

# IgE mediators, inflammatory mechanisms

## Selection of representative *Alternaria* strain groups on the basis of morphology, enzyme profile, and allergen content

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*In an attempt to identify representative strains, 12 strains of Alternaria alternata were contributed by four different research groups. Each was grown on two types of solid media and characterized with descriptions of pigmentation and morphology. Enzyme profiles were determined on both the cellular antigen and the culture filtrate material with use of a commercial kit. Concentrations of a previously described Alt a 1 and a 70 kd allergen, GP70, were measured by a two-antibody "sandwich" ELISA. Allergenic proteins were visualized by IgE immunoblots. With use of these criteria, four separate strains were identified that might serve as representative of Alternaria for further research. (J ALLERGY CLIN IMMUNOL 1993;91:773-82.)*

**Key words:** *Alternaria*, strains, enzymes, immunoblot, immunoassay

Extracts from various species of *Alternaria* prepared both in the laboratory or obtained commercially have been examined by many investigators. Significant interstrain differences in protein and allergen content have been demonstrated with use of techniques such as crossed immunoelectrophoresis,<sup>1, 2</sup> RAST,<sup>3-5</sup> and Western blot.<sup>6-9</sup> Many reasons exist for these differences. One is the changeable and adaptable nature of the organism when grown under various conditions. In addition, the time of extraction, the extraction buffer used for elution, and whether or not dialysis is performed may have an effect on the final product.

The resultant preparations derived from *Alternaria* are composed of a myriad of proteins and glycoproteins including both soluble cytoplasmic components as well as membrane- and cell-wall-bound compo-

### Abbreviations used

|           |                                                           |
|-----------|-----------------------------------------------------------|
| CF:       | Culture filtrate                                          |
| CA:       | Cellular antigen                                          |
| ELISA:    | Enzyme-linked immunosorbant assay                         |
| GP70:     | A 70 kd glycoprotein <i>Alternaria</i> allergen           |
| PBS:      | Phosphate-buffered saline                                 |
| PDA:      | Potato dextrose agar                                      |
| SDS-PAGE: | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| TBS:      | TRIS-buffered saline                                      |

### Enzymes used

|                |                         |
|----------------|-------------------------|
| LAP:           | Leucine amino peptidase |
| $\alpha$ -GLU: | Alpha-glucosidase       |
| $\beta$ -GAL:  | Beta-galactosidase      |
| $\beta$ -GLR:  | Beta-glucuronidase      |
| $\alpha$ -MAN: | Alpha-mannosidase       |

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nents. Given this situation, it has been very difficult for investigators who are working with different strains under different laboratory conditions to separate the effect of growth and extraction conditions from differences that derive from the particular strain of *Alternaria* that is used. In a recent study the effect of vigorous physical measures to break up the mold as well as the presence of protease inhibitors and phe-

**TABLE I.** Source of 12 *Alternaria* strains studied arranged in order of the investigator who submitted them

| <i>Alternaria</i> strain | Group no. | Contributing investigator | Pigment PDA | Pigment Sabouraud |
|--------------------------|-----------|---------------------------|-------------|-------------------|
| AL 001                   | II        | J. Portnoy*               | Low         | Medium            |
| AL 002                   | II        | J. Portnoy                | Medium      | Dark              |
| AL 003                   | III       | J. Portnoy                | None        | None              |
| AL 005                   | II        | J. Portnoy                | Low         | Medium            |
| 188698                   | I         | H. Vijay‡                 | Dark        | Dark              |
| 188699                   | I         | H. Vijay                  | Dark        | Dark              |
| 188700                   | I         | H. Vijay                  | Dark        | Dark              |
| 188701                   | I         | H. Vijay                  | Dark        | Dark              |
| ATCC 16086               | III       | R. Esch§                  | None        | None              |
| ATCC 11680               | IV        | R. Esch                   | Medium      | None              |
| CBS 103.33               | IV        | R. Esch                   | Dark        | None              |
| ATCC 46582               | III       | R. Bush                   | None        | None              |

Group numbers are defined in the text. Pigmentation was measured as none, low, medium, and dark.

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nol binding agents on the yield of the 31 kd allergen (probably *Alt a* I) was examined. Although these procedures did not result in significantly greater release of the desired allergen,<sup>8</sup> the same authors were able to increase its yield by using a different strain of *Alternaria* and adjusting the growth conditions.

To address this problem we have attempted to identify morphologic growth characteristics, enzymatic profiles, IgE immunoblot patterns, and patterns of specific allergen content to designate a few strains of *Alternaria* as being "representative" of the genus. We further intend that these strains of *Alternaria* be made available for routine use by investigators. To do this, 12 strains of *Alternaria* contributed by four different laboratories in North America were studied.

## METHODS

### Human studies

All clinical studies were performed after obtaining approval from the pediatrics institutional review board of the University of Missouri-Kansas City. Written consent was obtained before any experimental procedure was performed. Volunteers were tested for *Alternaria* sensitivity by prick skin test with use of three commercial *Alternaria* preparations (Greer Laboratories, Lenoir N.C.; Center Laboratories, Port Washington N.Y.; Hollister-Stier, Spokane, Wash.). Serum was obtained from those with 5 mm or greater wheals to one or more of the *Alternaria* extracts and screened for anti-*Alternaria* IgE by direct ELISA. Samples with significant specific IgE were pooled. The final serum pool containing IgE consisted of equal amounts of sera taken from 13 patients sensitive to *Alternaria*.

