

# Immunoprint analysis of *Calvatia cyathiformis* allergens

## I. Reactivity with individual sera\*

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*Basidiomycete allergens have received scant attention to date, compared to allergens from deuteromycetes. This is true even though in previous studies, 32% of atopic patients with respiratory allergies were skin test reactive to basidiospore extracts. Since sufficient quantities of Calvatia cyathiformis spores were available, their allergens were sequentially fractionated by gel filtration (GF) and hydrophobic interaction chromatography (HIC). Unfractionated extract (crude), GF, and HIC fractions were electrofocused in polyacrylamide gel (pH 3.5 to 9.5) and immunoprinted onto CNBr-activated nitrocellulose (0.2  $\mu$ m) filters. Sera from 19 skin prick test positive and 10 negative subjects, with comparable total IgE levels, were used to screen individual strips of each blot. Blots were reacted with  $^{125}$ I-labeled anti-IgE and analyzed by autoradiography. IgE from 79%, 89%, and 89% of the positive sera bound to crude extract, GF, and HIC, respectively; IgE from none of the negative sera bound to crude extract. A series of bands (pH 3.6 to 4.6) reacted with 63%, one band (pH 6.6) reacted with 68%, and one band (pH 9.3) reacted with 63% of the sera tested. These studies demonstrate at least three important groups of allergens in C. cyathiformis spores. This characterization allows assignment of initial C. cyathiformis allergen designations and development of a purification protocol. (J ALLERGY CLIN IMMUNOL 1989;83:784-92.)*

The subdivision Basidiomycotina (basidiomycetes) is the most conspicuous and familiar group of fungi and includes mushrooms and puffballs. Furthermore, they occur in such a variety of habitats that most all humans live in the vicinity of basidiomycetes. Since most basidiomycetes produce prolific numbers of airborne spores, most human environs presumably include basidiospores.

Air-sampling data demonstrate that basidiospores are prevalent in the air spora in many regions of the world<sup>1-6</sup> and have been demonstrated to be aller-

### Abbreviations used

|      |                                        |
|------|----------------------------------------|
| HIC: | Hydrophobic interaction chromatography |
| GF:  | Gel filtration                         |
| UV:  | Ultraviolet                            |
| PBS: | Phosphate-buffered saline              |
| pI:  | Isoelectric point                      |
| IEF: | Isoelectric focusing                   |

genic.<sup>6-12</sup> The allergenic components of basidiospores are, however, incompletely characterized. The present study extended the characterization of spore allergens from one basidiomycete.

Basidiospore extracts of *Calvatia cyathiformis* (Bosc) Morg. (known also as the lilac puffball and cup-shaped puffball) previously have been demonstrated to bind specific IgE from a pool of sera from skin test reactive, atopic individuals.<sup>13</sup> The present study was undertaken to characterize further the spore allergens of *C. cyathiformis*. Specifically, the purposes were (1) to determine which are the major allergens, as determined by reactivity with individual sera, (2) what is the pI of the major allergens, and

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(3) whether the same allergens are detected by immunoprint analysis in the different purification steps described earlier.<sup>13</sup>

## MATERIAL AND METHODS

### Patient population and skin testing

Volunteers with a personal history of atopic respiratory disease were skin prick tested with 12 common inhalant allergens (Greer Laboratories, Lenoir, N.C., or Hollister-Stier Laboratories, Spokane, Wash.) to confirm the presence of respiratory allergies. *C. cyathiformis* skin test reagents were prepared by resuspending lyophilized material in PBS. These solutions were passed through a 0.45  $\mu$ m filter and then diluted 1:1 with sterile glycerol to final concentrations of 10, 1, 0.1, and 0.01 mg/ml. Results were recorded at 20 minutes and considered positive if the mean wheal diameter was 2 mm greater than the negative control (PBS). Histamine diphosphate at 1 mg/ml was used as the positive control to demonstrate skin reactivity.

Sera were obtained from patients skin test reactive to *C. cyathiformis* for RAST and for immunoprinting. This panel of 19 sera is referred to as positive sera. Positive serum No. 20 is a pool of equivalent volumes of sera Nos. 4 to 8. Negative control sera were obtained from volunteers that were skin test reactive to at least four common inhalants, but not reactive to *C. cyathiformis*. This panel is referred to as negative sera.

### Spore collection and extraction

Basidiocarps (puffballs) were collected fresh in the Gulf South area and allowed to dry in the laboratory for 2 to 3 days. The spore mass was then removed from the interior of the puffball with a spatula, passed through a kitchen strainer, and stored in glass jars until it was used. Material thus obtained was about 85% spores, as determined by light microscopy, with the remainder being capillitium (sterile hyphae). Spores were defatted with ether (200 ml/gm of spores) before extraction. Defatted spores were allowed to air dry overnight and then were homogenized in 0.125 mol/L of  $\text{NH}_4\text{HCO}_3$  at pH 8.1 for 2 minutes in a Braun (B. Braun Melsungen, Melsungen, West Germany) homogenizer cooled with escaping  $\text{CO}_2$  gas from a cylinder of liquid  $\text{CO}_2$ . The homogenized suspension was centrifuged for 30 minutes at 80,000 g, and the supernatant was lyophilized. Lyophilized crude extract was stored under desiccation until it was used.

