

Role of *Alternaria* and *Penicillium* spores in the pathogenesis of asthma

Kenrick Licorish, M.D., Harold S. Novey, M.D., Peter Kozak, M.D.,†
Ronald D. Fairshier, M.D., and Archie F. Wilson, M.D., Ph.D. Irvine, Calif.

The ability to harvest spore-rich isolates of molds permitted quantitative studies of their role in the pathogenesis of asthma. Alternaria and Penicillium were selected as examples of ubiquitous molds that readily induce IgE antibodies and are of contrasting sizes. Extracts from those spores were prepared for skin tests and aerosol bronchial challenges. Intact spores were used in the same subjects in bronchial challenges delivered by a Spinhaler. Seven patients with a history of mild asthma received a total of 16 bronchial challenges with the mold to which they had been sensitized. Provocative doses in spore equivalents for a 35% drop in SG_{aw} , 20% drop in FEV_1 , or 25% drop in PEFr were sought for each challenge. Density dependence-flow rates were also determined. Environmental spore survey data were obtained and compared with the challenge doses for these spores. It was found that (1) immediate-type asthma was readily provoked by both whole spores and by their extracts, (2) in some subjects fewer intact than extracted spores were required, (3) delayed-type asthma occurred only after whole spore challenges, (4) SG_{aw} was the most sensitive and equally specific of the pulmonary function tests, and (5) provocative doses of spore equivalents were within natural exposure ranges. The study confirmed that Alternaria and Penicillium spores in relatively natural states and numbers were potent immunopathogens for asthma. (J ALLERGY CLIN IMMUNOL 76:819-25, 1985.)

Mold spores have long been considered causative agents for asthma, but few studies have been published with the use of mold spores for either skin tests or bronchial inhalation challenges. As early as 1873, Charles Blackley,¹ the originator of skin tests to pollens, described his own asthmatic response after inhalation of *Penicillium* mold. In 1930 Hopkins et al.² described a 37-year-old man whose asthma was associated with visits to damp and musty areas and in whom an attack was provoked by deliberate inhalational challenge to a ground-up isolate of *Alternaria*. Harris³ and Pennington⁴ independently reported in 1941 both immediate and late asthma in sensitized patients exposed to *Alternaria* spores released in a closed room or applied locally by nasal swabs. Most other studies of the role of molds in asthma have used extracts from mycelial components relatively devoid

Abbreviations used

SG_{aw} :	Specific airway conductance
PEFR:	Peak expiratory flow rate
$\Delta V_{E\max 50}$:	Density dependence of maximum expiratory flow at 50% of vital capacity
PD ₃₅ , PD ₂₀ , PD ₂₅ :	Provocative doses in mold spore equivalents required to drop SG_{aw} 35%, FEV_1 20%, or PEFr 25%, respectively
CFU:	Colony forming units, terminology used in spore surveys to denote both single spores and spore chains in the counting process

From the Department of Medicine, Divisions of Basic and Clinical Immunology and Pulmonary and Critical Care Medicine, University of California, Irvine, Calif.

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Reprint requests: Harold S. Novey, M.D., Department of Medicine, Division of Basic and Clinical Immunology, 101 City Dr. South, Orange, CA 92668.

†Dr. Kozak died August 5, 1985.

of spore content. Part of the reason for the difficulty in isolating the spores has been recently overcome, and spore-rich isolates are now available.

When natural material is used as an asthma inducer, particle size is one determinant of clinical response. Pollen grains usually associated with allergic asthma, e.g., are of a size ($>15 \mu m$ in diameter) precluded from entry into the lower airways⁵ and have not proved effective in provoking immediate asthma in their intact state.^{6, 7}

In this study two common mold spores of contrasting size were chosen to evaluate their role in the patho-



FIG. 1. Photomicrographs of spore material used in the study. Original magnification $\times 400$. **A**, *Alternaria alternata* spores with contaminating mycelial elements. Some portions of the isolate had less mycelia; other portions had more to average about 10%. **B**, *Alternaria* spores mixed with lactose granules in a 1:10 weight ratio for use in the Spinhaler delivery system demonstrating fair separation of elements. **C**, *Penicillium* spores with little mycelial contamination. The spores are spheres $3.0\ \mu\text{m}$ in diameter whose blurred margins are due to a layering effect of the spores and to focusing of the camera. **D**, *Penicillium* spores (arrows) separated by lactose granules in a weight ratio of 1:20 for use in the Spinhaler. **E**, Central insert is an example of typical *Alternaria* culture isolate used for commercial extracts, composed almost completely of mycelia, and largely devoid of spores.