### *Alternaria* source materials

Twelve strains of *Alternaria* were contributed by four different research groups from North America. Three of the strains are available from ATCC (American Type Culture Collection, Rockville, Md.), one of the strains is available from CBS (Centraalbureau voor Schimmel Cultures, Baarn, The Netherlands), and eight of the strains were derived from the wild or from other laboratory cultures. The designations of these are shown in Table I.

### Growth conditions

For morphologic studies, cultures were grown on two types of solid media: potato dextrose agar (PDA) pH 5.6, and Sabouraud dextrose agar, pH 5.6, containing 0.005% chloramphenicol (Blue Ridge Biologicals, Hickory N.C.). For preparation of extracts, cultures were also grown on two types of media. One was a high-nutrient peptone-based medium available from Greer Laboratories. The second was a low-nutrient synthetically defined medium containing only inorganic nitrogen, sucrose, and trace minerals.<sup>10</sup> All cultures were grown under 12 hours of light per day (GE Growlite; General Electric Lighting Group, Cleveland, Ohio) at 20° to 25° C for 2 weeks.

### Preparation of culture filtrate (CF) and cellular antigen (CA)

Fungal mats were defatted with acetone, air dried, and powdered with mortar and pestle. The resultant dry powder was next extracted in deionized water (1:20 weight per volume) at 4° C for 3 hours, filtered through a 0.45 filter (Millipore Corp., Bedford, Mass.), dialyzed against 50 mmol/L ammonium bicarbonate, and lyophilized. This material is referred to as CA. The spent culture medium was

decanted, clarified through an AP15 filter (Millipore), dialyzed with a hollow fiber cartridge (Amicon HP10 P5; Amicon Division of W. R. Grace and Co., Beverly, Mass.), and lyophilized. This material is the CF. All lyophilized materials were stored at  $-20^{\circ}\text{C}$  until used.

### Determination of enzyme activities

Enzyme activities of the *Alternaria* preparations were determined with use of a commercial kit (ApiZym, Analab Products, Plainview N.Y.). We followed the procedure as recommended by the manufacturer. Antigens were applied at a concentration of  $20\text{ }\mu\text{g}$  protein per well, and the reactions were read with a color chart supplied with the kit. The scale ranged from 0 to 5, representing no color change to a strong color change indicating the most active preparations.

### Monoclonal and polyclonal antibodies

Polyclonal rabbit antibody specific for **hypoallergen (30 kd)** was obtained from Dr. H. Vijay. The polyclonal sera has been shown to be directed to a material containing a dominant epitope of *Alt a I*<sup>11</sup> and has a similar *N*-terminal amino acid sequence to that given for *Alt a I* by another investigator.<sup>12</sup>

Monoclonal antibodies directed toward GP70 (a 70 kd glycoprotein *Alternaria* allergen) were produced by BALB/C mice with use of standard procedures described previously.<sup>13</sup> Polyclonal sera were collected and pooled from the *Alternaria*-immunized mice before fusion of their spleens. Cloned hybridoma cells were next injected into pristane-treated mice, and ascites fluids containing monoclonal antibodies were collected. These were tested for anti-*Alternaria* activity by means of direct ELISA. Monoclonal and polyclonal antibodies were purified with use of protein A before use. Purity was confirmed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions.

Pooled human serum containing IgE was diluted 1:2 in phosphate-buffered saline (PBS), and this was precipitated with 50% ammonium sulfate. After centrifuging, the precipitate was recovered and washed two times with 50% ammonium sulfate. The resulting pellet was then dissolved in TRIS-buffered saline (TBS) and dialyzed exhaustively against the same buffer at  $4^{\circ}\text{C}$ .

### Biotinylation of antibodies

For detection, secondary antibodies were labeled with biotin. Two milligrams of purified polyclonal mouse or human anti-*Alternaria* IgG or affinity purified lyophilized monoclonal antibody were dissolved in  $200\text{ }\mu\text{l}$  of 0.05 mol/L bicarbonate buffer (pH 7.5) and incubated at  $4^{\circ}\text{C}$  for 1 hour with  $3\text{ }\mu\text{l}$  of NHS-biotin (Bio-Rad Laboratories, Chemical Div., Richmond Calif.). At the end of this time, the IgG was dialyzed at  $4^{\circ}\text{C}$  overnight against 0.1 mol/L TRIS-buffered saline and stored at  $-20^{\circ}\text{C}$  in 50% glycerol at a protein concentration of 5 mg/ml. The activities of biotinylated antibodies were confirmed by direct ELISA

with use of 1:1000 avidin-alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) to detect them.

### Coating of microtiter plates

Polystyrene microtiter plates (Immulon 2; Dynatech Laboratories Inc., Chantilly, Va.) were coated by dissolving lyophilized materials in a 0.15 mol/L bicarbonate buffer (pH 9.6) and adding  $200\text{ }\mu\text{l}$  per well. The plates were then incubated at  $24^{\circ}\text{C}$  overnight. For direct ELISA with *Alternaria* in solid phase, a concentration of 10 mg dry weight/ml was used. The optimal adsorption concentrations were determined empirically for each monoclonal antibody as discussed below.

