Studies on Heat Resistance of Conidiospores of *Aspergillus fumigatus*

I. Qualitative Observation of the Resistance

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INTRODUCTION

As generally known, the majority of fungus spores of various types has no ability to survive thermal treatments which destroy the vegetative hyphae from which they arise. In early studies of the heat resistance, however, a certain type of fungal spores, i.e., ascospores of some *Neurospora* species were found resistant to heating at 50–60°C for more than 4 hours¹⁾²⁾. A higher susceptibility of conidia of *Neurospora tetrasperma* to injury by heat was also observed in the latter investigation.

The present study deals with the changes in the heat resistance of conidiospores of *Aspergillus fumigatus*, which is best known as one of the thermophilic fungi, during maturation and germination and various factors which may affect their heat resistance.

MATERIALS AND METHODS

Organism.

A stock culture of *A. fumigatus* strain N-1, isolated from sputum of a patient with pulmonary aspergillosis and maintained on Sabouraud's glucose agar at 27°C, was used.

Culture media.

The basal sporulating medium was the modification of Sabouraud's agar (pH 5.4–5.6), containing 1% Polypepton (Daigo Eiyokagaku Co., Ltd.) and 1% glucose.

Unless otherwise indicated, Sabouraud's broth was used to determine the viability of the heated conidia.

Preparation of spore suspension.

A dormant spore suspension prepared from a slant culture of the fungus was spread uniformly on to a sporulating medium and incubated at 27–37°C for 4–7 days. Ungerminated conidia developing on aerial hyphae of the fungus were harvested by floating on sterile distilled water. To remove spore clumps and contaminating short hyphae, the spore harvests were filtered through a sterile paper filter (No. 5A, Toyo Roshi Co., Ltd.), otherwise the unfiltered spore suspension showed considerable variations in the thermal tolerance. Conidiospores were then washed twice with sterile M/15 phosphate buffer (pH 7.0) by centrifugation and resuspended in it. The spore suspensions were standardized by cell number determined with a Thoma haemocytometer and diluted with phosphate buffer so as to contain a definite number of spores. The stock suspensions were stored in the refrigerator for not more than 7 days.

Determination of heat resistance.

A water-bath equipped with an electric thermostat and a stirrer was used for heat processing. The

pyrex test tubes containing the conidia were placed directly into the bath which was kept at the desired heating temperature. One ml of the standardized spore suspensions was pipetted into test tubes having an inside diameter of 18 mm, a length of 180 mm, and a thickness of 1 mm; 4 tubes were used for each heating interval when the heating increments were 5 min or less. After heating, the tubes were cooled quickly with running water. Ten ml of the recovery culture medium (Sabouraud's broth) was poured into each heated tube and incubated for 2 weeks at 27°C. Growth as observed every 48 hours was examined carefully. Time of heating at a given temperature which is enough to produce no growth of conidia in any of 4 tubes was designated the thermal death time.

Table 1 presents an example of the thermal resistance data obtained with spore suspension of *A. fumigatus* by 4-tube technique. Our experience has shown that replicated determination by single tube technique often provides data exhibiting considerable variation. For example, the 2nd and 3rd columns at 7 days of subculture in Table 1 show the "skips" in case of 1-tube method and difference in the thermal death time may be observed in the results between 1st and 2nd column at 14 days of recovery culture.

Table 1 Results of heat-resistance of *A. fumigatus* conidia by 4-tube method

Time heated at 60°C (min)	days of subculture																			
	3				5				7				10				14			
7	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	
8	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	
9	-	-	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	
10	-	-	-	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	
12	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+	+	+	+	
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Experimental conditions:

Spore age, 4-day-old on Sabouraud's agar; temperature of conidiation, 27°C; pH of suspending medium (phosphate buffer solution), 7.0; spore concentration, 10 millions per ml.

+ = positive growth

- = no growth

Conidia were killed off by 5-min exposure to 70°C, but survived more than 3 hours at 50°C.

RESULTS

Effect of Concentrations of Spore Suspension

Different densities of conidiospores produced on Sabouraud's agar were first tested for their heat resistance. Table 2 indicates that the greater the density of the conidia within the limits examined, the greater is the heat resistance of the conidia. However, a lower density of the spores (less than 10^7 cells per ml) showed the same thermal tolerance; they survived equally 12 min at 60°C. For this reason spore suspension containing 10^7 cells/ml was standardized by means of a haemocytometer and used in the further experiments.