genesis of asthma. *Alternaria alternata* produces pyriform-shaped spores with an average length of $18\ \mu\text{m}$ and diameter of $5\ \mu\text{m}$, and *Penicillium* species have spherical spores averaging $3\ \mu\text{m}$ in diameter. The goals were to learn if these spores could indeed induce asthma, what type of asthma might be induced, e.g., large or small airway, immediate or delayed, or some

combination, and if the quantity of spores required was comparable to natural exposures.

SUBJECTS AND METHODS

Subjects

Patients were selected on the basis of a history of asthma, strongly positive immediate-type reaction by the prick

method with *Alternaria* and *Penicillium* commercial extracts, and consent to undergo bronchial inhalation challenges.

Three female patients, ranging in ages from 18 to 23 years, and four male patients, ranging in ages from 18 to 39 years, were studied. They had histories of sporadic asthma, minimal to mild in nature. The patients had increases of at least 15% in FEV₁ after inhalation of a β -adrenergic aerosol as confirmation of the diagnosis of asthma. Only the 18-year-old woman used as many as two bronchodilators daily. None required steroids or hospitalization for asthma or were ever on immunotherapy.

The control subjects, a female, aged 46, and a male, aged 48, were in normal health. They served as safety controls for toxic or irritant effect of the challenge materials.

Preparation and quantitation of mold materials

Local isolates of *Alternaria alternata* strain 523 (speciated and confirmed independently by Commonwealth Mycological Institute, Kew, Surrey, England, and by Dr. Paul Neergaard, Danish Government Institute, Hellerup, Copenhagen, Denmark) and *Penicillium* species were grown on solid-phase synthetic media (Flow Laboratories, Rockville, Md.)⁸ with a porous barrier on the surface of the media. When maximum growth had occurred, the barrier with attached spores and mycelia was removed. The mold material was removed from the barrier, and the spores and mycelia were further separated by sieving (process for obtaining spores, United States patent No. 4,280,000).

The Alt-I content of the *Alternaria* strain extract used for these challenges was determined by David Marsh (Johns Hopkins University, personal communication) to be less than 50 arbitrary units per milliliter for the 1/50 dilution. Aliquots of the spore-rich *Alternaria* and *Penicillium* were examined microscopically, and the number of mycelial fragments and spores per area was determined. The spore-rich material contained <10% of mycelial fragments (Fig. 1, A and C). This was further confirmed by counting the number of spores and mycelial fragments present in the 1:25 v/v dilution of each extract.

In an effort to maximize safety for human use, the spore-rich material was subjected to electron beam radiation (IRT Corp., San Diego, Calif.) in order to render the material nonviable.⁹ The nonviability was confirmed by lack of growth on culture media.

Solutions of varying concentration were prepared from the spores for skin and bronchial inhalation testing by extracting the spore isolate in phosphate-buffered saline containing 0.1% phenol and 0.2% Tween 80 at 4° C for 48 hours. The concentrate was kept refrigerated at 4° C, and dilutions were prepared within 1 week of use.

Whole spore preparations for bronchial inhalation challenges were made by mixing weighted portions with weighted lactose. Lactose (USP lactose; Foremost Food Co., San Francisco, Calif.) was first sieved through a 325-mesh filter (45 μ m diameter) to obtain material of relatively small size. Portions were resieved several times to obtain a more

homogenous material. The spore-rich material was weighed and sieved into a pan containing measured amounts of lactose. This mixture of lactose and spores was resieved three additional times in an attempt to obtain a uniform distribution of the spores in the lactose (Fig. 1, B and D). Dilutions of the spore-lactose mixture were prepared for both *Alternaria* and *Penicillium*. A weighed aliquot of the 1:20 dilution was mixed with a tenfold weight of lactose to prepare a 1/200 dilution. Further tenfold dilutions were prepared in a similar manner. Aliquots of the initial three serial dilutions of dry spore materials in lactose were diluted with extraction solution, and the number of spores in each gram of material was determined by counting in nine wells of a hemacytometer and obtaining an average value. The variation in counts was $\pm 11\%$. It was determined that there were 2×10^9 *Alternaria* spores per gram of dry weight and 6×10^{10} *Penicillium* spores per gram of dry weight. This information was used to quantitate the number of spores in each challenge with extract and whole spore material. The spore-lactose mixtures were placed in sealed glass jars, and these were stored at room temperature in a vacuum-sealed desiccator.

