INFECTION AND IMMUNITY, Apr. 1970, p. 345-350

Vol. 1, No. 4
Printed in U.S.A.

Interaction of *Aspergillus fumigatus* Spores with Human Leukocytes and Serum

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Received for publication 18 December 1969

Serum was necessary for optimal phagocytosis of *Aspergillus fumigatus* spores by human leukocytes, and its opsonic capacity was greatly diminished by heat inactivation (56 C, 30 min). A germination assay, described in this report, was developed to study the fate of phagocytized spores. After incubation for 3 hr with normal leukocytes and serum, spores ingested by peripheral blood neutrophils and monocytes remained viable. Since we had previously found that myeloperoxidase (MPO), a lysosomal enzyme of human neutrophils and monocytes, exerted fungicidal activity against *Candida albicans* when combined with H_2O_2 and chloride or iodide, the effects of these substances on *A. fumigatus* spores were examined. Spore viability was not impaired by MPO alone, H_2O_2 alone, or KI alone, but high concentrations of KI and H_2O_2 in combination caused marked inhibition of subsequent germination. MPO imparted fungicidal activity to concentrations of KI and H_2O_2 that lacked any effect in its absence. NaCl, in combination with MPO and H_2O_2, was far less effective than the iodide salt against *A. fumigatus*. The relative ineffectiveness of chloride in this system could underly the apparent inability of human neutrophils to kill ingested *A. fumigatus* spores, despite their competence to kill *C. albicans*.

Phagocytic cells are prominent in the defense mechanisms of mammalian hosts against fungal and bacterial infection. Most studies of the microbicidal process in mammalian leukocytes have employed vegetative-phase microorganisms, and relatively little is known of the fate of ingested bacterial or fungal spores. The significant incidence of aspergillosis among patients receiving immunosuppressive or cytotoxic therapy (3, 5) warrants the identification and characterization of the host factors which normally prevent disease after exposure to these ubiquitous spores. In this report, we describe a quantitative assay to determine the viability of *Aspergillus fumigatus* spores and its application to a continuing study of the fungicidal properties of human leukocytes.

MATERIALS AND METHODS

Organism. *A. fumigatus* (strain 14-7) was selected for abundant sporulation and high spore viability from six clinical isolates kindly provided by Carlyn Halde. The organism was maintained on Sabouraud dextrose-agar slants at room temperature and transferred every 4 months. Test organisms were cultured on Sabouraud slants at room temperature for 1 to 6 weeks after a preliminary 72-hr incubation at 33 C. Mature spores were collected by gently pipetting several milliliters of Hanks balanced salt solution (BSS) containing 0.1% Tween 80 over the surface of the slant. The recovered spores were washed twice in Hanks BSS containing 0.01% Tween 80 (BSS-T) and counted in a hemocytometer. Approximately 88% of the recovered spores were solitary, 10% were present in pairs, and the remainder occurred in chains of three or four. Once harvested and washed, the spores withstood storage at 4 C in BSS-T without germination or loss of viability for at least 4 weeks.

Germination assay. Germination was assessed morphologically by the general criteria established by Yanagita in his studies of *A. niger* (21). The germination medium was composed of medium 199-Hanks base (Grand Island Biological Co., Berkeley, Calif.) supplemented with a final concentration of 1% agar (Difco), 8% normal group AB human serum, and 200 units of penicillin G and 200 μg of streptomycin per ml of medium. This medium allowed complete and relatively synchronous germination of viable spores and retarded subsequent mycelial proliferation sufficiently to permit observations to be made without interference by mycelial overgrowth. Assays were performed by placing one or two drops (approximately 25 to 50 μliters) of the suspension to be tested on a sterile glass microscope slide (7.6 by 2.5 cm), adding three or four drops of germination medium (kept liquid at 48 C), and tilting the slide to distribute the spores within the rapidly solidifying thin agar medium.

The slide cultures were placed on glass supports in covered petri dishes containing moist pads and incubated at 33 C for 15 to 18 hr. A segment of the agar was then transferred to a new microscope slide and pressed beneath a cover glass. A 200-spore sample was counted and examined microscopically to establish the percentage which had germinated. In preliminary experiments, we found that the presence of 0.5% sodium deoxycholate in the germination medium did not alter the germination process. We therefore utilized this concentration of sodium deoxycholate to lyse leukocytes and erythrocytes in some of the studies to be described.

