Irreversible Block of the Mycelial-to-Yeast Phase Transition of *Histoplasma capsulatum*

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p-Chloromercuriphenylsulfonic acid (PCMS), a sulfhydryl inhibitor, prevented the mycelial-to-yeast transition of the dimorphic fungal pathogen, *Histoplasma capsulatum*. The effect of PCMS was specific for the mycelial-to-yeast transformation; it had no effect on growth of either the yeast or mycelial forms or on the yeast-to-mycelial transition. The failure of PCMS-treated mycelia to transform to yeast was permanent and irreversible. PCMS-treated mycelia could not infect mice but could stimulate resistance to infection by a pathogenic strain of *Histoplasma capsulatum*. These results suggest a new general strategy for vaccine development in diseases caused by dimorphic pathogens.

MANY PATHOGENS CHANGE FORM OR PHASE DURING AN infection. For example, some dimorphic fungi shift from mycelia in the soil to yeast in the blood or tissues of infected individuals (*1*). It is now possible to block this transition in several fungi, thereby producing organisms that are noninfective but still capable of vaccinating the host against the pathogen. These results suggest a new general strategy for vaccine development for diseases due to dimorphic pathogens.

Most of our studies have been done with the Downs strain (*2*) of the dimorphic pathogenic fungus *Histoplasma capsulatum*, which grows as a multicellular mycelium at 25°C but as a yeast at 37°C. Temperature shifts between 25° and 37°C trigger the transition from one phase to the other. Three distinct stages in the normal mycelial-to-yeast transition of the Downs strain of *H. capsulatum* have been defined (*2*). Immediately after the temperature shift, there is a rapid decline in intracellular adenosine triphosphate (ATP) that follows uncoupling of oxidative phosphorylation (*3*). There is then a progressive decrease in respiration rate (stage 1). After 24 to 40 hours, the cells enter a dormant period (stage 2) that lasts 4 to 6 days. Stage 2 cells have sharply decreased concentrations of mitochondrial electron transport components. Stage 3 is characterized by restoration of cytochrome components, resumption of normal respiration, induction of the yeast phase-specific cysteine oxidase, and completion of the transition to yeast morphology (*4*).

Previous results have suggested that sulfhydryl compounds are involved in the mycelial-to-yeast transition (*5*). Specifically, cysteine was shown to have at least two roles in completion of the mycelial-to-yeast transition of the Downs strain of *H. capsulatum*: (i) cysteine and other sulfhydryl-containing compounds function to maintain respiration and ATP levels by stimulating the shunt pathways that bypass the temporary block in cytochrome components during stage 2 of the mycelial-to-yeast transition (*4*); and (ii) since yeast are auxotrophic for cysteine, probably because enzymes involved in cysteine biosynthesis are repressed after mycelial phase cells are shifted from 25° to 37°C (*6*), the cellular requirement for cysteine becomes specific later in the transition (stage 3).

It was therefore not surprising to find that a sulfhydryl blocking agent, *p*-chloromercuriphenylsulfonic acid (PCMS), prevented the mycelial-to-yeast transition of *H. capsulatum*. Unexpectedly, however, the block was irreversible and appeared to be inherited. Moreover, inoculating mice with the organisms blocked in the mycelial phase protected them against subsequent infection with a virulent strain of *H. capsulatum*.

To understand the blockage induced by PCMS, the growth characteristics of control and PCMS-treated mycelia were compared. Exposure to 100 μM PCMS for 24 hours had no effect on subsequent growth of mycelia washed free of the PCMS and incubated at 25°C; these mycelial cultures grew in subcultures in the absence of PCMS at the same rate as control mycelia at 25°C (Fig. 1). When control cultures were switched to 37°C, mycelia grew for 24 hours, then showed a characteristic lag before further growth and transformation to yeast. Mycelia treated with PCMS for 24 hours and then washed free of the sulfhydryl inhibitor also showed an initial brief growth period at 37°C; by contrast, however, their subsequent lag was indefinite. They did not transform to yeast and after several weeks of incubation at 37°C they had become vacuolated and lysed.