### Chromatography

Sephadex G-75 GF and HIC with phenyl-Sepharose were performed as previously described.<sup>13</sup> Briefly, GF was conducted in a 5 by 100 cm column with Sephadex G-75 in 0.125 mol/L of  $\text{NH}_4\text{HCO}_3$  (pH 8.1) at 4° C. Sample was applied in 10 to 15 ml volume. Ten milliliter fractions were collected, and UV absorbance at 280 nm was recorded. Fraction pools were established to include peaks and interpeak areas of the UV absorbance curve. Pools were lyophilized and stored under desiccation until they were used.

HIC was conducted with the RAST-active Sephadex

G-75 pool. Lyophilized material from this pool was resuspended in 0.05 mol/L of Tris buffer, pH 7.4, with 4 mol/L of NaCl (Tris per 4 mol/L) for application to the column. Elution with Tris per 4 mol/L was continued until the UV absorbance (280 nm) returned to baseline. Further stepwise elutions were made in a similar fashion with Tris per 1 mol/L of NaCl, water, and finally with 20% (vol/vol) ethylene glycol. HIC pools were dialyzed exhaustively against 0.05 mol/L of  $\text{NH}_4\text{HCO}_3$  at 4° C and lyophilized.

### RAST and total serum IgE determinations

RAST was performed as previously described.<sup>13</sup> CNBr-activated paper disks (Whatman No. 1, Whatman, Inc., Clifton, N.J.) were coated with antigen resuspended in 0.1 mol/L of borate buffer (pH 8.0) at 20 mg/ml. Duplicate disks were then incubated overnight in 100  $\mu$ l of serum and were washed free of unreacted serum with three changes (2 ml) of saline (0.9% NaCl) before <sup>125</sup>I-labeled anti-IgE (approximately 25,000 cpm) (Kallestad Laboratories, Austin, Texas) was added. The free-labeled antibody was washed off with three changes of saline, and residual counts were determined in a Beckman model 5500 gamma counter (Beckman Instruments, Inc., Irvine, Calif.).

RAST results were expressed as the percent of total label added that was bound to the disks. A positive RAST was considered to be >3 SD above the mean of the negative panel (mean, 0.96; SD, 0.334). Thus, values >2% bound were considered a positive RAST reaction.

Total serum IgE determinations were conducted in duplicate with Quanticlone kits (Kallestad Laboratories) according to the manufacturer's recommendations.

### IEF

Protein samples were focused in thin-layer polyacrylamide gels (5%T/3%C) according to the manufacturer's recommendations for power limits and total time (Pagplates, LKB, Bromma, Sweden). Samples for immunoprinting and for staining were run in duplicate on adjacent portions of the same gel. Portions of gels to be stained were first fixed in a solution of 3.5% sulfosalicylic acid and 11.5% trichloroacetic acid. Fixed gels were equilibrated in staining solvent (3:1:9 methanol:acetic acid:water) for 20 minutes and then stained in 0.1% Coomassie brilliant blue R 250 in staining solvent at 60° C (initially) for 20 minutes. Gels were destained with washes of staining solvent.

### Immunoprinting

Focused proteins were blotted by passive transfer to 0.2  $\mu$ m pore size nitrocellulose membranes previously activated with cyanogen bromide by the method of Demeulemester et al.<sup>14</sup> After nitrocellulose membranes were blotted, they were removed from the gel by soaking in 50% glycerol in 0.1 mol/L of phosphate buffer at pH 7.5. Stockblocking (quenching) buffer was prepared with 0.2 mol/L phosphate buffer (pH 7.5) with 2% Tween 20 and 5% bovine serum albumin. This stockblocking buffer was diluted 1:5 with ion-depleted water for a 1-hour incubation of the blotted