Effect of pH of Spore Suspending Medium

A. fumigatus conidia grown on Sabouraud's agar and washed in sterile deionized water were suspended in M/15 phosphate buffer solutions of various pH values. The thermal death time was determined by

Table 2 Effect of concentrations of *A. fumigatus* conidia on their heat-resistance

survival time at 60°C (min)				
number of conidia ($\times 10^6$ /ml)				
1/10	1	10	50	100
12	12	12	14	18

Experimental conditions:

Spore age, 4-day-old on Sabouraud's agar; temperature at which spores were formed, 27°C; pH of suspending medium (M/15 phosphate buffer), 7.0.

Table 3 Effect of pH of suspending medium on heat-resistance of *A. fumigatus* conidia

Survival time at 60°C (min)	pH of suspending medium				
	4.5	5.9	7.0	7.7	8.3
alive	12	12	12	16	16
dead	14	14	14	18	18

Experimental conditions are all the same as those described in Table 1, except for the pH of spore suspending medium.

the same manner as described above. The data shown in Table 3 reach the conclusion that *A. fumigatus* conidia may survive rather longer in the alkaline solutions at pH 7.7–8.3 than in the neutral to acidic pH range (7.0–4.5), but there is no difference in the thermal tolerance.

Effect of Temperature for Spore Formation

Experiments were conducted to investigate whether the thermal resistance of *A. fumigatus* spores is influenced by the temperature of sporulation. For this purpose 4 different temperatures from 23 through 27, 30, and 37°C were used. As shown in Table 4, the conidiospores grown at 37°C survived 60 min at 60°C, while those grown at lower temperatures of 23, 27, and 30°C survived only 12 min. These results suggest that conidia formed at 37°C are more heat-stable than those formed at lower temperature.

Table 4 Effect of temperature of conidiation on heat-resistance of *A. fumigatus* conidia

Survival time at 60°C (min)	temperature of conidiation			
	23°C	27°C	30°C	37°C
alive	12	12	12	60
dead	14	14	14	70

Experimental conditions are all the same as those described in Table 1, except for the temperature at which conidia were formed.

Effect of Age of Conidiospores

Strain N-1 of *A. fumigatus* employed in this study usually begins to yield conidia on Sabouraud's agar after 40 hours of incubation. Thereafter, maturation of the spores may proceed progressively; the temperature of spore formation gives a considerable effect upon the maturity rate of the spores. Table 5 indicates the relationship between the thermal tolerance of the conidia and their age when conidiation occurs at 27 and 37°C. The most heat stable conidia were obtained from a 4- to 7-day old culture kept at 37°C. They survived 60 min at 60°C. The older conidia of 10-21 days of age rather decreased in the resistance.

Table 5 Effect of spore age on heat-resistance of *A. fumigatus* conidiospores

Temperature of sporulation	Survival time at 60°C (min)	spore age in days				
		4	7	10	14	21
37°C	alive	60	60	30	30	30
	dead	70	70	40	40	40
27°C	alive	12	40	20	12	12
	dead	14	50	25	14	14

Experimental conditions:

Spore concentration, 10 millions per ml; pH of suspending medium (phosphate buffer solution), 7.0.

The heat resistance of spores grown at 27°C was somewhat lower throughout their whole age than spores grown at 37°C. The young conidia of 4-day age were the least resistant to heat at 60°C. However, the matured conidia obtained from a 7-day old culture showed a marked increase in the heat resistance. They survived 40 min at 60°C.

It is noted from the results obtained above that the temperature of conidiation and the age of conidia are of important factors which are directly concerned with their heat resistance.

Effect of Sporulating Medium

For the purpose of studying the variations in the heat resistance of *A. fumigatus* conidia ascribed to the kind of conidiating medium, the conidia were grown on (1) malt extract agar, with 1% glucose,

Table 6 Effect of the kind of conidiating media on heat-resistance of *A. fumigatus* conidia

Conidiating medium	survival time at 60°C (min)	
	alive	dead
Sabouraud's glucose agar	60	70
malt extract agar	60	70
soil extract agar	45	50
Czapek's agar	70	80

Experimental conditions:

Spore age, 5-day-old; temperature of conidiation, 37°C; spore concentration, 10 millions per ml; pH of suspending medium (phosphate buffer solution), 7.0.