### Direct ELISA

Plastic microtiter plates (Immulon 2, Dynatech) were coated with  $200\text{ }\mu\text{l}$ /well of  $10\text{ }\mu\text{g/ml}$  antigen overnight at room temperature. The plates were washed three times with PBS-Tween 20 with 0.01% sodium azide and 0.1% gelatin. For IgE determination,  $200\text{ }\mu\text{l}$ /well of antibody-containing serum diluted 1:10 was added. After a 2-hour incubation and washing,  $100\text{ }\mu\text{l}$ /well of 1:1000 rabbit anti-human IgE (Sigma) was added followed by  $100\text{ }\mu\text{l}$ /well 1:1000 alkaline phosphatase-conjugated goat antirabbit IgG (Sigma). For IgG determinations,  $200\text{ }\mu\text{l}$ /well of 1:100 serum was added for 2 hours followed by  $100\text{ }\mu\text{l}$ /well of 1:1000 alkaline phosphatase-conjugated goat antihuman IgG (Sigma). Enzyme activities were detected with 1 mg/ml p-nitrophenyl phosphate, and optical densities at 405 nanometers were measured with a Dynatech MR7000 plate reader.

### Purification of GP70

The 70 kd glycoprotein was purified by affinity chromatography with use of a specific monoclonal antibody in solid phase as described previously.<sup>13</sup> In brief, lyophilized *Alternaria* extract was dissolved in TRIS-buffered saline at a concentration of 10 mg/ml. This was applied to an affinity column that was subsequently washed until the optical density at 280 nm fell to baseline. The bound antigen was then eluted with 0.1 mol/L acetate buffer (pH 3.0), dialyzed against 50 mmol/L ammonium bicarbonate, and lyophilized. Purity was confirmed by SDS-PAGE.

### Sandwich ELISA

Individual allergens were measured with an antigen-capture or "sandwich" ELISA. Measurement of GP70 was performed by first coating microtiter plates with  $10\text{ }\mu\text{g/ml}$  of a monoclonal antibody directed at GP70 designated F8F11, which has been described previously.<sup>13</sup> The plates were coated with polyclonal rabbit anti-*Alt a I* for the *Alt a I* assay. Coated plates were washed and blocked with 0.5% gelatin (Fisher Scientific Co., Pittsburgh, Pa.) for 1 hour at  $37^{\circ}\text{C}$  to reduce background. Serial dilutions of sample dissolved in PBS Tween-20 azide buffer were added to each well and incubated for 2 hours at  $24^{\circ}\text{C}$ . After washing, the plates were incubated with biotinylated monoclonal (F8F11) or polyclonal antibody (rabbit polyclonal) for 2 hours. The

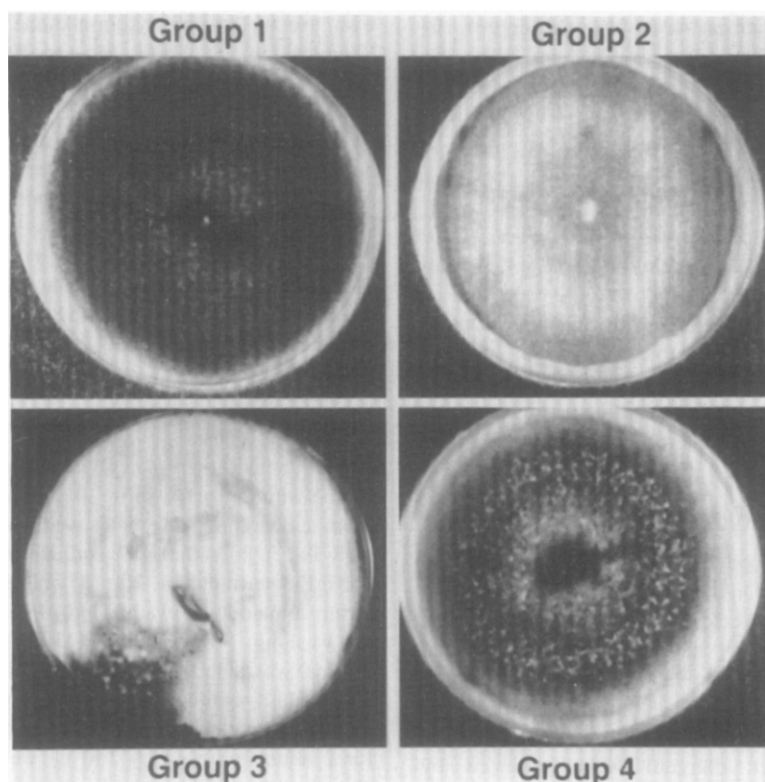


**FIG. 1.** Macroscopic appearance of *Alternaria* that are representative of groups I, II, III, and IV. Notice that the group III representative exhibited "sectoring" in that a section of it mutated to a pigment-forming strain as it grew from the center.

plates were again washed and incubated for 1 hour with 1:1000 avidin-conjugated alkaline phosphatase (Sigma). After addition of 1 mg/ml p-nitrophenyl phosphate, the optical density at 405 nm was read with a Dynatech MR7000 plate reader. Purified GP70 was used to standardize the sandwich ELISA for GP70 in micrograms of dry weight per milliliter. Because we do not yet have purified *Alt a* I available, this assay was standardized by assigning 1000 units of activity to one of the strains (ATCC 11680) and measuring *Alt a* I concentrations relative to it.