Skin and bronchial challenge tests

Prick skin testing was used initially to identify potential candidates for the study. Intracutaneous titrations were then performed to determine relative sensitivity for those selected. End point titration was defined as the concentration of the extract that first produces a reaction equal to or greater than the histamine control.

Patient preparation, pulmonary function tests, and safety procedures were based on guidelines of the subcommittee on Bronchial Inhalation Challenges, American Thoracic Society, 1979.

Two kinds of challenge material were used, aqueous extracts and intact spores. The initial concentration of extract was based on the results of serial titration skin testing by the intracutaneous method, and starting challenge doses for the intact spores were based on the aqueous challenge results.

The delivery system for the aqueous extracts was described by Ryan et al.¹⁰ The Wright nebulizer was calibrated, and the aerosol generated was adjusted to provide a volume of 0.26 ml/2 min.

The intact spore delivery system used the Spinhaler (Fisons Corp., Bedford, Mass.) method of Wilson et al.⁵ Before the challenges with the active ingredient, each subject was challenged with diluent by nebulizer and lactose by the Spinhaler in order to detect nonspecific reactions to the media used for the dilution of mold material.

The following pulmonary function variables were measured: SG_{aw} by body plethysmography,¹¹ FEV₁, forced vital capacity, PEFR, and forced expiratory flow from 25% to 75% of forced vital capacity.¹² $\Delta \dot{V}_{E, \max 50}$ was performed according to the method described by Fairshir et al.¹²

The criteria for a positive bronchial challenge were as follows: FEV₁, 20% or more decrease from baseline; PEFR, 25% or more from baseline; or SG_{aw}, 35% or more from

TABLE I. Comparison of immediate skin and bronchial sensitivity to *Alternaria* in spore equivalents

Patient	Skin	Bronchial (PD ₃₅)	
	IC positive	Extract	Whole
K. L.	2×10^3	1×10^6	1×10^5
D. G.	7×10^3	6×10^4	4×10^5
N. J.	7×10^3	8×10^4	2×10^5
D. M.	4×10^4	3×10^5	9×10^4

IC = intracutaneous.

baseline; and clinical evidence of asthma. From these tests the respective provocative doses based on the quantity of spores inhaled (see Preparation and quantitation of mold materials) termed PD₂₀, PD₂₅, and PD₃₅ were obtained.¹³ The number of inhaled spores per aerosol challenge was calculated by multiplying the dilution of the extract by the number of milliliters of aerosol generated during 2 minutes times the percent of the subject's inspiration cycle. For the whole spores, their numbers were calculated by subtracting the weight of the Spinhaler unit with its capsule after the challenge from the weight before challenge. Weight in milligrams (mean of 0.1 mg was obtained) was multiplied by the dilution of spores in order to obtain the number of spores per challenge. Pulmonary function tests were plotted against cumulative doses of spores on a semilogarithmic scale in order to obtain PD.

The patients were monitored in the laboratory for 6 hours. In order to detect even later asthmatic reactions, the patients continued to record their PEFs at home with a mini-Wright flow meter (Armstrong Industries, Inc., Northbrook, Ill.) for at least another 12 hours.

Spore surveys

Spores were collected and counted in multiple environmental settings. An Andersen sampler (Andersen Samplers, Inc., Atlanta, Ga.) was used for collections of viable spores in undisturbed outdoors, disturbed outdoors, and indoor settings as previously described by Kozak et al.¹⁴ The counts were recorded as CFU per cubic meter of air. Collection samples were made at more than 400 residences during a 5-year period. From these collections 50 surveys were selected that contained the highest numbers of *Alternaria* and *Penicillium* spores. The results were compared to the spore quantitations obtained in the bronchial challenges in order to assess the relationship of the challenge doses to the number of spores encountered during natural exposures.

