8.3, with 0.1% SDS. After the electrophoresis, the proteins were stained in 0.125% (weight per volume) Coomassie blue R-250 prepared in 50% methanol and 10% acetic acid. *Right lane*, purified cytochrome c; *left lane*, molecular weight markers from bottom to top: horse heart cytochrome c (11,700), soybean trypsin inhibitor (21,500), ovalbumin (45,000), and bovine serum albumin (68,000).

ragweed<sup>7</sup> and Kentucky bluegrass<sup>9</sup> were also suggested to be allergenically active in allergic individuals. Thus, in a preliminary communication, Goodfriend et al.<sup>7</sup> observed positive skin test reactions "at dosages of 0.1 µg/ml" of ragweed cytochrome c in ragweed-allergic subjects. (The numbers of patients tested and found positive were not mentioned.) Ekramoddoullah et al.<sup>9</sup> found two of three patients demonstrating positive skin reactions with 20 µl of Kentucky bluegrass cytochrome c (1 µg/ml) in intradermal skin tests. Ekramoddoullah et al.<sup>10</sup> found three patients positive to ryegrass cytochrome c with 1 µg/ml in

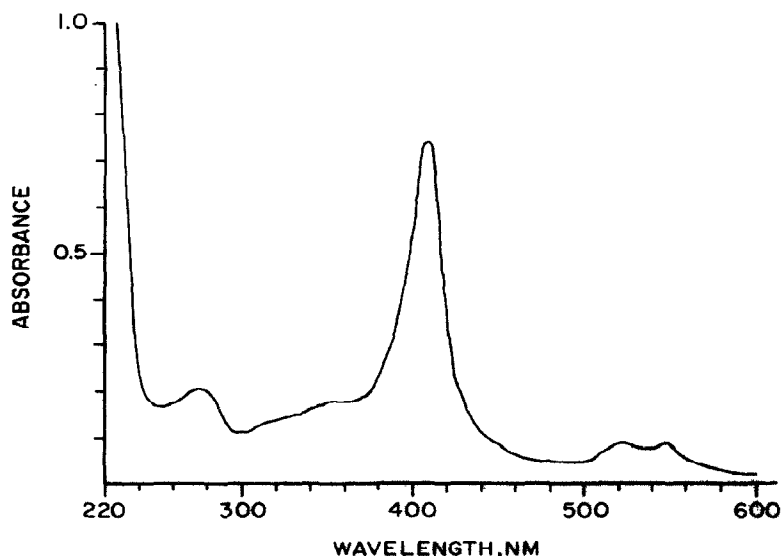


FIG. 4. Absorption spectrum of purified *Lol p* cytochrome c in 0.1 mol/L of ammonium bicarbonate, pH 8.0.

TABLE I. Amino acid composition of rye cytochrome c

| Amino acid                   | Residues per 12,000 daltons |                                     |
|------------------------------|-----------------------------|-------------------------------------|
|                              | Present work                | Ekramoddoullah et al. <sup>10</sup> |
| Alanine                      | 14                          | 13                                  |
| Arginine                     | 4                           | 3                                   |
| Aspartic acid and asparagine | 6                           | 8                                   |
| Cysteine                     | nd                          | 2                                   |
| Glutamic acid and glutamine  | 10                          | 9                                   |
| Glycine                      | 12                          | 12                                  |
| Histidine                    | 2                           | 2                                   |
| Isoleucine                   | 3                           | 4                                   |
| Leucine                      | 8                           | 8                                   |
| Lysine                       | 13                          | 11*                                 |
| Methionine                   | 1                           | 2                                   |
| Phenylalanine                | 4                           | 4                                   |
| Proline                      | 8                           | 8                                   |
| Serine                       | 7                           | 7                                   |
| Threonine                    | 8                           | 7                                   |
| Tryptophan                   | nd                          | 2                                   |
| Tyrosine                     | 5                           | 5                                   |
| Valine                       | 3                           | 5                                   |

nd = Not done.

\*Includes one tetramethyl lysine.

intradermal skin tests of three grass-sensitive subjects. The intradermal skin test is highly sensitive and elicits positive reactions in highly allergic subjects at allergen concentrations of  $10^{-3}$  to  $10^{-4}$   $\mu\text{g/ml}$ .<sup>19</sup> At a concen-

tration of 1  $\mu\text{g/ml}$ , a highly purified test allergen with a purity as high as 99.9% may contain several highly allergenic impurities at the level of  $10^{-3}$   $\mu\text{g/ml}$ . *Lol p* III (Rye III) that, like cytochrome c, is a basic protein and has a molecular weight of 11,000 daltons (cytochrome c has 12,000 daltons), is a possible contaminant of cytochrome c. We have found<sup>20</sup> that a significant proportion (about 43/184; 23.4%) of grass-allergic individuals have IgE and IgG Abs to *Lol p* III. Thus, it is possible that intradermal skin tests with 1  $\mu\text{g/ml}$  of cytochrome c could elicit positive reactions because of the presence of low levels of highly allergenically active molecules.

The technique of DARIA used in the present study, although it is highly sensitive for detecting low levels of Abs, is relatively insensitive to low levels of contamination in the allergen preparation. Thus, if the allergen preparation used for radioiodination contained 0.1% contaminant and the patient's serum contained Ab to the contaminant only, then only about 0.1% of the total counts per minute added to the test would bind to the Ab, which would go undetected.

It has been suggested that cytochromes c are poor immunogens because they have such a highly conserved overall structure and maintain the same polypeptide backbone structure.<sup>21, 22</sup> The observed low frequency of human immune response to ryegrass cytochrome c is perhaps not surprising, since homologues are present in all eukaryotes, and cytochrome c represents a minor constituent of ryegrass pollen. In contrast to the findings of Goodfriend et al.<sup>7</sup> and Ekramoddoullah et al.,<sup>9, 10</sup> our human data

are consistent with previous findings in animals, and we conclude that the prevalence of human IgE and IgG Ab responses to cytochrome c is low.

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