Phagocytosis. Leukocytes were prepared from samples of peripheral venous blood from normal adult volunteers and were suspended at a concentration of 10^7 neutrophils/ml in BSS as described previously (13). Equal volumes (0.25 ml) of autologous serum, BSS, leukocyte suspension, and spores (2×10^7/ml or 5×10^7/ml in BSS-T) were added to sterile plastic tubes (12 by 75 mm; Falcon Plastics, Los Angeles, Calif.) and incubated at 37 C in air with rotation (30 rev/min). In some experiments, the serum component had been heated at 56 C for 30 min. After 30, 60, and 120 min, samples were taken and diluted with BSS containing 0.1% human serum albumin; slides were prepared with a Cytocentrifuge (Shandon Scientific Co., London, England). The slides were air-dried, fixed in absolute methanol, and stained with Giemsa. The percentage of phagocytized spores was determined by examining 200 consecutive randomly encountered spores on these slides and noting if they were phagocytized or free. In selected studies, an additional 1,000 consecutively encountered phagocytized spores were examined and classified according to the phagocytic cell type.

To determine the fate of the ingested spores, incubation mixtures were prepared as described at a spore to neutrophil ratio of 2:1. Control tubes, which contained spores in BSS or in BSS with 25% autologous serum, were included in each experiment. After 3 hr, 0.25 ml of 2.5% sodium deoxycholate (*p*H 8.7) was added to all tubes, and slide cultures were prepared as described above to assess the viability of the spores.

Myeloperoxidase (MPO) system. Highly purified human MPO was the generous gift of J. Schultz. Its preparation and characteristics have been previously described (19). The preparation employed in these studies had an activity of 28,000 *o*-anisidine units/mg (14). Hydrogen peroxide solutions were prepared by diluting 30% H_2O_2 (J. T. Baker Chemical Co., Phillipsburg, N.J.) and verifying the concentrations by measuring their absorbancy at 240 nm. NaCl, KI, and KBr were of reagent grade (Baker). The spore suspension was washed twice (1,000 × *g*) with distilled water containing 0.01% gelatin before addition of 5×10^5 spores (50 μliters) to the incubation mixture, which contained 20 μmoles of *p*H 5 citrate-phosphate buffer, various combinations of MPO, H_2O_2, and halide, and sufficient water to replace any component omitted and to bring the final volume to 0.5 ml. H_2O_2 was the last component to be added, after which the tubes were incubated in a water bath at 37 C for 30 min and slide cultures were prepared.

RESULTS

Germination. The ungerminated spores of *A. fumigatus* (strain 14-7) had a mean diameter of 3.2 μm (range, 2.6 to 4.2 μm) when initially harvested and during storage in BSS-T. After slide culture, the first morphological alteration accepted as indicating germination was spore swelling. When measured by a calibrated eyepiece micrometer, the mean spore diameter increased to 5.9 μm (range, 4.9 to 6.5). With experience, we learned to distinguish the swollen and nonswollen spores by inspection and had to measure only the relatively few spores in the intermediate size range. With continued incubation, swollen spores soon sprouted and developed one or more germ tubes which rapidly enlarged, branched, and ultimately gave rise to macroscopically visible mycelia (Fig. 1).

Phagocytosis. When spores and neutrophils, at a ratio of 5:1, were incubated together in autologous serum, 73 ± 5% (five normal subjects, eight experiments) of the added spores were ingested within 30 min, with a gradual increase to a maximum of 80 to 90% at 2 hr. At a spore to neutrophil ratio of 2:1, over 90% of the added spores were ingested within 30 min. Microscopic examination of unstained wet mounts confirmed the intracellular location of added spores. Examination of the cellular distribution of ingested spores in the stained slides (Table 1) consistently revealed monocytes to be more avidly phagocytic than neutrophils, whereas eosinophils were, by comparison, minimally effective. However, since neutrophils far outnumbered monocytes, they accounted for about 80% of the ingested spores in most studies. Phagocytized spores within neutrophils and monocytes showed no change in size, morphological characteristics, or staining characteristics during incubation periods as long as 3 hr.