(2) soil extract agar plus 1% glucose, (3) Czapek's agar supplemented with 0.5% peptone, and (4) Sabouraud's agar as the reference medium. All media were adjusted to pH 6.0 and incubation was 37°C for 5 days. Table 6 summarizes the results of heat-stability of spore crops produced on each sporulating medium. There was no remarkable difference in heat resistance among spores produced on these media. The conidia grown on soil extract agar, which may be regarded as being poor in certain organic constituents, were of slightly lower resistance than those grown on the other media.

Differences in Heat Resistance of Conidia of A. fumigatus Due to Its Strains

Eight strains of *A. fumigatus* of different sources have been cultivated on Czapek's agar at different temperatures, and the resistance to heat of the conidia harvested from each culture compared. As indicated in Table 7, the strains were of unequal heat resistance, and the degree of the responses to increased temperature likewise were unequal. However, most strains were revealed to increase resistance with increasing temperature at which conidia were formed. Also, there was no evidence suggesting that the conidia of the strains isolated from clinical sources, e.g., sputum and lung, have an extremely high resistance.

The mature conidia of all strains grown at 37°C survived 60 min or more at 60°C.

Table 7 Differences in heat-resistance of conidia of *A. fumigatus* due to its strains

Strain	Source	survival time at 60°C (min)			
		temperature of spore formation and spore age			
		at 27°C for 7 days		at 37°C for 4 days	
		alive	dead	alive	dead
N-1	sputum	40	45	70	80
N-2	sputum	90	120	90	120
N-3	sputum	80	90	120	?
0-3-1	lung	70	80	70	80
111-5-3	lung	90	120	90	120
NHL-5009	rice	35	40	60	70
IFO 4399	soil	90	120	90	120
IFO 4400	soil	70	80	120	?

Experimental conditions:

Spore concentration, 10 millions per ml, heated in phosphate buffer solution (pH 7.0) at 60°C; conidiating medium, Czapek's agar plus 0.5% peptone.

? not examined more than 120 minutes

Heat Resistance of Conidiospores of Several Aspergilli

It was noted from the foregoing results that the mature conidia of *A. fumigatus* are considerably resistant to heat; they may resist moist heat at 60°C for an hour or more. To confirm whether this resistance is peculiar only to the spores of the fungus, a comparative study was undertaken with 7 species of *Aspergillus* group. Spore suspensions were not standardized by cell number, but by cell density represented by optical absorbency with a spectrophotometer, because of differences in spore sizes between different species of *Aspergilli*. Heat experiments were repeated 3 times to insure the accuracy of the data recorded. As is evident from the data of Table 8, the mature conidia of *A. fumigatus* have a far higher

Table 8 Heat-resistance of conidiospores of several *Aspergilli*

Species and strain	survival time at 60°C (min)			
	temperature of spore formation and spore age			
	at 27°C for 7 days		at 37°C for 4 days	
	alive	dead	alive	dead
<i>A. flavus</i> , IFO 6343	5	6	6	7
<i>A. fumigatus</i> , N-1	40	45	70	80
<i>A. nidulans</i> , T-1	3	4	5	6
<i>A. niger</i> , N-1	3	4	7	8
<i>A. sydowi</i> , IFO 7531	2	3	2	3
<i>A. tamarii</i> , IFO 7465	7	8	6	7
<i>A. terreus</i> , IFO 6123	12	14	16	18

The concentration of conidiospores was standardized by optical absorbency with a spectrophotometer at 600 mμ wavelength and was adjusted with additional neutral phosphate buffer so as to obtain a final optical density of 0.14 at which the spore suspension of *A. fumigatus* contained 10 millions cells per ml. Czapek's agar plus 0.5% peptone was used for conidiation.

heat-stability than those of other *Aspergilli*. This fact leads to the conclusion that the conidia of *A. fumigatus* only have the ability to survive heat treatment which may kill off the conidia of other *Aspergillus* species within 10 min at 60°C.