### Electrophoresis

SDS-PAGE was performed on 10% vertical slab gels (Bio-Rad) with the method of Laemmli.<sup>14</sup> Molecular weight markers (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany) were included with each run. Lyophilized samples were reconstituted in distilled water at a concentration of 1 mg/ml, and 10  $\mu$ l of each sample diluted in 30  $\mu$ l of TRIS-SDS (pH 6.8) with beta-mercaptoethanol and 25% glycerol were applied to each lane. SDS-PAGE gels were stained for glycoprotein with a silver stain according to the method of Wray et al.<sup>15</sup>

### Immunoblotting

Proteins from SDS-PAGE gels were transferred electrophoretically to nitrocellulose membranes (Bio-Rad TransBlot) in 25 mmol/L TRIS and 192 mmol/L glycine

buffer, pH 8.8, containing 20% methanol at 30 V overnight.<sup>16</sup> Proteins were detected either with a gold stain (Bio-Rad) to confirm completeness of the transfer, or they were blocked overnight with 5% nonfat dry milk (Carnation Co., Los Angeles, Calif.) in TBS. Blots were subsequently probed overnight with the 1:10 pooled human serum that contained IgE to *Alternaria*. Blots next were incubated for 2 hours with 1:1000 rabbit antihuman IgE washed with TBS containing 0.3% TWEEN and incubated for 2 hours with 1:1000 alkaline phosphatase-conjugated goat anti-rabbit IgG. After washing in TBS TWEEN the blots were developed with alkaline phosphatase substrate until bands were well visualized.

For storage and labeling, immunoblots were scanned into a Macintosh IICx (Apple Computer Corp., Cupertino, Calif.) computer a Microtek 300 ZX (Microtek Medical, Inc., Columbus, Miss.) scanner. Photographs for publication were taken directly from a 21-inch gray scale monitor with use of a Polaroid (Polaroid Corp. Medical Products, Cambridge, Mass.) camera. Molecular weights were determined by linear regression of the log (molecular weight) of the standards versus position of the sample bands.

## RESULTS

### Morphology

The classic description of *Alternaria* describes the organism as follows: Conidiophores dark, septate,

**TABLE II.** Enzyme activities of 12 strains of *Alternaria* grown on defined medium arranged by proposed morphologic group number

| Group no. | <i>Alternaria</i> strain | Alk Phos |    | $\alpha$ -GLU |    | LAP |    | $\alpha$ -GAL |    | $\beta$ -GAL |    | $\beta$ -GLR |    | $\alpha$ -MAN |    |
|-----------|--------------------------|----------|----|---------------|----|-----|----|---------------|----|--------------|----|--------------|----|---------------|----|
|           |                          | CA       | CF | CA            | CF | CA  | CF | CA            | CF | CA           | CF | CA           | CF | CA            | CF |
| I         | 188698                   | 5        | 3  | 3             | 4  | 1   | 1  | 5             | 1  | 5            | 2  | 3            | 0  | 2             | 0  |
| I         | 188699                   | 5        | 5  | 4             | 4  | 1   | 1  | 5             | 2  | 4            | 1  | 3            | 0  | 1             | 0  |
| I         | 188700                   | 4        | 5  | 4             | 4  | 1   | 1  | 5             | 4  | 4            | 1  | 3            | 0  | 3             | 0  |
| I         | 188701                   | 5        | 5  | 3             | 4  | 0   | 3  | 5             | 2  | 4            | 4  | 3            | 0  | 2             | 0  |
| II        | AL 001                   | 3        | 5  | 3             | 3  | 3   | 2  | 3             | 2  | 3            | 3  | 1            | 0  | 3             | 0  |
| II        | AL 002                   | 3        | 5  | 4             | 4  | 1   | 4  | 3             | 2  | 3            | 4  | 0            | 0  | 2             | 0  |
| II        | AL 005                   | 3        | 1  | 3             | 4  | 1   | 0  | 3             | 2  | 3            | 4  | 3            | 0  | 1             | 0  |
| III       | AL 003                   | 5        | 3  | 5             | 4  | 2   | 1  | 4             | 0  | 3            | 0  | 1            | 0  | 0             | 0  |
| III       | ATCC 16086               | 2        | 5  | 4             | 3  | 0   | 0  | 2             | 0  | 1            | 0  | 0            | 0  | 1             | 0  |
| III       | ATCC 46582               | 4        | 1  | 4             | 3  | 0   | 0  | 0             | 0  | 0            | 0  | 0            | 0  | 0             | 0  |
| IV        | ATCC 11680               | 3        | 5  | 3             | 4  | 1   | 3  | 4             | 1  | 2            | 0  | 0            | 0  | 2             | 0  |
| IV        | CBS 103.33               | 3        | 3  | 2             | 2  | 0   | 0  | 3             | 0  | 3            | 0  | 1            | 0  | 1             | 0  |