The lethal effect of incubation at 37°C was established within 24 hours. Subcultures from the PCMS-treated mycelial cultures 24 hours after the 25° to 37°C shift-up in temperature no longer grew on solid or in liquid medium at either 37° or 25°C, even in the absence of PCMS. Furthermore, PCMS prevented the transition of normal mycelia to yeast if it was added within 48 hours of the temperature shift-up. Mycelia treated with PCMS more than 48 hours after the shift to 37°C, however, formed yeast cells normally even in the presence of PCMS. Such yeast grew normally and could be transformed back to mycelia by shifting the temperature back to 25°C. Nevertheless, the PCMS had had an effect because subsequent transformation of these mycelia back to yeast was now blocked, and the cells died when incubated at 37°C. Thus, a 24-hour exposure to PCMS under standard conditions did not affect the growth of yeast at 37°C, the growth of mycelia at 25°C, or the transition of yeast to mycelia at 25°C. However, when PCMS-treated yeast had once transformed to mycelia at 25°C, they were then unable to transform back to yeast at 37°C. This inability was permanent and irreversible

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despite multiple passages of mycelia in medium without PCMS.

Protein synthesis paralleled the growth of cultures. In control cultures shifted to 37°C, protein synthesis fell to undetectable levels after 24 hours and resumed after 72 hours. In contrast, in mycelia treated with PCMS protein synthesis dropped to zero after 24 hours but never resumed.

Concentrations of PCMS as low as 10 μM and incubations for only 3 hours inhibited the mycelial-to-yeast transition, but the most reproducible and consistent results were seen with a standard treatment of 100 μM PCMS for 24 hours.

Studies of energy metabolism in the control and PCMS cultures show that after the shift from 25° to 37°C (Fig. 2) an almost immediate uncoupling of oxidative phosphorylation took place in both the control (A and B) and PCMS-treated mycelia (C and D). Coupling of oxidative phosphorylation was assayed indirectly by the ability of an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (Cl-CCP), to stimulate respiration in the presence of oligomycin, an inhibitor of ATP synthetase (*3*). Cell respiration assays were carried out in the presence of salicylhydroxamic acid (SHAM) to inhibit the alternate oxidase and force electron flux through the cytochrome systems (*7*). At 25°C, addition of oligomycin to control or PCMS-treated mycelia (Fig. 2, A and C) resulted in a modest inhibition of respiration, suggesting that the rate was already close to state IV. Subsequent addition of the uncoupler (Cl-CCP) resulted in a four- to sixfold increase in respiration rates in both cultures, indicating that respiration was coupled to oxidative phosphorylation. By contrast, when both mycelial cultures were incubated at 25°C and then shifted to 37°C, there was an increase in respiration rate, no inhibition by oligomycin, and no stimulation by the subsequent addition of uncoupler (Fig. 2, B and D). This new pattern, indicative of uncoupled respiration, began immediately after the temperature shift and was complete by 5 minutes. It included a decrease in intracellular ATP in both cultures. By 5 minutes after the shift-up in temperature, ATP in both control and PCMS-treated mycelia had declined from 0.18 to less than 0.01 μmol per gram of mycelia.

The uncoupling of oxidative phosphorylation and the decrease in ATP were followed by a more gradual decrease in respiration (stage 1), which fell to zero during the first 24 hours (stage 2). Stage 2 persisted for 3 to 6 days in control cultures, after which respiration returned and the cells transformed to yeast (stage 3). In contrast to the control cultures, the PCMS-treated mycelia switched from 25° to 37°C remained in a dormant state (stage 2) and never entered stage 3. Once respiration in these cultures ceased, it never reappeared for as long as the cultures were observed (14 days in the experiment shown in Fig. 3).

The changes in ATP levels and respiration in PCMS-treated mycelia shifted to 37°C were correlated with a decrease in the cytochrome components. Cytochrome spectra of PCMS-treated mycelia were identical to those we reported in normal cells (*4*). Table 1 summarizes the content of electron transport components of mycelia at different times after the shift-up, calculated from low-temperature difference spectra of dithionite-reduced minus oxidized mitochondria. In control cells, the concentrations of electron transport components declined over the first 24 hours after the temperature shift. After about 3 days, cytochromes c and aa_3 were virtually undetectable and cytochrome b was decreased to approximately one fourth of the initial level. The residual cytochrome b was not reduced by succinate, suggesting that it was inactive in respiration. The minimum concentration of electron transport components occurred at about 6 days after the temperature shift. After that, the concentrations of cytochrome components increased to normal levels as yeast began to appear in the cultures, and the transition was completed by 12 to 13 days. Unlike the normally transforming