**TABLE 1.** Characterization of sera from subjects used to characterize *C. cyathiformis* spore allergens

| Subject   | ST† | Total IgE‡ | RAST* |      |      |     |
|-----------|-----|------------|-------|------|------|-----|
|           |     |            | Crude | GF   | HIC  | HSA |
| 1         | +   | >1000      | 3.6   | 3.3  | 3.7  | 1.3 |
| 2         | +   | 950        | 5.6   | 10.0 | 9.7  | 1.6 |
| 3         | +   | 630        | 26.0  | 47.9 | 17.2 | 1.1 |
| 4         | +   | 80         | 10.3  | 23.1 | 3.5  | 0.6 |
| 5         | +   | 370        | 15.5  | 12.8 | 4.6  | 0.7 |
| 6         | +   | 630        | 14.7  | 24.7 | 11.0 | 0.9 |
| 7         | +   | 270        | 4.0   | 8.0  | 3.2  | 1.2 |
| 8         | +   | >1000      | 13.7  | 28.8 | 13.6 | 2.1 |
| 9         | +   | 630        | 29.8  | 47.6 | 17.6 | 1.3 |
| 10        | +   | 180        | 6.2   | 16.6 | 6.5  | 0.6 |
| 11        | +   | 54         | 3.0   | 6.6  | 1.9  | 5.7 |
| 12        | +   | 80         | 3.6   | 8.5  | 1.2  | 0.6 |
| 13        | +   | 280        | 2.7   | 1.3  | 1.2  | 0.6 |
| 14        | +   | >1000      | 2.5   | 3.6  | 2.8  | 1.0 |
| 15        | +   | 190        | 0.9   | 0.9  | 1.0  | 0.5 |
| 16        | +   | 360        | 2.6   | 3.4  | 1.5  | 0.9 |
| 17        | +   | 190        | 2.3   | 4.6  | 1.2  | 0.7 |
| 18        | +   | 850        | 3.5   | 3.2  | 4.0  | 1.3 |
| 19        | +   | 106        | 0.8   | 0.8  | 0.8  | 0.6 |
| 20 (pool) | NA§ | 630        | 12.9  | 25.1 | 9.2  | 1.2 |
| 21        | —   | 260        | 0.8   | NA   |      | 0.5 |
| 22        | —   | 50         | 0.7   |      |      | 0.5 |
| 23        | —   | 250        | 1.0   |      |      | 0.6 |
| 24        | —   | 33         | 0.4   |      |      | 0.5 |
| 25        | —   | 600        | 1.1   |      |      | 1.2 |
| 26        | —   | 950        | 0.9   |      |      | 1.8 |
| 27        | —   | 740        | 0.9   |      |      | 0.9 |
| 28        | —   | 1000       | 1.1   |      |      | 0.7 |
| 29        | —   | 1000       | 1.0   |      |      | 0.5 |
| 30        | —   | 320        | 1.7   |      |      | 0.9 |

\*RAST values are percent bound of total activity added. Disks were coupled with crude extract, RAST-active GF fraction, RAST-inhibiting HIC fraction, or human serum albumin.

†Skin prick test.

‡Values are international units per milliliter.

§Number 20 is a pool of equal volumes of Nos. 4 to 8.

||Sera 21 to 30 were only tested by RAST versus crude extract and human serum albumin.

nitrocellulose after removal from the gel. Stockblocking buffer was diluted 1:2 to dilute serum or radiolabeled anti-IgE to working strength. Strips were incubated overnight sequentially in serum and radiolabeled anti-IgE. Radiolabel concentration was adjusted to approximately 100,000 cpm per strip. Three washes in PBS with 0.05% Tween 20 followed each step.

The positions of pI standards (Sigma Chemical Co., St. Louis, Mo.) used on each gel were recorded relative to the anodal wick. Distance from the wick was regressed on pI, and the regression equation was used to demarcate pH intervals. The number of radiostained bands per interval

were recorded for each serum and compiled into isoelectric distribution tables (allergograms) for each of the three blotted antigen preparations (crude extract, GF fraction, and HIC fraction).

Membranes were blotted with crude extract and overlaid with the positive sera pool to determine reactivity with other immunoglobulin classes. These membranes were subsequently incubated with affinity-purified, peroxidase-labeled antibody conjugates (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) to detect bound IgA, IgG, and IgE. Bound antibodies were visualized with the chromogen 2,4-diaminobenzidine. Serum dilutions used were 1:4 for IgE,