Increasing enzyme activities recorded on a scale from 0 to 5.

sometimes inconspicuous, simple or branched; muriform conidia, ovate to obclavate, with tapering apex cell; porospores solitary or more often produced in acropetal succession to form simple or branched chains; darkly pigmented conidia with smooth-to-roughened texture.<sup>17</sup> Classic macroscopic morphology would describe the organism as obverse being olive green, velvety, slight aerial hyphae, and hyaline-to-colorless peripheral growth; reverse black color.<sup>18</sup>

Four of the 12 strains submitted had distinctive morphologic patterns that we divided into groups I, II, III, and IV (Table I). Representative strains of each group are shown in Fig. 1. Group I strains (188698, 188699, 188700, and 188701) most closely resembled the classic morphology described above. These four strains exhibited dark pigmentation on both media. Group II strains (AL 001, AL 002, and AL 005) deviated from the classic morphology by macroscopically having a more golden brown color on both media, with slightly more aerial hyphae. Group III strains (AL 003, ATCC 16086, and ATCC 46582) deviated from the classic morphology by macroscopically having little to no pigmentation on either medium. Group IV strains (CBS 103.33 and ATCC 11680) showed dark pigmentation on PDA but not on Sabouraud. Microscopically, strains from all four groups had typical dark colored, muriform conidia confirming their identity as *Alternaria*. There was significant reduction in the amount of sporulation in Group III, whereas groups I and II and IV had approximately equal amounts. Sectoring, which is the tendency of a single strain to mutate as it grows from the center of a culture plate, was observed in one strain (AL 003) grown on Sabouraud but not on PDA (Fig. 1).

### Enzyme activities

The activity of selected enzymes from the API-ZYM profile for the 12 strains is shown in Table II. The enzymes presented are those with the highest activities and greatest interstrain variability. Some enzymes as  $\alpha$ -glucuronidase were consistently present in all strains with approximately equivalent activity levels. Other enzymes, such as the protease leucine amino peptidase (LAP) and the glycosidases,  $\alpha$ -glucosidase ( $\alpha$ -GLU),  $\beta$ -galactosidase ( $\beta$ -GAL),  $\beta$ -glucuronidase ( $\beta$ -GLR), and  $\alpha$ -mannosidase ( $\alpha$ -MAN) showed a great deal of variability from strain to strain. The LAP activity tended to be greater in the CF than in the CA. Higher glycosidase activities tended to reside in the CA material. The greatest variability was seen with  $\beta$ -GAL, with activities ranging from none in ATCC 46582 to a high level in the 188698 strain.

Mean enzyme activities were highest in group I strains for alk phos,  $\alpha$ -GAL,  $\beta$ -GAL, and the CA of  $\beta$ -GLR. Group II strains had high activities of alk phos and  $\alpha$ -GLU but very little others. Group III strains displayed only alk phos and  $\alpha$ -GLU activities. The two group IV strains did not have unique enzyme patterns that distinguished them from the other *Alternaria* strain groups.

### Immunoblots

Fig. 2 shows the IgE-immunoblots of the CA from 12 *Alternaria* strains grown on defined medium and separated by SDS-PAGE under reducing conditions. Consistently recognized bands are seen at positions corresponding to molecular weights of 14, 36, 41, 45, 64, and 100 kd (arrows 1 through 6). At least two

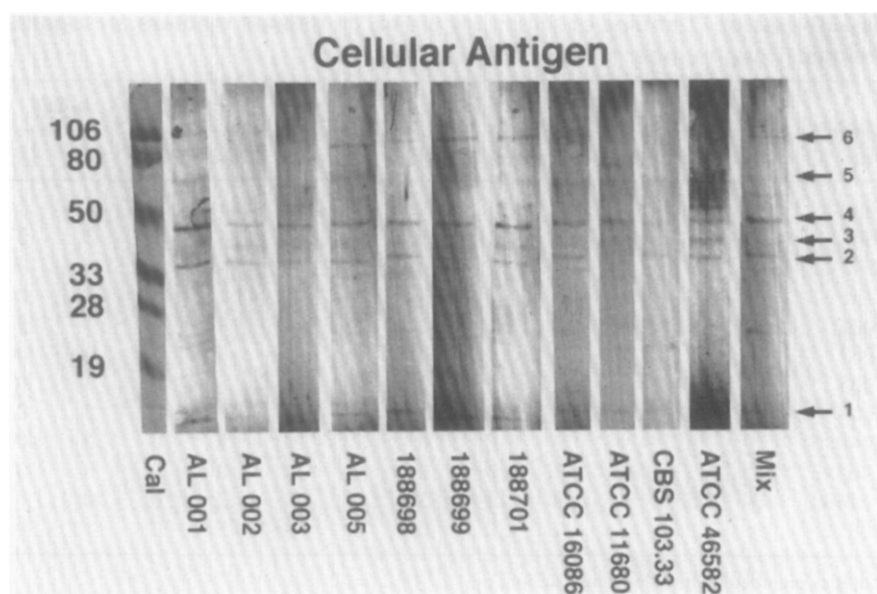


FIG. 2. IgE immunoblot of CAs grown on defined medium. Strain 188700 was identical to 188701.