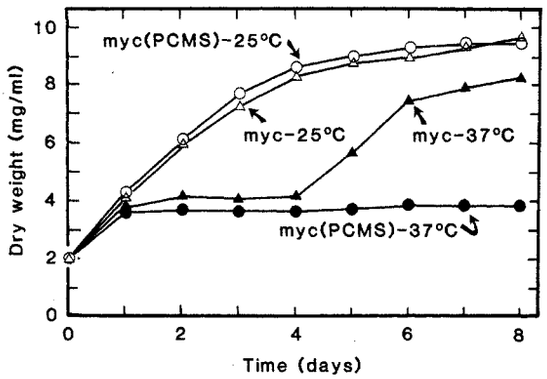

Fig. 1. Growth of control and PCMS-treated cultures of mycelia of the Downs strain of *H. capsulatum* incubated at 25° and 37°C. Cells were grown in 2 percent glucose plus 1 percent yeast extract (*4*). The cultures were started with a constant inoculum of cells and were grown to mid-log phase, which occurred after 48 hours of incubation at 25°C. PCMS (Sigma) was then added to one set for 24 hours and then removed from the cultures by filtration and washing with medium. The cultures were then diluted 1:5 in fresh medium, divided among several flasks, and incubated at 25° or 37°C. The contents of the flasks were filtered and dried at 42°C to determine dry weights. For this and the other figures, the results shown are typical of at least three independent experiments.

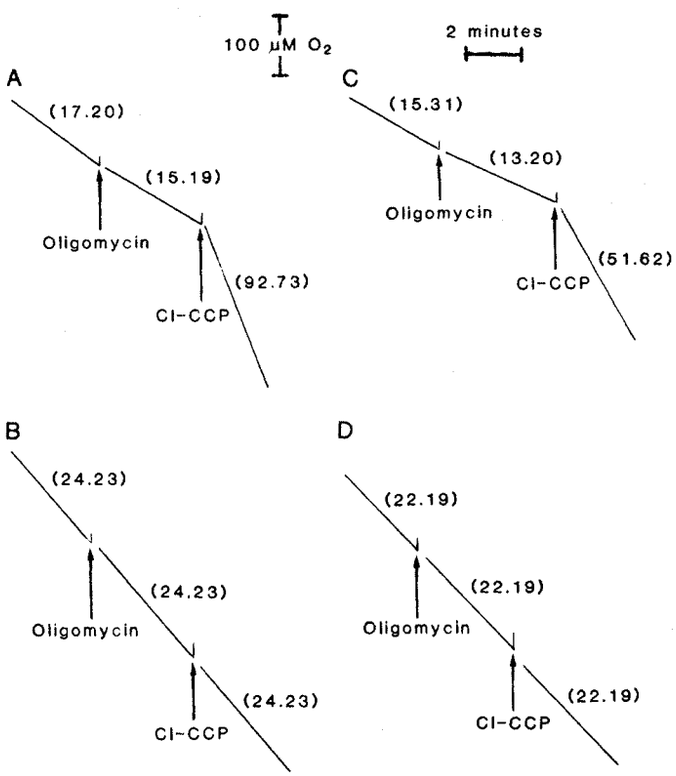

Fig. 2. Oxygen electrode recordings of respiration of mycelial- and yeast-phase cells. Cells [0.5 ml, 0.5 to 0.7 mg (dry weight) per milliliter] were resuspended in respiration buffer containing 1 percent mannose, 1 mM $CaCl_2$, and 1 mM dimethylglutaric acid (*p*H 7.2). Oxygen concentrations were measured polarographically with a KIC oxygraph equipped with a Clark-type oxygen electron (Gilson). The values in parentheses are rates of oxygen consumption [microliters of O_2 per hour per milligram (dry weight)]. Additions were of oligomycin (5 μg/ml) and Cl-CCP (0.1 mM) (both from Sigma). They were dissolved in absolute ethanol and were prepared freshly for each experiment. Cell respiration assays were carried out in the presence of 0.5 mM SHAM (*7*). The experiment was repeated six times with independent cultures and essentially identical results were obtained. (A and C) Mycelia at 25°C; (B and D) mycelia 5 minutes after shift from 25° to 37°C; (A and B) control mycelia; (C and D) PCMS-treated mycelia.