**TABLE II.** Isoelectric distribution of allergenic protein bands from *C. cyathiformis* crude spore extract

| Subject | pH Zones     |              |              |              |              |              |              |              |              |              |              |      |
|---------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|------|
|         | >3.5<br>-4.0 | >4.0<br>-4.5 | >4.5<br>-5.0 | >5.0<br>-5.5 | >5.5<br>-6.0 | >6.0<br>-6.5 | >6.5<br>-7.0 | >7.0<br>-7.5 | >7.5<br>-8.0 | >8.0<br>-8.5 | >8.5<br>-9.0 | >9.0 |
| 1       |              | x*           | x            |              |              |              | x            |              |              |              |              |      |
| 2       |              | x            | 2            |              |              |              |              |              |              |              |              |      |
| 3       |              |              | x            | x            | 2            | 3            | 2            | x            | 2            |              |              | x    |
| 4       |              |              |              |              |              |              | x            |              |              |              |              | x    |
| 5       |              |              |              |              |              |              | x            |              |              |              |              | x    |
| 6       |              | 3            | x            |              | x            |              | x            |              |              |              |              | x    |
| 7       |              |              |              |              |              |              | x            |              |              |              |              | x    |
| 8       |              | x            |              |              | x            | x            | x            |              |              |              |              | x    |
| 9       | x            |              |              | x            | 2            | x            | 2            | x            | x            |              |              | x    |
| 10      |              |              |              |              |              |              | x            |              |              |              |              | x    |
| 11      |              | x            |              |              |              |              | x            |              |              |              |              | x    |
| 12      |              |              |              |              |              |              |              |              |              |              |              | x    |
| 13      |              | x            | 2            |              |              |              |              |              |              |              |              |      |
| 14      | x            | x            |              |              |              |              |              |              |              |              |              |      |
| 15      |              |              |              |              |              |              |              |              |              |              |              |      |
| 16      |              |              |              |              |              |              |              |              |              |              |              | x    |
| 17      |              |              |              |              |              |              |              |              |              |              |              |      |
| 18      |              |              |              |              |              |              |              |              |              |              |              |      |
| 19      |              |              |              |              |              |              |              |              |              |              |              |      |
| 20      |              | 3            | x            |              | 2            |              | x            |              |              |              |              | x    |
|         | 11%†         | 37%          | 26%          | 11%          | 21%          | 16%          | 53%          | 11%          | 11%          | 0%           | 0%           | 58%  |

\* A single band in a zone is indicated by x; more than a single band is indicated by a numeral.

†Percentage of subjects (1 to 19) reactive to bands in this pH zone.

1:64 for IgA, and 1:1024 for IgG. Antibody peroxidase conjugates were diluted 1:500 for anti-IgE and 1:1000 for anti-IgG and anti-IgA.

### Immunoprint inhibition

Nitrocellulose membranes were blotted with crude spore extract after IEF. Reaction with the positive sera pool (diluted 1:8) was inhibited with an equivalent volume of resuspended crude extract yielding a 1:16 final concentration of pooled sera. Resuspended extract concentrations were adjusted to 1.0, 0.1, and 0.01 mg/ml for inhibitions. Subsequent washes and labeling were done as above.

### RESULTS

Two panels of sera samples were assembled with generally comparable levels of total IgE. One panel was from subjects skin test positive, and the other panel was from subjects skin test negative to *C. cyathiformis* (Table I). Seventeen of 19 positive sera were RAST positive to at least one *C. cyathiformis* extract or fraction (Table I). For most sera, the GF fraction had the greatest RAST activity. None of the

negative sera reacted with crude *C. cyathiformis* by RAST or by immunoprinting.

In immunoprint analysis, crude spore extract bound IgE from 15 of 19 (79%) individual positive sera tested (Table II). The number of bands detected by autoradiography varied from 0 to 13 among individual positive sera (Fig. 1).

The GF fraction bound IgE from 17 of 19 (89%) positive sera (Table III). The number of bands detected varied from 0 to 21 among individual positive sera (Fig. 2).

The HIC fraction bound IgE from 17 of 19 (89%) positive sera (Table IV). The number of bands detected varied from 0 to 16 among individual positive sera (Fig. 3).

The two skin test-positive sera that were RAST negative were the only two skin test-positive sera, Nos. 15 and 19, that failed to bind any bands from any of the three immunoprint analyses.

Three allergens were found in *C. cyathiformis* spore extracts that reacted with a majority of positive sera

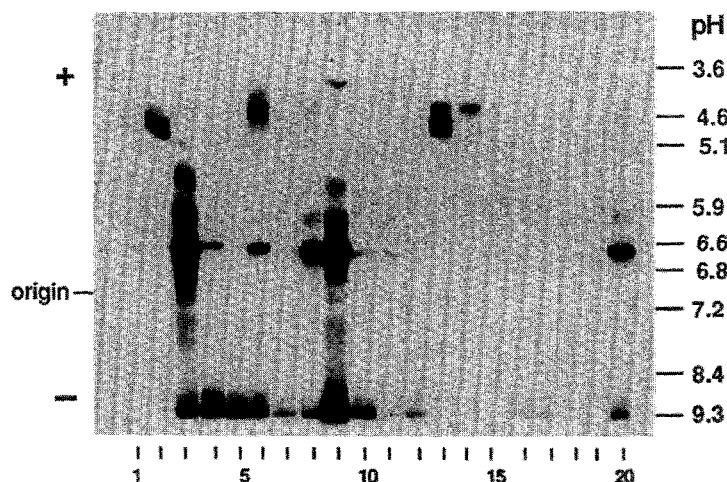


FIG. 1. Immunoprinting of *C. cyathiformis* crude spore allergens with 19 individual sera (skin test positive) and a positive pool. Origin indicates sample application point. Lane numbers at bottom correspond to subject number in Table I.