In two studies at a 2:1 spore ratio, we measured the rate of phagocytosis in heat-inactivated serum, and found the following mean percentages of ingested spores: 30 min, 26%; 60 min, 59%; 120 min, 65%; and 180 min, 83%.

Fate of ingested spores. Spores were incubated with leukocytes and serum for 3 hr and then tested for viability by culture on slides. It was apparent (Table 2) that the viability of the spores was not impaired by serum or after phagocytosis by normal neutrophils and monocytes. The value given for percentage of germination in Table 2 includes both swollen and sprouted (with germ tubes) spores. In all but one of this series of experiments, the "swollen" category comprised 3% or less of the total.

Effect of the MPO system. Since leukocytes

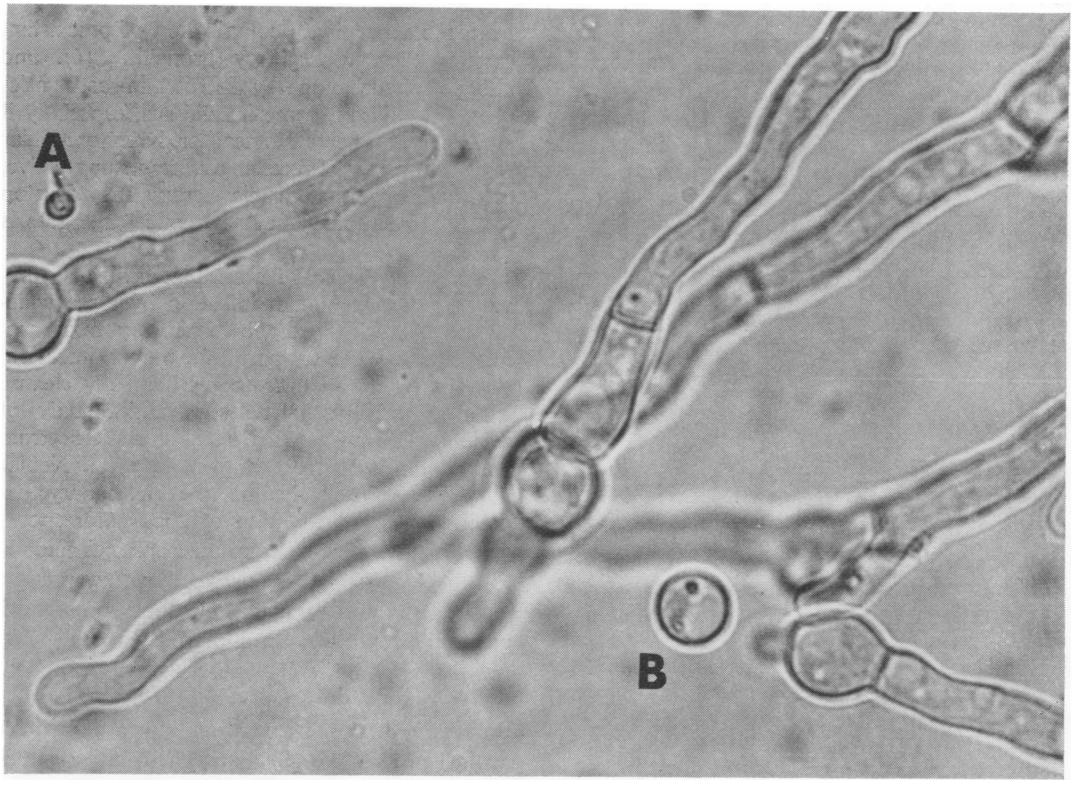

FIG. 1. *Germination of Aspergillus fumigatus spores in slide culture. (A) An ungerminated spore. (B) A spore which has swollen but not yet sprouted. Other spores show formation of germ tubes. Phase-contrast microscopy, × 1,250.*

TABLE 1. *Phagocytosis of Aspergillus fumigatus spores by human leukocytes*[a]