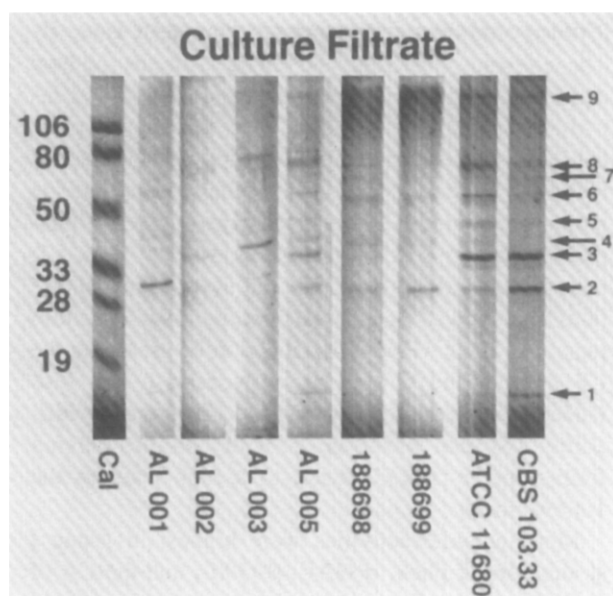


FIG. 3. IgE immunoblot of CFs grown on defined medium.

darkly stained allergen bands were seen in each of the strains. The 14 kd band was clearly seen in four strains: AL 001, AL 005, 188698, and 188699. The most consistent bands were seen at positions corresponding to arrows 2, 4, and 5.

The CFs grown in the same medium (Fig. 3) most consistently contained bands at 15, 31, 36, 41, 45, 55, 67, 74, and 132 kd (arrows 1 through 9). The CAs showed a fair amount of interstrain consistency,

whereas the CFs varied considerably. In particular, ATCC 11680 and CBS 103.33 showed the darkest 36 kd band, and the latter also showed a very dark 31 kd band. Two strains (AL 001 and 188698) contained only the 31 kd band in their CFs.

When the strains were grown in protein-rich medium (Fig. 4), the IgE-recognized patterns became far more complex. In particular, a greater amount of "background" was evident in all of the samples. A prominent 12 kd band was seen in three strains as were 67 and 70 kd bands. It was difficult to identify individual bands in the higher molecular weight regions for several strains (188699, 188700, 188701, ATCC 46582) because of the intense staining.

### Measurement of specific allergens

The concentrations of *Alt a* I and GP70 grown on protein-rich or protein-poor defined media is shown in Fig. 5. *Alt a* I was detectable in all of the strains. *Alternaria* grown in protein-rich medium had more of this allergen in the CA. Mold grown in protein-poor medium had less CA *Alt a* I and about the same amount in the CF. There was no consistent pattern of *Alt a* I concentrations by morphologic groupings. The highest concentration of *Alt a* I in CA was found in strain 188701, which is approximately 10 times higher than that of the lowest strains ATCC16086 and 188699. No correlation was apparent between the *Alt a* I content in the CA and CF materials. It was present primarily in the CF for AL 003 and in the CA for strains 188701 and ATCC 16086. The strain with the highest

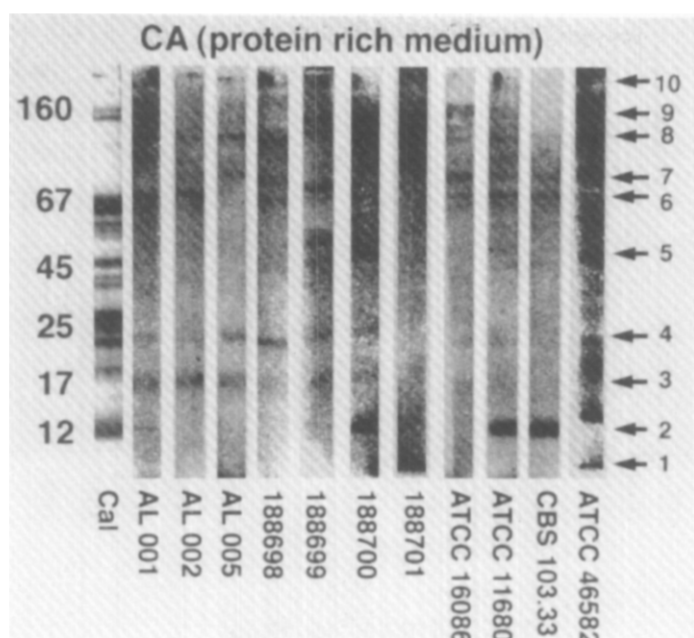


**FIG. 4.** IgE immunoblot of CAs grown in protein-rich medium. **AL 003 is not shown because it would not grow in protein-rich medium.**

*Alt a* I in the CF, ATCC 11680, contained approximately eight times the amount of the lowest, ATCC 46582.