TABLE III. Isoelectric distribution of allergenic protein bands from *C. cyathiformis* GF fraction

| Subject | pH Zones     |              |              |              |              |              |              |              |              |              |              |      |
|---------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|------|
|         | >3.5<br>-4.0 | >4.0<br>-4.5 | >4.5<br>-5.0 | >5.0<br>-5.5 | >5.5<br>-6.0 | >6.0<br>-6.5 | >6.5<br>-7.0 | >7.0<br>-7.5 | >7.5<br>-8.0 | >8.0<br>-8.5 | >8.5<br>-9.0 | >9.0 |
| 1       | x*           | x            | x            |              | x            |              | x            |              |              |              |              |      |
| 2       | x            | x            | 3            |              | 3            |              |              |              |              |              |              |      |
| 3       |              | x            | x            | 2            | 3            | 2            | 4            | 4            | 3            | 2            |              | x    |
| 4       |              |              |              |              | x            | x            | x            |              |              |              |              | x    |
| 5       |              |              |              |              | x            | 2            | x            |              |              |              | x            | x    |
| 6       |              | x            | x            | 2            | x            | 2            | x            |              |              | x            |              | x    |
| 7       |              |              |              |              |              |              | x            |              |              |              |              | x    |
| 8       |              | x            | 3            |              | x            | 2            | x            |              |              |              |              | x    |
| 9       |              | 3            |              | x            | x            | 2            | 4            | 4            | x            | x            |              | x    |
| 10      |              | 2            |              |              |              | x            | x            |              |              |              |              | x    |
| 11      |              | 2            |              |              |              | x            | x            |              |              |              |              | x    |
| 12      |              | x            |              |              |              |              | x            |              |              |              |              | x    |
| 13      |              | 2            |              |              |              |              |              |              |              |              |              |      |
| 14      |              | 2            | x            |              |              |              |              |              |              |              |              |      |
| 15      |              |              |              |              |              |              |              |              |              |              |              |      |
| 16      |              |              |              |              | x            |              | x            |              |              |              |              | x    |
| 17      |              |              |              |              |              |              | x            |              |              |              |              | x    |
| 18      |              |              |              |              | x            |              |              |              |              |              |              |      |
| 19      |              |              |              |              |              |              |              |              |              |              |              |      |
| 20      |              | x            | x            |              | x            | 2            | x            |              |              |              |              | x    |
|         | 11%†         | 58%          | 32%          | 16%          | 53%          | 42%          | 68%          | 11%          | 11%          | 21%          | 5%           | 63%  |

\*A single band in a zone is indicated by x; more than a single band is indicated by a numeral.

†Percentage of subjects (1 to 19) reactive to bands in this pH zone.

in at least two of the immunoprint analyses (Table V). These allergens were a series of bands focusing between pH 3.6 to 4.6, a band which focused at pH 6.6, and another band at pH 9.3. Reactivity of positive

test sera versus these three allergens were 63%, 68%, and 63%, respectively, based on all three blots (crude extract, GF fraction, and HIC fraction).

Two other pH zones contained allergens that bound

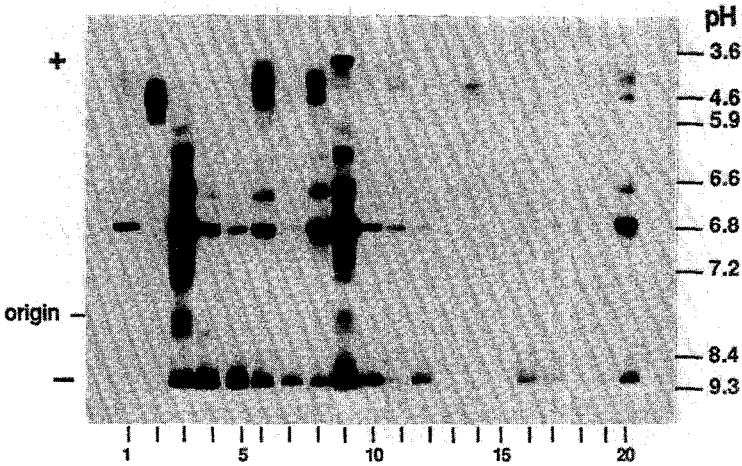


**FIG. 2.** Immunoprinting of *C. cyathiformis* GF spore allergens with 19 individual sera (skin test positive) and a positive pool. Origin indicates sample application point, Lane numbers at bottom correspond to subject number in Table I.

