Spore germination increases allergen release from *Alternaria*

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Allergen released from individual spores of the fungus *Alternaria* has not been investigated. Germination of spores has been suggested to increase allergen release. This study examined allergen released from individual spores and the effect of germination on allergen availability. Allergen release was determined with the Halogen (Inhalix, Sydney, Australia) immunoassay, by use of serum IgE from *Alternaria*-sensitized subjects and 3 Alt a 1–specific antibodies. Not all spores released allergen. Germination of the spores significantly increased the proportion that released allergen ($P < .0001$ for all antibodies). Alt a 1 may be a minor contributor to the total allergen released from spores except when spores have germinated. How these results reflect the allergen content of spores in the air that we breathe requires investigation. (J Allergy Clin Immunol 2001;107:388-90.)

Key words: *Allergen, Alt a 1, Alternaria, germination, Halogen, immunoassay*

The fungus *Alternaria* is strongly associated with allergic disease, particularly with asthma.[1] Whereas allergens of *Alternaria* have been identified from both spore and mycelial extracts, allergen released from individual spores and hyphal fragments has not been investigated. The viability of spores may influence the amount of allergen released because spores of *Aspergillus* are reported to release more allergen when germinated.[2]

Allergen released from individual spores can be determined with the Halogen (Inhalix, Sydney, Australia) immunoassay, which captures any immunostains allergen released on a protein-binding membrane. We aimed to examine allergen release from individual spores and to test the influence of germination.

METHODS

Alternaria alternata was isolated from wheat and grown on vegetable juice agar for 14 to 18 days. Spores were collected onto polyvinylidene difluoride (PVDF) protein-binding membrane by touching the membrane directly to the culture plate. The spores were pressed with a second membrane to ensure separation. To germinate spores, we placed samples prepared on PVDF (without adhesive) in a humid box at room temperature for 14 hours. Both germinated and ungerminated spore samples were permanently laminated between the membrane and a clear water-based adhesive film (Inhalix, Sydney, Australia).

Abbreviations used
pAb: Polyclonal antibody
PVDF: Polyvinylidene difluoride

Serum IgE was pooled from 30 subjects who had positive reactions to RAST and skin prick tests for *Alternaria*. Serum was diluted 1:3 in 2% skim milk/PBS/0.05% Tween 20. At this concentration, detection of the allergen released was optimal in approximately 60% of spores (data not presented). Pooled serum (n = 4) from subjects who, by RAST, had allergic reactions to fungi but not to *Alternaria* was used as a negative control.

Two mouse mAbs directed against Alt a 1 were used (mAb 1 was a gift from INDOOR Biotechnologies Ltd, Charlottesville, Va; mAb 2 was a gift from Prof Jay Portnoy and associates, The Children's Mercy Hospital, Kansas City, Mo). Both mAbs were diluted 1:500. Higher concentrations, including doubling of either mAb, had no effect (data not presented). mAbs were tested individually and in combination. Prof Jay Portnoy also provided a rabbit polyclonal antibody (pAb), which was a gift from Dr H. Vijay (Health Canada, Toronto, Canada). This polyclonal antisera was directed against Alt a 1 and was used diluted 1:4000.

Antibodies were initially selected by positive detection of antigens from an extract of *Alternaria*. The extract was made from 4 isolates of *A alternata* (isolated from wheat and cotton plants). Heavily sporulating cultures were harvested and macerated. Elution was overnight in borate buffer (pH 8.2). The supernatant was dialyzed, lyophilized, and then reconstituted to contain 256 μg protein per milliliter, as measured by the BCA Protein Assay (Pierce Chemical Co, Rockford, Ill). Serial dilutions of the extract were dotted onto a nitrocellulose membrane.

Dot blots were incubated overnight with the positive IgE serum pool, mAbs or pAb; washed; and incubated for 1.5 hours with either biotinylated goat antihuman IgE (Kirkegaard & Perry Laboratories, Gaithersburg, Md) or biotinylated goat antimouse IgG (Sigma Chemical Co, St Louis, Mo), as appropriate, diluted 1:500. Samples were incubated for 1.5 hours with ExtrAvidin alkaline phosphatase conjugate (Sigma) diluted 1:1000, followed by incubation with NBT/BCIP substrate (Pierce). Dot blots stained purple where allergen was detected.

In the Halogen assay,[3] spores that had been permanently laminated between the membrane and adhesive film were incubated for 4 hours in borate buffer (pH 8.2) to enable allergens to elute and bind to the PVDF. The laminates were then progressed through the same incubation steps as the dot blots. Positive detection of allergen produced a "halo" of stain around the spore.