RESULTS

Skin and bronchial challenge tests

The aerosol challenges with aqueous extracts of *Penicillium* and *Alternaria* spores induced immediate asthma in six of seven of the highly sensitized patients. The single exception had the least skin sensitivity to the *Penicillium* spore extract, at least 2 log orders of

TABLE II. Comparison of immediate skin and bronchial sensitivity to *Penicillium* in spore equivalents

Patient	Skin	Bronchial	
	IC positive	Extract	Whole
F. P.	8×10^1	5×10^6	5×10^7
P. S.	6×10^5	1×10^7	6×10^9
N. J.	7×10^7	NEG	NEG

IC = intracutaneous; NEG = negative.

magnitude less than the next least sensitive subject (N. J. in Table II). Table I compares the quantity of *Alternaria* spores required to elicit a positive immediate skin test by end point intracutaneous titration and a positive bronchial challenge (PD₃₅) by the aqueous extract and by the intact spores. Table II provides the same data for the *Penicillium* spores.

All patients with a positive bronchial challenge to the extract also developed asthma from inhalation of whole spores. *Alternaria* spores were at least as effective as the smaller *Penicillium* spores. Our subjects, who were more sensitive to *Alternaria* (except subject D. M.) than *Penicillium* required correspondingly lesser doses of *Alternaria* spores to induce asthma. It is noteworthy that asthma was caused by smaller numbers of spores by the whole spore challenge than the aerosol challenge in some subjects, notably K. L., D. M. (Table I), and P. S. (Table II). Three patients developed a dual asthma pattern, meaning an almost immediate asthma that subsided during 1 hour only to return 6 to 8 hours later. This dual reaction occurred only after the intact spore exposures.

The three types of PD challenges are compared in Table III for *Alternaria* and in Table IV for *Penicillium*. The PD₃₅ was the most sensitive indicator of asthma, since the PD of spores was the lowest in nine of the 12 positive challenges. For the remaining three challenges, it was the change in FEV₁ that occurred with the smallest PD of spores.

Table V depicts the changes in $\Delta V_{E, \max 50}$ before challenge, during the height of change in SG_{aw}, and during the recovery state. The values compare those for challenges with extract against whole spores regardless of the mold source *Alternaria* or *Penicillium*. The results were variable and indicated individual subject-specificity. For example, patients T. P. and N. J. had increases in values for both extract and whole spore challenges, whereas the others had either a large, or in two cases, a minimal decrease (K. L., extract; P. S., spores) in their values. There was no difference when the spore sources were considered separately.

TABLE III. Quantity of *Alternaria* spores provoking a positive bronchial inhalation challenge

Patient	PFTs	Aqueous extract	Whole spore
K. L.	PD ₃₅	1×10^6	1×10^5 (L)
	PD ₂₅	NEG	8×10^6
	PD ₂₀	NEG	2×10^7
D. G.	PD ₃₅	6×10^4	4×10^5
	PD ₂₅	4×10^5	4×10^5
	PD ₂₀	2×10^5	3×10^5
D. M.	PD ₃₅	3×10^5	9×10^4
	PD ₂₅	NEG	1×10^7
	PD ₂₀	NEG	2×10^6
N. J.	PD ₃₅	8×10^4	2×10^5 (L)
	PD ₂₅	8×10^4	NEG
	PD ₂₀	5×10^4	1×10^7

PFTs = pulmonary function tests; (L) denotes late asthma in addition to immediate asthma; NEG = negative.

The two nonasthma control subjects were subjected to challenge doses at least 2 log orders of magnitude higher than any of the asthma patients. These doses did not produce either immediate or delayed pulmonary function abnormalities, immediate fever, or any other untoward responses. One subject did, however, develop a transient fever of 37° 8 hours after the whole spore challenge, together with malaise and aching. Whether this reaction was the result of the mold exposure or a concomitant viral infection could not be established.