**TABLE IV.** Isoelectric distribution of allergenic protein bands from *C. cyathiformis* HIC fraction

| Subject | pH Zones |      |      |      |      |      |      |      |      |      |      |      |
|---------|----------|------|------|------|------|------|------|------|------|------|------|------|
|         | >3.5     | >4.0 | >4.5 | >5.0 | >5.5 | >6.0 | >6.5 | >7.0 | >7.5 | >8.0 | >8.5 | >9.0 |
|         | -4.0     | -4.5 | -5.0 | -5.5 | -6.0 | -6.5 | -7.0 | -7.5 | -8.0 | -8.5 | -9.0 |      |
| 1       | x*       | 2    | x    |      |      |      |      |      |      |      |      |      |
| 2       |          | 2    | x    | 2    |      |      |      |      |      |      |      |      |
| 3       | 2        | x    | x    | 2    | 2    | x    | x    | x    | x    | x    | 2    | x    |
| 4       |          |      |      |      |      | 2    | x    |      |      |      | x    | x    |
| 5       |          | x    | x    |      |      |      | x    |      |      |      | x    | x    |
| 6       |          | 2    | x    |      |      | x    | x    |      |      |      | x    | x    |
| 7       |          | 2    | x    |      |      |      | x    |      |      |      |      | x    |
| 8       |          | 2    | x    | x    |      | 2    | x    |      |      |      |      | x    |
| 9       |          | x    | x    | x    | x    | 2    | x    |      | x    |      | x    | x    |
| 10      |          | x    | 2    |      |      | x    | x    | x    |      |      |      | x    |
| 11      |          | x    | 2    |      |      |      | x    |      |      |      |      | x    |
| 12      |          | x    | x    |      |      |      |      |      |      |      |      | x    |
| 13      |          | x    | x    |      |      |      |      |      |      |      |      |      |
| 14      |          |      | 2    | x    | x    |      |      |      |      |      |      |      |
| 15      |          |      |      |      |      |      |      |      |      |      |      |      |
| 16      |          |      |      |      |      |      |      |      |      |      | x    | x    |
| 17      |          |      |      |      |      |      |      |      |      |      | x    |      |
| 18      |          |      | x    | x    |      |      |      |      |      |      |      |      |
| 19      |          |      |      |      |      |      |      |      |      |      |      |      |
| 20      |          |      | 2    | 3    |      | 2    | x    |      |      |      | x    | x    |
|         | 11%†     | 63%  | 74%  | 32%  | 16%  | 32%  | 47%  | 11%  | 11%  | 5%   | 37%  | 58%  |

\*A single band in a zone is indicated by x; more than a single band is indicated by a numeral.  
†Percentage of subjects (1 to 19) reactive to bands in this pH zone.

IgE from a majority of positive sera in one of the immunoprints. These were pH 4.5 to 5.0 in the HIC blot, and 5.5 to 6.0 in the GF blot (Table V).

The bands with pI 3.6 to 4.6 from crude extract, GF fraction, and HIC fraction bound specific IgE from 37%, 58%, and 63%, respectively, of the positive test sera. The pI 6.6 band from crude extract, GF fraction, and HIC fraction bound IgE from 53%, 68%, and 47% of the test sera, respectively. The pI 9.3 band from crude extract, GF fraction, and HIC fraction

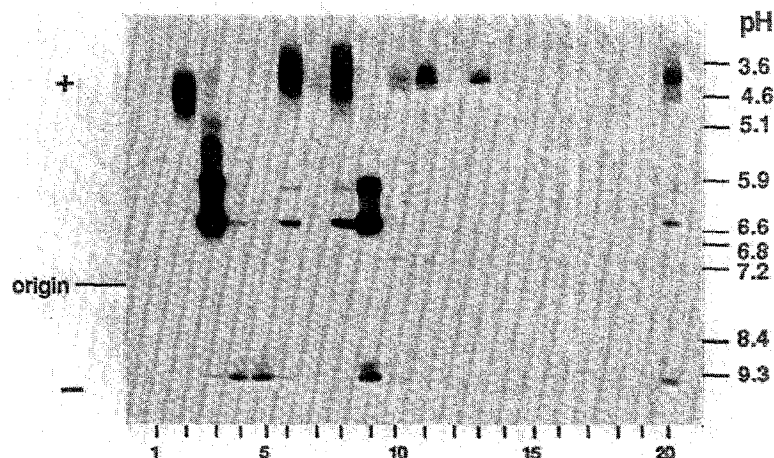


FIG. 3. Immunoprinting of *C. cyathiformis* HIC spore allergens with 19 individual sera (skin test positive) and a positive pool. Origin indicates sample application point. Lane numbers at bottom correspond to subject number in Table I.