Subject	Ingested fraction			Relative phagocytic index		
	Neutrophils	Monocytes	Eosinophils	Neutrophils	Monocytes	Eosinophils
1	0.836	0.161	0.003	1.05	1.79	0.03
2	0.772	0.226	0.002	0.91	1.82	0.08
3	0.816	0.172	0.012	0.91	2.32	0.40
4	0.787	0.213	0.000	1.02	1.33	0.00
5	0.788	0.210	0.002	0.91	1.91	0.08

[a] Leukocytes from five normal subjects were incubated with spores in autologous serum for 60 min at a spore to neutrophil ratio of 2:1. Ingested fraction is the distribution by phagocytic cell type of 1,000 ingested spores. Relative phagocytic index was calculated for each cell type by dividing its ingested fraction by the frequency of the cell in the total phagocyte population, as determined by a 500-cell differential count.

have been shown to kill yeast-phase *Candida albicans* (13) and there is evidence that this ability depends on the action of the lysosomal enzyme MPO (12, 13, 14), we examined the effects of human MPO on *Aspergillus* spores by using the slide germination technique. The results of these studies are shown in Table 3. *Aspergillus* spores were resistant to MPO, KI, and H_2O_2 individually in the concentrations tested. At the highest concentrations examined, the combination of iodide and H_2O_2 reduced spore viability to approximately 14%. The addition of MPO to concentrations of iodide and H_2O_2 that were themselves ineffective introduced a marked inhibitory effect (0 to 2% of spores were viable). When chloride replaced iodide in the presence of MPO and H_2O_2, the results were variable, but suggested that chloride was relatively ineffective as an inhibitory cofactor. Our limited supply of MPO precluded efforts to identify the sources of variability in the chloride-MPO-H_2O_2 systems, but the presence of iodide (0.001%) as a contaminant in the reagent grade sodium chloride may have been a contributing factor.

DISCUSSION

Aspergilli may coexist with man in many ways. Probably, they are most often present as transient pulmonary residents, reflecting both their

abundance in the aerial spora (2) and the efficient retention by the human lung of airborne particles the size of *Aspergillus* spores (1, 6). Aspergilli may be associated with several pulmonary disease patterns including mycetoma formation resulting from the saprophytic colonization of preexistent cavities (20), aspergillary bronchitis (4), and allergic pneumonopathy in certain asthmatics (18). In addition, some individuals, especially if afflicted with hematological or lymphoreticular malignancies, or if receiving immunosuppressive, cytotoxic, or antineoplastic therapy, may develop invasive pulmonary or systemic aspergillosis (3, 5).

What are the host factors that ordinarily prevent significant human infection by these widespread spores? Since the respiratory tract provides the major entry portal for human infection, the ability of the lung to mechanically cleanse itself of retained particles is undoubtedly of major importance (1). There is evidence from animal experimentation that phagocytic alveolar macrophages help defend the pulmonary parenchyma after inhalation of *Aspergillus* spores (6, 8, 15). Whether alveolar macrophages actually kill the ingested spores or whether they prevent germination and participate in their mechanical removal has not, in our opinion, been established by these studies. The observations of Ford, Baker, and Friedman (7) serve to illustrate that the inhibition of spore germination could protect against progressive infection. These investigators

TABLE 2. *Effect of human leukocytes and serum on the viability of Aspergillus fumigatus spores*[a]

Subject	Germination of *A. fumigatus* spores cultured after 180 min of incubation with		
	BSS	BSS + autologous serum	BSS + autologous serum + leukocytes
	%	%	%
1	100	100	99.0
1	98.5	98.0	99.0
1	93.0	92.5	89.5
2	99.5	98.5	99.5
2	96.0	96.0	99.5
3	96.0	62.0	96.0
4	96.0	91.0	88.0
5	99.5	99.5	99.5

[a] The differences between BSS and BSS + serum ($P > 0.2$) and between BSS and BSS + serum + cells ($P > 0.5$) are not significant by the paired t test.