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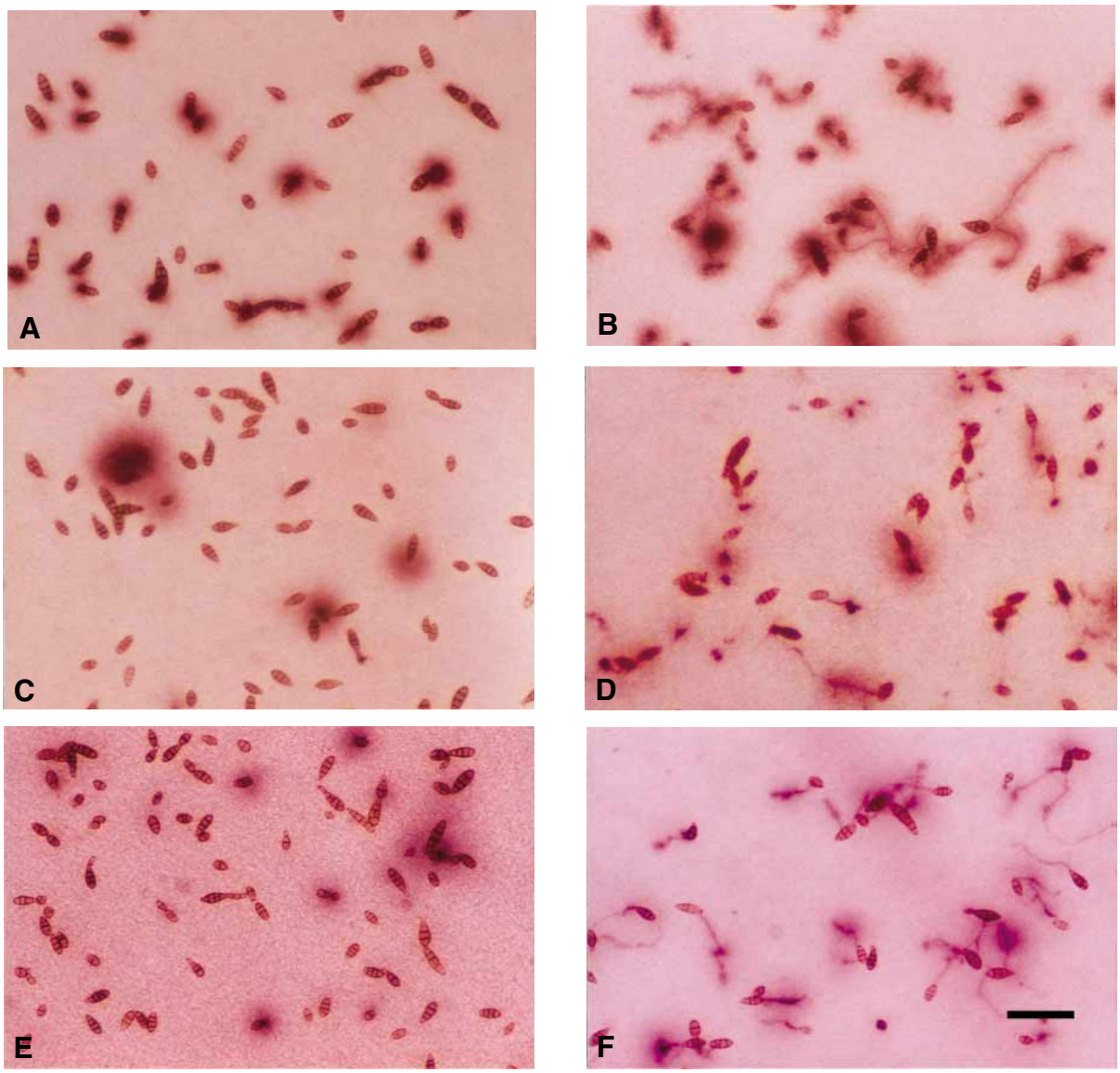

FIG 1. Individual *Alternaria* spores immunostained with different primary antibodies. **A,** IgE and ungerminated spores. **B,** IgE and germinated spores. **C,** Alt a 1 mAb2 and ungerminated spores. **D,** Alt a 1 mAb2 and germinated spores. **E,** Alt a 1 pAb and ungerminated spores. **F,** Alt a 1 pAb and germinated spores. (Scale bar = 50 μm.)

Spores were viewed by light microscopy at a magnification of 250×. At least 100 mature spores were observed for haloes from both ungerminated and germinated samples. Counts were expressed as percentages. Five replicates were assessed per treatment, and the experiment was repeated. The effect of germination for each antibody was tested statistically with independent samples *t* tests by use of Analyse-It for Microsoft Excel (Analyse-It Software Ltd, Leeds, UK).