When grown on the protein-rich media, only three of the strains (188698, 188700, and 188701) had substantial amounts of GP70 in the CA material. The CF of seven of the strains (the above, 188698, 188699, 188700, and 188701, AL 001, AL 002, and AL 005) contained large quantities of this allergen, suggesting that it is excreted into the medium during growth. The absolute amounts of GP70 per gram of dry weight, when present, were much greater in the protein-rich medium. Note that strain 188701 tended to produce large amounts of both *Alt a* I and GP70; whereas other strains are more selective, such as ATCC 46582, which produces only *Alt a* I. Also, the CA concentrations do not vary as much in the defined medium as they do in the protein-rich media, but there are detectable levels in more of the strains. The lower limit of detection for GP70 is approximately 10  $\mu$ g/gm dry weight. It is surprising to note that no apparent correlation existed between measured concentrations of *Alt a* I and GP70 and the appearance of their corresponding bands on the IgE immunoblots.

## DISCUSSION

Because of the complexity of *Alternaria* extracts, the multiplicity of its major allergens, and the extreme variability of allergen production between strains, it was our desire to identify a few groups of strains based

on morphology, enzyme patterns, IgE immunoblot, and concentrations of a few major allergens. This would ideally reduce the need to study numerous strains every time a characteristic of *Alternaria* is evaluated. Morphologically, the strains tended to divide into four major groups that corresponded to pigment production. Group I was closest to the classic description of *Alternaria*, and group III was the furthest from that description.

Strains of *Alternaria* that were assigned to group I (188698, 188699, 188700, 188701) tended to be more darkly pigmented and more stable on repeated culture. They also had similar enzyme profiles in that each had high amounts of  $\beta$ -GAL and  $\beta$ -GAL activities. Three of them also produced high amounts of GP70 in the CA fraction and intermediate amounts of *Alt a* I in protein-rich medium. Cellular antigen immunoblots of strains in this group showed more of the high molecular weight allergen and greater "background" when grown in protein-rich medium.

Group II strains (AL 001, AL 002, and AL 005) displayed intermediate amounts of pigmentation. Their enzyme activities tended to include increased LAP activity but were otherwise indistinct. These were among the highest producers of *Alt a* I in their CA's, and they each tended to secrete large amounts of GP70 into their CFs. Immunoblots of these strains showed the same bands (arrows 3 and 8 in Fig. 4); however, their patterns were quite different in Fig. 3.