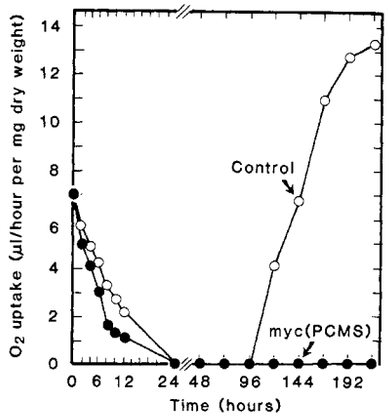

Fig. 3. Oxygen uptake after temperature shift from 25° to 37°C. The cells were suspended in cell respiration buffer containing 1 percent mannose, 1 m*M* $CaCl_2$ and 1 m*M* dimethylglutaric acid (*p*H 7.2). Oxygen concentrations were measured polarographically using a KIC oxygraph. Cell respiration rates were expressed as microliters of O_2 per hour per milligram of cells (dry weight) at 37°C.

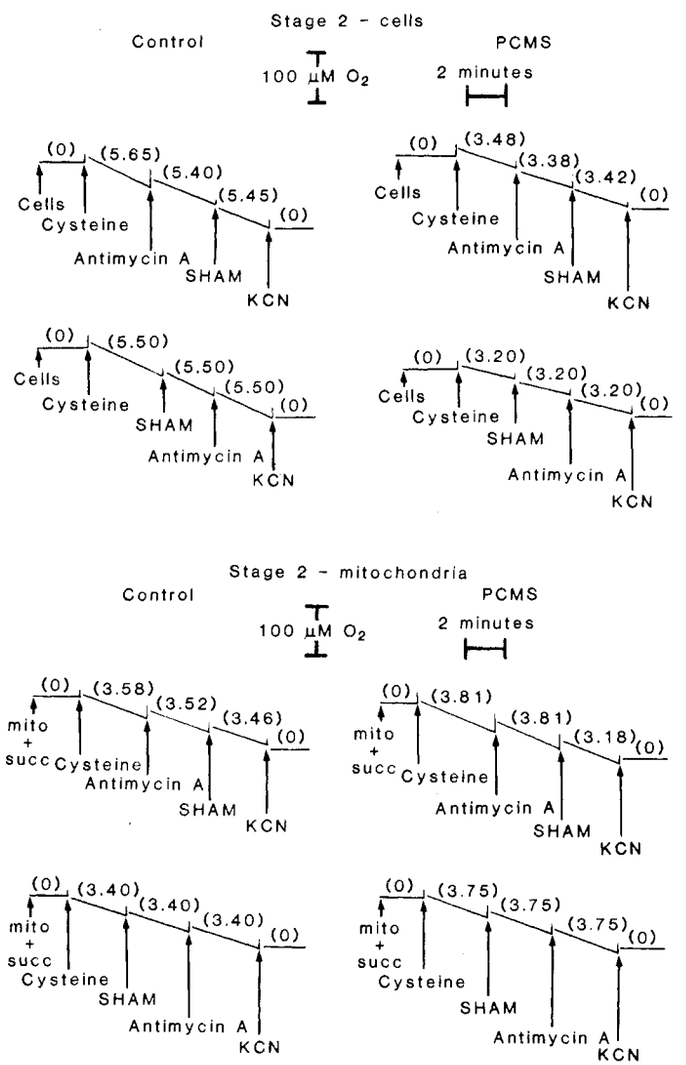

Fig. 4. Sulfhydryl shunt pathways in control and PCMS-treated cells and mitochondria 24 hours after temperature shift from 25° to 37°C (stage 2) demonstrated by O_2 electrode recordings of respiration of cells and mitochondria. Additions to the mycelia [0.5 ml; 0.7 mg (dry weight) per milliliter] and mitochondria (1.0 mg/ml) were KCN (Sigma), 0.5 m*M*; SHAM (Aldrich), 0.5 m*M*; antimycin (Sigma), 1.0 µg/ml; and cysteine, 1.6 m*M*. The values in parentheses are rates of oxygen consumption in microliters of O_2 per hour per milligram of cells (dry weight) and microatoms of O per minute per milligram of mitochondria.

mycelia, cytochrome levels in the PCMS-treated cultures never returned to normal.