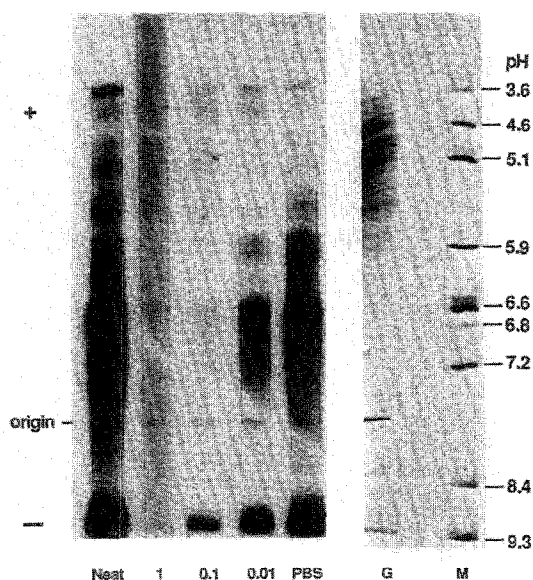


FIG. 4. Immunoprint inhibition of crude *C. cyathiformis* spore allergens. Immunoprinted nitrocellulose strips were reacted with a serum pool, and the reaction was inhibited with crude spore extract (1:1, vol:vol) at the indicated concentration. A strip incubated with serum without inhibition (neat) and with PBS as the inhibitor (dilution control) were included. To the right is a section of the gel from which the blots were made with focused extract (G) and the calibration markers (M).

reacted with 58%, 63%, and 58% of the test sera, respectively.

The specificity of the IgE binding/radiostaining reaction was demonstrated by the blot-inhibition activity at 0.1 mg/ml (Fig. 4). Although the inhibition was not complete at this concentration, the higher concentration, 1.0 mg/ml, demonstrated irreversible and

TABLE V. Percentage of subjects reactive to *C. cyathiformis* crude spore extract and fractions in selected pH zones

| Source | pH Zones     |              |              |              |      |
|--------|--------------|--------------|--------------|--------------|------|
|        | >4.0<br>-4.5 | >4.5<br>-5.0 | >5.5<br>-6.0 | >6.5<br>-7.0 | >9.0 |
| Crude  | 37           | 26           | 21           | 53           | 58   |
| GF     | 58           | 32           | 53           | 68           | 63   |
| HIC    | 63           | 74           | 16           | 47           | 58   |

nonspecific binding of the radiolabel to the nitrocellulose. Incubation of serum with inhibitor solution at concentrations  $\leq 0.01$  mg/ml resulted in radiostaining comparable to the PBS control (Horner WE. Unpublished data).

Peroxidase labeling indicated that components of crude extract bound IgE, IgG, and IgA (Fig. 5). Comparison of IgE binding patterns revealed by the peroxidase and the isotope-labeled antibodies demonstrated good agreement between the two probes, as illustrated in lanes I and E in Fig. 5. A total of nine bands bound antibodies from more than one class. Bands with pI 3.8, 4.3, two bands very close to pI 5.9, and bands with pI 6.6 and 9.3 bound both IgG and IgE. A band with pI 5.1 bound both IgA and IgG, a band with pI 5.2 bound IgA and IgE, and a band with pI 5.5 bound antibodies from all three classes.

## DISCUSSION

We considered any allergen to be important that reacted with a majority of positive sera in at least two of the immunoprint analyses (crude extract, GF frac-

tion, and HIC fraction). Three allergens present in *C. cyathiformis* spores met this criterion and should be considered important. Even without full characterization, we believe that the reproducibility of these assays allows designation of two allergens from *C. cyathiformis*.

Since the spore-extract components were resolved by their pI, and in accordance with the recommendations of the committee of the World Health Organization on allergen nomenclature,<sup>15</sup> these preliminary designations are based on the pI of the IgE binding bands. Thus, *Cal c* Bd6.6 is the allergen with pI 6.6 and *Cal c* Bd9.3 is the allergen with pI 9.3.

The classification of a third important allergen, or group of allergens, with pI values between 3.6 to 4.6, is more complex. The wide pH range of these separations (pH 3.5 to 9.5) prevented adequate resolution over the narrow pH range in which these bands occurred. Further elucidation of the IgE binding components in this pH range is advised before conferring allergen designations. These bands are therefore referred to simply as Bds 3.6 to 4.6.

Two other allergens, or allergen groups, reacted with a majority of positive sera in at least one immunoprint analysis. These were in the pH range >4.5 to 5.0 in the HIC fraction immunoprint and in the pH range >5.5 to 6.0 in the GF fraction immunoprint. We believe that the presence of additional important allergens is thus indicated, but, as above, increased resolution is required before characterization is sufficient for allergen designations.

The loss of reactivity in the blot inhibition indicates that the reactions observed in these immunoprint analyses are antibody-specific binding reactions. This was confirmed by negative results from the RAST and immunoprint incubations in atopic, skin test-negative sera.