RESULTS

No staining of any spores was observed with the control serum. For all test antibodies, the percentage of spores immunostained was higher in germinated spores than in ungerminated spores (Table I). Of the antibodies, IgE stained the most spores and with greatest intensity (Fig 1). mAb 1 stained no ungerminated spores, whereas mAb 2 stained a mean of 5.7% ungerminated spores. When the mAbs were combined, a greater proportion of spores stained than by the mAbs individually. The Alt a 1 pAb stained more spores than the mAbs but fewer than IgE.

DISCUSSION

This is the first study to examine the release of allergen from individual spores of *Alternaria.* Not all spores released allergen, and by germination of spores of *Alternaria,* a greater number of spores released allergens, including the major allergen Alt a 1.

TABLE I. Percentage of individual *Alternaria* spores that released allergen (as shown by immunostaining) when ungerminated and germinated, with different primary antibodies used for detection

Primary antibody	Mean % haloed spores (95% CI)	*P* value
Control serum		
Ungerminated	0	
Germinated	0	
IgE to *Alternaria*		
Ungerminated	68.0 (64.5-71.5)	<0.0001
Germinated	94.4 (91.6-97.1)	
Alt a 1 mAb 1		
Ungerminated	7.4 (5.1-9.7)	<0.0001
Germinated	37.4 (26.0-48.7)	
Alt a 1 mAb 2		
Ungerminated	0	<0.0001
Germinated	51.3 (41.7-60.9)	
Combined mAbs		
Ungerminated	7.5 (5.1-9.8)	<0.0001
Germinated	61.1 (53.8-68.4)	
Alt a 1 pAb		
Ungerminated	16.9 (11.5-22.3)	<0.0001
Germinated	86.5 (77.7-95.2)	

Control serum was collected from atopic subjects who tested negative by RAST to *Alternaria*.

Variation in the allergen content between individual spores was expected. Allergen content can vary between strains of the same fungus[4] and between components of the same isolate (spore vs hyphae).[5] The difference in detection of Alt a 1 allergen by the mAbs suggests that the mAbs are not binding to the same epitope on the allergen. The amount of spores stained with polyclonal Alt a 1 antibody was greater than with the mAbs combined, which suggests that multiple epitopes exist.

From the Alt a 1–specific antibodies, it was evident that either a minority of ungerminated spores released Alt a 1 or that considerable variation exists in the quantity of Alt a 1 allergen released by different spores. Because IgE would detect all eluted allergens, the difference in proportion of stained spores between IgE and mAb antibodies suggests Alt a 1 is a minor contributor to the total allergen released, except when spores have germinated.

The results clearly demonstrate the importance of the period of germination in the amount of allergen detected from *Alternaria.* Alt a 1 is genetically similar to secreted hydrophobic proteins implicated in the formation of conidial rodlets.[6] Our results support the hypothesis that Alt a 1 is secreted or released from growing germ tubes, and more so than from ungerminated spores, as found elsewhere with *Aspergillus.*[2] The growing hyphal tip has been demonstrated to carry allergenic proteins, as shown by gold labeling of IgE against *Aspergillus,*[7] and intense staining of allergen was frequently observed at hyphal tips of germinated spores of *Alternaria* in this study.

There were a number of limitations to this study. Although the assay is probably detecting picogram amounts of allergen per spore, it does not exclude the possibility that unstained spores have not released any allergen. We are currently attempting to develop image-analysis techniques to quantify the amount of allergen per spore. We are also unsure of the effect of the 14-hour period of germination on allergen release; however, the halo intensities of spores that had been incubated but did not germinate appeared similar to those of ungerminated spores. We hypothesize that the allergens require immersion in liquid for the proteins to be solubilized.

It is unclear what dose of spores is required to elicit allergic reactions. However, if our results reflect the proportion of airborne spores that release allergen, the results suggest that a large number of spores may be required to elicit an allergic reaction, particularly given that most spores of *Alternaria* are too large to penetrate past the oropharynx in the respiratory system.[8]

Alternatively, it has been suggested that fungal spores germinate in the respiratory tract, releasing a greater quantity of allergen through the germ tubes than through the spore wall.[2] Spores can remain in the respiratory tract for a sufficient time to germinate.[8] *Alternaria* has been demonstrated to germinate and grow in the sinonasal cavities of immunocompromised patients.[9] However, currently there is no supporting evidence that spores germinate in the respiratory tract of healthy or allergic individuals before clearance. This requires investigation.

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