Spore surveys

A summary of the number of *Alternaria* and *Penicillium* CFU per cubic meter of air collected under various conditions are summarized in Table VI. In order to compare these surveys with the doses inhaled by our patients, we transposed the collection data into the number of spores an adult subject could inspire in 24 hours. An adult man at rest inspires about 6500 ml of air per minute (500 ml tidal volume times 13 breaths per minute)¹⁵ or 3.9×10^5 ml per hour that is equivalent to 0.4 m³ of air per hour or about 10 m³ of air daily. If we assume that he was exposed to the maximum number in our table for the entire 24 hours and inspired these from the ambient air, then in rounded numbers, 2×10^4 *Alternaria* spores and 7×10^5 *Penicillium* spores would be inhaled by an adult man daily. Our PD₃₅ results in spore equivalents were comparable.

DISCUSSION

This study demonstrated the feasibility of provoking asthma with intact mold spores in addition to aero-

TABLE IV. Quantity of *Penicillium* spores provoking a positive bronchial inhalation challenge

Patient	PFTs	Aqueous extract	Whole spore
P. S.	PD ₃₅	1×10^7	6×10^4 (L)
	PD ₂₅	NEG	6×10^4
	PD ₂₀	NEG	1×10^4
T. P.	PD ₃₅	5×10^6	5×10^7
	PD ₂₅	NEG	4×10^8
	PD ₂₀	NEG	NEG
N. J.	PD ₃₅	NEG	NEG
	PD ₂₅	NEG	NEG
	PD ₂₀	NEG	NEG

PFTs = pulmonary function tests; (L) denotes late asthma in addition to immediate asthma; NEG = negative.

sol challenges with spore extracts. Although there was no attempt to observe direct deposition of spores in the lower airways as in other experiments with intact pollen,³ it can be safely assumed that some deposition probably occurred in order to account for the presence of immediate asthma. Initially, we were surprised that the larger *Alternaria* spores produced acute asthma. It was expected that their length of more than 15 µm would not allow entry into the airways. Further reflection uncovered at least two factors that modified the size component of *Alternaria* spores in airway deposition. The pyriform or cigarlike configuration could have permitted entrance by a vertical presentation in which the diameter rarely exceeded 5 µm, and thus, this spore could behave aerodynamically like a small particle. Additionally, all spores were not equally mature, resulting in varied sizes. The more distal younger spores released from the hyphae are perhaps one half the size of their more mature sisters (see Fig. 1) and more likely to be inhaled. An additional factor cannot be wholly excluded. Despite only approximately 10% contamination by mycelial elements, their deposition might have contributed to the allergenicity of the spore material. Another study with equivalent amounts of mycelial fragments alone should define their role.

The mold spore challenges produced both qualitative and quantitative differences from the extracts. In half the subjects with positive challenges, less spore material was required from the whole spore than from the extract challenges. Since the aerosol form would be expected to produce a deeper and more diffuse penetration of allergen into the airways than the larger particle form, any lesser requirement by intact spores suggests that they may contain potent allergens not fully removed in the extraction process. The dual asthma reactions limited to the whole spore challenges

TABLE V. $\Delta\dot{V}_{E\max_{50}}$ values at baseline, time of maximum SG_{aw} response, and recovery (mean $\pm$ SD)

Patient	Extract			Whole spores		
	Baseline	Max SG_{aw}		Baseline	Max SG_{aw}	
		Response	Recovery		Response	Recovery
T. P.	39.1	45.5	36.6	36	40	45
N. J.	37.5	43.7	ND	25.9	28	38.5
D. M.	—	ND	ND	27.6	13.3	31.3
P. S.	21.7	14.6	29.3	27.6	25	34.9
D. G.	36.8	5.3	33.3	21.7	0	9.7
K. L.	42.6	37.9	32.5	42.2	29.7	40.9
	35.5 $\pm$ 8.05	29.4 $\pm$ 18.3	32.9 $\pm$ 3.0	30.2 $\pm$ 7.5	22.7 $\pm$ 14.0	33.4 $\pm$ 12.5

ND = not done, usually because of technical problems.