Two sera in our positive panel, Nos. 15 and 19, were skin test positive and yet RAST and immunoprint negative. This may indicate false positive skin tests or that the skin test was more sensitive than the in vitro assays. The possibility of IgG involvement remains unknown since IgG reactivity was not assessed for individual sera.

The scheme used to partially purify these allergens, with GF and HIC enhanced the activity of the Bds3.6 to 4.6 allergen (group) more than the activity of *Cal c* Bd6.6 or *Cal c* Bd9.3. This can especially be observed from the summary data in Table V in which the percentage of positive reactors for Bds3.6 to 4.6 increases with each purification step. Thus, the HIC procedure may prove especially valuable in attempts to purify this (group of) allergen(s).

A comparison of the binding patterns yielded by

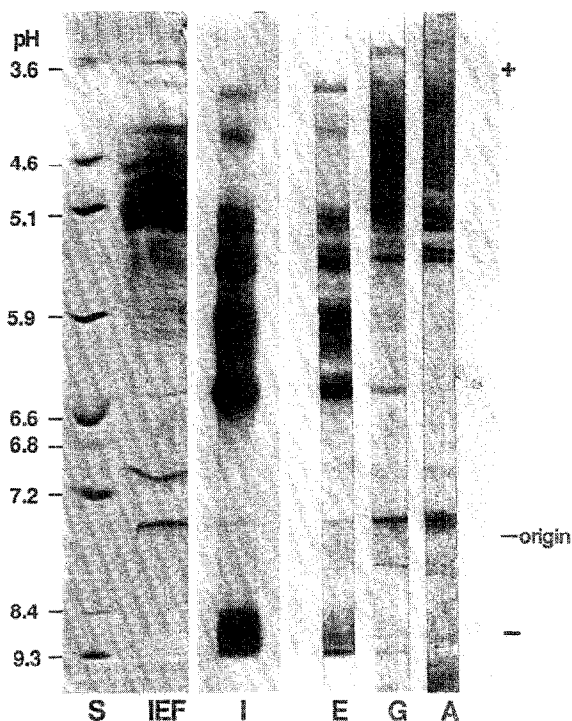


FIG. 5. Binding of IgG, IgE, and IgA from a serum pool to blots of crude *C. cyathiformis* spore extract after IEF. S, lane of the gel with pI standards; IEF, section of the gel from which blots were made; I, blot with bound IgE detected by autoradiography; E, G, and A, blots with bound IgE, IgG, and IgA detected by peroxidase-labeled antibodies.

the radiolabeled anti-IgE and the peroxidase-labeled anti-IgE revealed good agreement in number and position of bands between the two detection reagents. The radiolabeled antibody appeared to be more sensitive, but the peroxidase-labeled antibody produced more discrete banding patterns.

The present study demonstrates that *C. cyathiformis* spore allergens also induce IgG responses (seven common bands), and to a lesser degree, IgA responses (two common bands). The antibodies conjugated to the peroxidase in this study were not specific to any of the IgG subclasses. Thus, we are not able to address whether IgG<sub>4</sub> is involved in the IgG response to *C. cyathiformis* spore allergens.

Data in Table I reveal that the sera panels used in this study were generally comparable in terms of total IgE. Both panels were selected from a sera bank obtained from individuals with a history of respiratory allergy and who were skin test reactive to at least four common inhalant allergens, for example, pollens, animal danders, or other fungal spore allergens. The RAST data indicate that the GF fraction yields the highest activity in most cases. The GF fraction is

probably more active than crude extract as a result of removal of inert material during the GF. The reduction of activity in the subsequent purification step (HIC) may be due to removal by fractionation of some of the allergens or reduced stability of some or all of the allergens in the more purified state. The presence of all major bands in the blots in Figs. 2 to 4 (autoradiographs of crude extract, GF fraction, and HIC fraction) argue against the former. Further work is continuing regarding this question.

Although not all the positive sera tested reacted with one of the three important allergens, IgE in all strongly reacting sera bound to at least one. Assuming that our panel was a representative panel of sera, it suggests that these three allergens may be useful as internal standards for allergen extract preparation. The use of defined individual allergens to monitor batch potency and/or consistency would represent significant progress toward a standardized allergen extract.

The pI values of these allergens cover a broad range. Allergen *Cal c* Bd6.6 and *Cal c* Bd9.3 each appear as a reasonably discrete band, especially *Cal c* Bd6.6. The acidic allergens, Bds3.6 to 4.6, however, appear heterogeneous. They are presumably either related allergens, that is, isoallergens, or discrete allergens with pI values proximal to one another.

*C. cyathiformis* spore extracts contain at least three major allergens; one of these three allergens possibly is a group of allergens. It is now possible to assign preliminary designations to two of these allergens, *Cal c* Bd6.6 and *Cal c* Bd9.3. These allergens are present in all three purification stages reported earlier.<sup>13</sup> Further work to purify these allergens is continuing.

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