Group III strains (AL 003, ATCC 16086, and ATCC

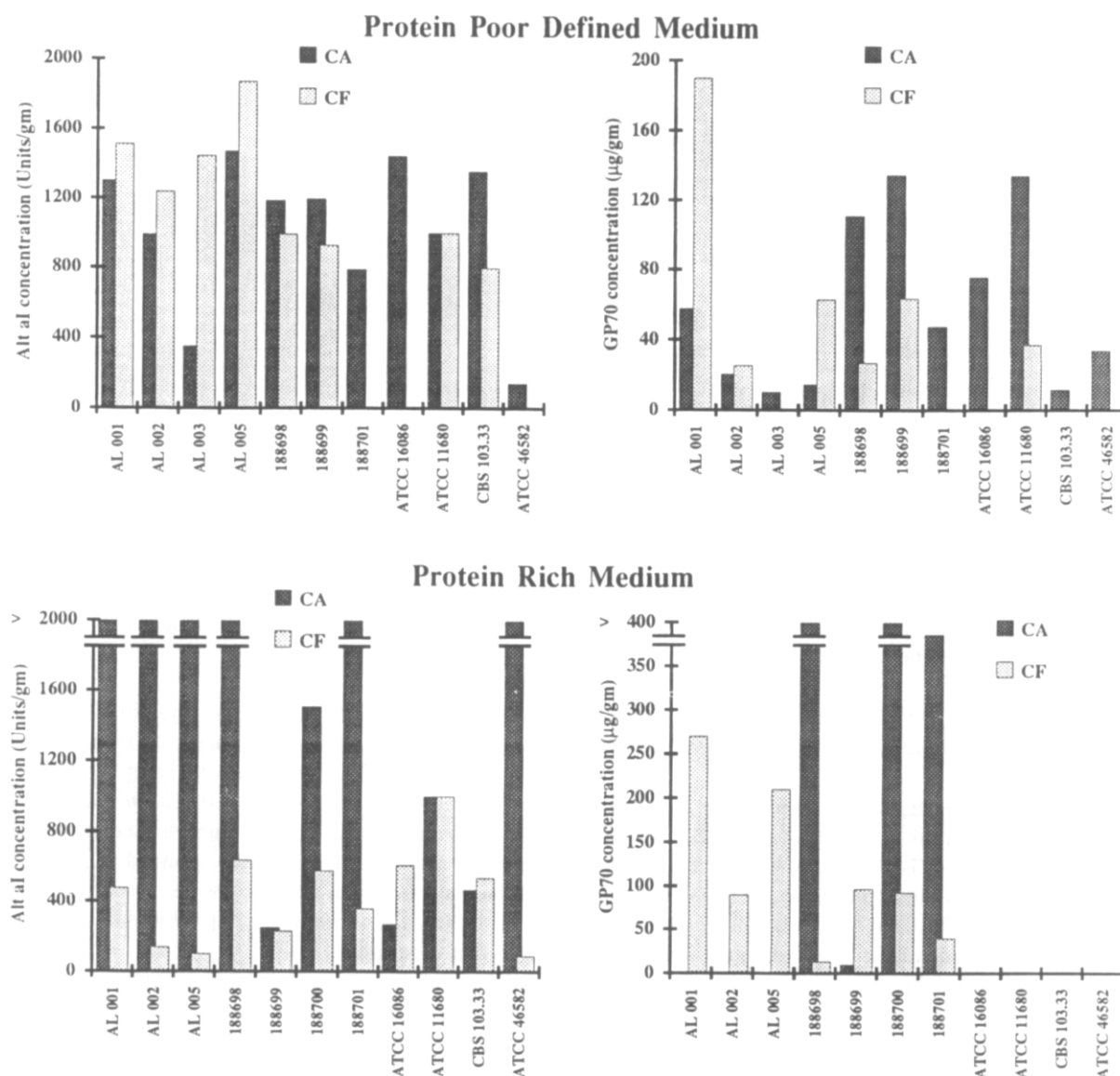


**FIG. 5.** *Alt a I* and GP70 concentrations in CA or CFs from different strains of *Alternaria* grown in protein-rich or protein-poor medium.

46582) were combined because they displayed very little pigmentation. Also, they tended to have very little LAP,  $\alpha$ -Gal,  $\beta$ -Gal,  $\beta$ -GLR, and  $\alpha$ -MAN activities. Their production of *Alt a I* and GP70 was extremely variable under each of the growth conditions; however, they did tend to produce very little GP70. On IgE immunoblot, the first two strains are quite similar; however, ATCC 46582 has a unique banding pattern that does not fit into any of the other groupings. This is particularly apparent in Fig. 2 where this strain produces a number of allergens around 70 kd and a large amount of an allergen at arrow 3.

The two group IV strains (ATCC 11680 and CBS 103.33) are uniquely identified by their tendency to produce darkly stained allergen at 12 kd (Fig. 4, arrow 2), and at 33 kd (Fig. 3, arrow 3). They also tend to produce very little GP70 and *Alt a I*. Their enzyme patterns are not distinguishable by any criteria. Morphologically they produce dark pigmentation on PDA and none on Sabouraud, which is unique.

Morphology and enzyme patterns did not seem to be associated with the specific allergen content for the *Alt a I*. The most likely reason is because this allergen is present in all of the strains. The measured *Alt a I*

concentrations do correlate with the content of low molecular weight allergens in the immunoblot. Strain 188701, which has the highest *Alt a* I concentration, displays intense staining in the low molecular weight range but no sharp bands are present. The four strains with the most clearly defined low molecular weight bands, AL 001, AL 002, 188700, and ATCC 46582, all showed moderate to high *Alt a* I content.

The significance of this grouping system is that with it, investigators may need only to study a few strains of *Alternaria alternata* to identify a characteristics of this mold. It is also notable that the strains within a particular group tended to come from a single investigator, that is, group I strains from Dr. Vijay, and so on. As a result, it is possible that information reported in the literature about *Alternaria* allergy from different laboratories might differ as a result of this difference in source materials. We also do not know whether every possible strain grouping of *Alternaria* has been identified. Had we included strains from other investigators in this research, further unique groups of *Alternaria alternata* might still be identified.

For standardization of *Alternaria* extracts, it may be sufficient to include representative strains from each of these groups, once they are all identified. Given the quantitative differences between them, it is clear that a single strain will not be adequate as a diagnostic or therapeutic representative of this species for this purpose. One could speculate that use of extracts prepared from a single strain of *Alternaria* for diagnosis of mold allergy will tend to select for patients who are sensitive to its allergens to the exclusion of patients who may be allergic only to components contained in others.

Access to defined groups of *Alternaria* strains should also make commercial production of purified allergens simpler. For example, if production of *Alt a* I is desired, certain strains such as 188701 grown in protein-rich medium should be chosen as the source material. In a similar manner, Dr. Paris determined that a particular strain of *Alternaria* grown under particular conditions produced a large amount of *Alt a* I.<sup>19</sup> For production of GP70, one should start with strains 188698, 188700, or 188701 and grow the mold in protein-rich medium. One group, for example, has been producing this allergen from a strain with relatively little GP70 in it (AL 003). A great deal of effort could have been avoided if we had known that the GP70 content of the other strains was higher.

For this study we did not attempt to exhaustively survey all available strains of *Alternaria*. There are, for example, 54 strains of *Alternaria alternata* listed in the 1991 ATCC catalogue of filamentous fungi.

Complete analysis of all of them would have been beyond the scope of this investigation. By selecting those strains used by prominent investigators in the field, we have begun the work of defining strain groupings. As further strains are evaluated, they will undoubtedly fall either into one of the previously described groups or they will define new ones. It is our hope that further work in this area will result in a more complete description of allergenic fungi from the genus *Alternaria*.

## CONCLUSIONS

*Alternaria alternata* is a complex species that exhibits significant interstrain differences in morphology, enzyme profile, and allergen content when grown under various conditions. Because a number of investigators are involved in studying allergy related to this fungus, it is critical that a consistent supply of source materials be used by each group. To reduce the need for examining numerous strains, we have attempted to identify a few groups of strains so that "representative" members from each group can be used for mold research. It is our intent that with standardization of source materials, progress in *Alternaria* allergen research can proceed in a more systematic manner than has been possible in the past. Given the significant quantitative and qualitative differences in allergen content among the groups, *Alternaria* extracts should not be considered to be interchangeable until classification of the source materials is made.

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