TABLE VI. Environmental sampling of *Alternaria* and *Penicillium* spores

CFU per cubic meter of air by Andersen sampler in 50 southern California homes containing maximum counts		
	Outdoor range	Indoor range
<i>Alternaria</i>	177 to 1589	200 to 1389
<i>Penicillium</i>	424 to 5791	753 to 73,345

also suggest more potent allergens were present in this form than in the extract. Allergens extracted from the spores may be unstable or degrade over time in solution, whereas intact spores could contain sequestered antigens that solubilize only after time. The factor of longer duration of activity must also be considered. The larger particulates would not be removed from their deposition sites as readily as the more soluble aerosol form. Such a dual reaction has been produced in rabbits sensitized and challenged with *Alternaria* and was considered mediated by IgE antibodies alone.¹⁶ However, in that study an aerosol extract and not whole spores was used in the challenge.

In order to better confirm a positive bronchial challenge, multiple spirometric tests were performed. For three fourths of the challenges, the test for SG_{aw} with body plethysmography was the most sensitive indicator of airway obstruction. The test also appeared to be specific. We believe certain true asthma was being measured by this test for three reasons: (1) The patient volunteered that an attack was developing. (2) Wheezes were auscultated. (3) Where higher challenge doses were attempted, e.g., patients N. J., K. L., D. M., and T. P., more severe asthma resulted, and other test parameters became positive. Several articles have recommended as a standard test for di-

agnosing asthma a 20% drop in FEV_1 .¹⁷⁻¹⁸ When whole spore material was used as the challenge agent in subject N. J. (Table III), nearly 100 times the dose was required to affect the FEV_1 change than the SG_{aw} measurements. The higher dose produced severe asthma that continued for several days despite bronchodilator and steroid therapy. It is thus conceivable that reliance on the FEV_1 alone or with as much as a 20% drop as the criterion for a positive reaction could similarly impair the challenged patient.

The differences in sensitivity of SG_{aw} and FEV_1 in detecting a bronchial provocative response were not directly investigated in this study. However, it is known that a deep inspiration may attenuate the effects of a bronchoconstrictor agent in normal subjects and many subjects with asthma.^{19,21} In one study of 71 patients with asthma, a prior deep inspiration produced bronchodilation in 75%. Since FEV_1 determinations were preceded by inspiration to total lung capacity and SG_{aw} measurements were not, this technical factor could account for some of the differences in sensitivity of the two measurements in this study.

Changes in density dependence ($\Delta\dot{V}_{E\max_{50}}$) have been demonstrated to reflect the predominant site of airway obstruction from methacholine in healthy subjects.²² Thus, a decrease in density dependence is consistent with a predominantly peripheral airway effect. Although the extract would be anticipated to penetrate more effectively small airways, the spore-rich material produced decreases in $\Delta\dot{V}_{E\max_{50}}$ in the same number of subjects. In fact, the density-dependence response appeared to be more a function of individual variability tested than the type of material (extract versus various spores) inhaled (Table V). Although interpretation of density-dependence values is controversial,²³ the large decrements in $\Delta\dot{V}_{E\max_{50}}$ in some of our patients suggest that both extracts and spores

are capable of causing peripheral airway constriction.

It is important to know whether antigen challenges mimic natural exposures; otherwise, their relevance to clinical settings is in question. Most positive challenges with ragweed-pollen extracts, e.g., require quantities encountered only after an entire season's exposure or some 6 weeks.²⁴ When we equated random spore collection data with our challenge doses, it was found that asthma was evoked by cumulative spore doses that would be inhaled over a 24-hour period. If there were unusually high mold concentrations in the environment, less time would be required to cause asthma. The sampling device used in our spore surveys, the Andersen sampler, requires viable and culturable spores and tends to undercount the actual number of CFUs in the ambient air. Natural mold exposure is neither constant nor uniform. Exposures frequently occur in bursts, as verified by studies of Hirst spore trap (Burkard Manufacturing Co., Ltd., Rickmansworth, Hertfordshire, England) studies during 24-hour periods.¹⁴ Another factor that emphasizes the potency of these mold spores for producing asthma arises from our patient selection. They were all relatively young and asymptomatic and had had only mild asthma in the past. Older patients or patients with more severe asthma might require less exposure to molds to either elicit or perpetuate their asthma.

These studies tend to confirm early²⁵ as well as contemporary investigators²⁶ who have advocated the importance of molds and fungi in the pathogenesis of asthma.

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