TABLE 3. *Effects of myeloperoxidase, hydrogen peroxide, and halides on the germinative ability of Aspergillus fumigatus spores*[a]

Components	Concn		No. of expt	Germination
	Halide	H_2O_2		
	M	M		%
MPO + H_2O_2		8×10^{-4}	3	97.5 ± 1.2
MPO + KI	10^{-4}		4	73.3 ± 16.1
MPO + NaCl	10^{-1}		3	97.3 ± 2.3
KI + H_2O_2	10^{-4}	8×10^{-4}	5	13.6 ± 6.4
	10^{-4}	8×10^{-5}	3	98.8 ± 0.9
	3.2×10^{-5}	8×10^{-4}	3	82.8 ± 9.3
	3.2×10^{-5}	8×10^{-5}	3	99.7 ± 0.3
MPO + KI + H_2O_2	10^{-4}	8×10^{-4}	3	1.2 ± 0.9
	10^{-4}	8×10^{-5}	2	0
	3.2×10^{-5}	8×10^{-4}	3	1.7 ± 1.4
	3.2×10^{-5}	8×10^{-5}	2	1.3
NaCl + H_2O_2	1	8×10^{-4}	3	99.2 ± 0.4
	10^{-1}	8×10^{-4}	4	87.9 ± 12.4
MPO + NaCl + H_2O_2	1	8×10^{-4}	3	75.5 ± 15.0
	10^{-1}	8×10^{-4}	3	51.5 ± 21.6
MPO			3	100
KI	10^{-4}		3	97.3 ± 1.6
NaCl	1		1	100
H_2O_2		4×10^{-2}	1	96.5
		8×10^{-3}	1	99.5
		8×10^{-4}	3	97.8 ± 1.9

[a] Myeloperoxidase (MPO), when present, was at a concentration of 2 μg/ml. Germination is expressed as the mean value observed in the number of experiments shown. SEM was calculated when three or more experiments were performed. Spores were considered to have germinated if they showed swelling or sprouting after culture on slides.

noted mice to be highly resistant to infection after intravenous inoculation of *A. clavatus* spores, yet the injected spores remained viable without germinating for prolonged periods in the livers and spleens of these animals.

The data presented here indicate that human neutrophils in vitro and under the conditions of our assay do not kill phagocytized *A. fumigatus* spores during a 3-hr period. Under identical in vitro conditions, human leukocytes do kill many species of bacteria and the fungus *C. albicans*. We believe that this reflects a functional limitation of human neutrophils rather than the effect of artificial assay conditions.

In view of their ability to kill ingested *C. albicans*, the failure of human neutrophils to kill *A. fumigatus* spores was unexpected. There is convincing evidence that neutrophils produce H_2O_2 after particle ingestion (9, 17, 22) and that H_2O_2 can act in concert with leukocyte MPO and a halide to form an effective in vitro bactericidal (10, 11) and fungicidal (12) system. We have previously presented evidence associating these substances with the fungicidal activity of leukocytes against *C. albicans* (13, 14; R. I. Lehrer, Clin. Res. **17**:331, 1969).

The intracellular cofactor in the MPO-H_2O_2-linked fungicidal activity of neutrophils against *C. albicans* has not been identified but is presumed to be a halide (10, 11, 12). The present investigations revealed that *Aspergillus* spores were relatively resistant to MPO, H_2O_2, and chloride in vitro, although *C. albicans* is highly sensitive under identical conditions of testing (12). If one postulates that chloride serves as the halide cofactor for MPO and H_2O_2 under intracellular conditions, and that the intracellular fungicidal activity of MPO is somewhat less than that measured by the in vitro assays, then the ability of leukocytes to kill *Candida* cells but not *Aspergillus* spores could be explained. Perhaps the dissimilar effects of chloride-MPO-H_2O_2 systems and iodide-MPO-H_2O_2 systems against different fungi also underly the clinically established efficacy of iodides in the therapy of certain fungal diseases.

Many facets of the pathogenicity of *Aspergillus* species for man remain to be elucidated. We have found that *A. fumigatus* spores are relatively resistant to the fungicidal processes of human neutrophils and monocytes. This resistance may attain significance when the organism escapes from the confines of the respiratory air passages and behaves as a pathogen.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant CA-11067 from the National Cancer Institute. R. G. J. was supported by Public Health Service Summer Student Fellowship CA-08054 from the National Cancer Institute.

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