Changes in Heat Resistance of A. fumigatus Conidia during Germination

Germinants were obtained by inoculating dormant conidia which have a high resistance to heat into Sabouraud's broth. After incubation at 27°C for 6–9 hours under shaking, the cells were collected by centrifugation and filtered through a sterile paper filter so as to separate the ungerminated conidia. Young germinants were harvested both by pouring M/15 neutral phosphate buffer on to the filter which retains germinants and by floating on it. The concentrations of germinants and conidia were standardized by optical absorbency with a spectrophotometer. Heat resistance of both cells was then compared in the same manner as described above. Data presented in Table 9 indicate a rapid loss of heat resistance of the conidia occurring during germination. The young germinants arising from the conidia were killed off by short exposure, less than 10 min, to 60°C.

Table 9 Changes in heat-resistance of *A. fumigatus* conidia during germination

Survival time at 60°C (min)	Conidia 4-day-old on Sabouraud's glucose agar at 37°C	Germinant 1 obtained by incubating the conidia for 6 hours at 30°C	Germinant 2 obtained by incubating the conidia for 9 hours at 30°C
alive	60	9	8
dead	70	10	9

The concentration of conidia and germinants was standardized by the use of a spectrophotometer and adjusted to 0.14 optical density at 600 mμ wavelength.

DISCUSSION

Few reports have been published on the resistance of fungus spores to heat. Difficulties to obtain a great number of spores in a pure state considerably prevent the study of their physiology and biochemistry. Since Faul (1930)¹⁾ pointed out the susceptibility of conidiospores of *Neurospora crassa* to injury by heat, fungus spores, in general, have been considered as they have no heat resistance or little, if any, except for a certain type of species, i.e., ascospores of *N. crassa*²⁾³⁾. Recently, Tada⁴⁾ found that conidia of *A. fumigatus* remained viable at 60°C for more than 3 hours. Kitamura, Suenaga & Nishio⁵⁾ also reported that chlamydospores and macroconidia of *Epidermophyton floccosum* subjected to 55–60°C survived longer than vegetative hyphae. These results may be interpreted as indicating a part of the peculiar physico-chemical aspects of fungus spores, which are different from parent hyphae. The previous publications, however, omitted any mention or illustration of the detailed experimental conditions and the factors affecting the heat-stability of fungus spores.

It is long known that *A. fumigatus* is thermotolerant and can grow and sporulate over a range of temperature of 25–50°C. This temperature “resistance” also serves as a basis for its isolation and identification from clinical materials⁶⁾. Thus, resistance of *A. fumigatus* conidia to heat may be expected to a certain extent. In the present paper, the heat resistance of conidia of *A. fumigatus* grown under varying conidiating conditions have been investigated by 4-tube method. Also, the resistance to heat of the conidia harvested from 6 other species of *Aspergillus* has been compared. It was suggested that only conidia of *A. fumigatus* strains show a notably high resistance to heat. Further, the heat resistance was influenced by the conditions of growth, age, and other factors. The age of conidia and the temperature of conidiation are two important factors which decide their heat-stability. However, it is assumed that these findings would have been more available if the data had been expressed in percent germination of conidia rather than visible growth. Observation of appearance of mycelial growth up to 2 weeks after heat treatments, in tubes that contained 10⁷ or more conidia, could be rather misleading. Even a single surviving conidium can germinate and produce visible growth in a few days after treatments. Therefore, quantitative data based on the percent germination of heated conidia will be required for the confirmation of heat resistance. A comparative and statistical study of the heat resistance by a certain parameter is now under way in the author's laboratory.

SUMMARY

The heat resistance of conidiospores of *A. fumigatus* grown under varying sporulating conditions has been studied. To obtain a reliable and reproducible result, spore clumps were removed by filtration through a sterile paper filter and thermal death time was determined by 4-tube method. Matured conidia of *A. fumigatus* formed at 37°C were of the highest heat tolerance among those produced by seven species of *Aspergillus* used. They survived heating for more than 60 min at 60°C, whereas both their parent hyphae and the conidia of other *Aspergillus* species were killed off by less than 10-min exposure to 60°C. The age of conidia and the temperature of conidiation were of two dominant factors which affect their heat resistance.

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