Thus, PCMS-treated mycelia appeared to pass through stages 1 and 2 of the transition but never entered stage 3. The point at which PCMS became lethal was correlated with the point at which cysteine became required (*2, 4*). A part of the response to cysteine, however, remained intact. Normally transforming stage 2 cells are dependent on a sulfhydryl shunt for maintenance of respiration and generation of a low level of ATP (*4*). Both normal and PCMS-treated mycelia 24 hours after the 25° to 37°C temperature shift (stage 2) did not have measurable respiration without added cysteine (Fig. 4). In addition, mitochondria isolated from these cells also showed no measurable respiration in the absence of cysteine. Like the normal sulfhydryl shunt pathways (*4*), the stimulation of respiration by cysteine in the control and PCMS-treated cells and mitochondria required the presence of a respiratory substrate and was resistant to the mitochondrial respiration inhibitors SHAM plus antimycin and sensitive to SHAM plus KCN. However, the induction of cysteine oxidase activity, specific to the yeast phase, did not occur (*8*). Cysteine was able to stimulate respiration in PCMS-treated cells and mitochondria for about 12 to 14 days after the temperature shift-up. Therefore, these cells were probably in a dormant state (stage 2), unable to complete the transition to yeast or return to the mycelial form when the temperature was returned to 25°C. Adding excess cysteine to the medium could not reverse the effect of PCMS and force the cells to transform to yeast. Uptake of cysteine into PCMS-treated cells took place and, in fact, early in stage 2 uptake was greater in PCMS-treated cells than in control cells.

There appeared to be no difference in the virulence of normal and PCMS-treated yeast after intravenous injection into CD-1 mice. The LD_{50} values of each were approximately 20×10^6 to 22×10^6 yeast per milliliter and spleens of infected animals were positive for *H. capsulatum* both by microscopic examination and by culturing organisms.

In contrast, as had been seen in vitro, PCMS-treated mycelia did not convert to yeast after intraperitoneal injections of 0.2 to 2 mg (dry weight) of cells into CD-1 mice. Cultures of spleens taken from mice killed 1 to 4 weeks after infection were negative. Histopathologic examination of hematoxylin-eosin stained sections of spleens revealed mild inflammation without organized granulomas or multinucleated giant cells, and fungi were not seen in sections stained by the Gomori methenamine-silver procedure (*9*). As expected, granulomas and yeast cells were seen microscopically in sections of splenic tissue removed from control mice injected intraperitoneally with untreated mycelia and cultures of this tissue were positive for *H. capsulatum*. The abortive infection with PCMS-treated Downs mycelia was sufficient to provoke a protective immune response in the mice. A range of dosages of PCMS-treated Downs mycelia could significantly increase survival of mice after infection with yeast of the virulent G186B strain of *H. capsulatum*, and the increase in survival was proportional to the dose of the immunizing inoculum (Fig. 5).

Three distinct stages in the normal mycelial-to-yeast transition of the Downs strain of *H. capsulatum* have been described (*2*). The results in the present study indicate that the behavior of mycelial cultures treated with the suylfhydryl inhibitor PCMS was aberrant when they were switched to 37°C. They progressed through stage 1 and entered the dormant state (stage 2) but never progressed to the recovery phase in which yeast cells appeared (stage 3). Since sulfhydryl-containing compounds themselves can drive the phase transition in stage 2 (*10*), PCMS affected at least some of the regulatory changes, presumably by blocking a particular sulfhydryl-sensitive step.

The specific function inhibited by PCMS in stage 2 is unknown,

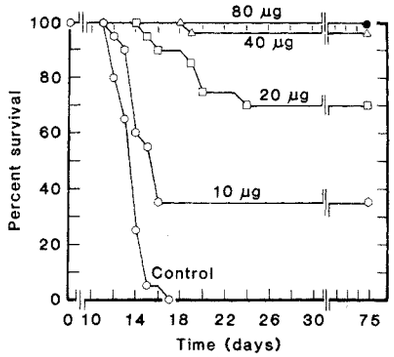

Fig. 5. Survival of CD-1 mice after immunization with several different concentrations of viable PCMS-treated mycelia (given three times intraperitoneally at weekly intervals) and challenged with 1×10^7 yeast cells given through a tail vein in 0.1 ml of buffer 24 hours after the last immunization. Each group contained ten mice.

although the sulfhydryl blocking agent is believed to react with proteins on the external surface of the cell membrane (*11*). It has been shown that PCMS can cause covalent modification of one or more sulfhydryl groups affecting receptors for estrogen and glucocorticoid uptake into cells (*12*), and can stimulate chemically induced differentiated Friend erythroleukemia cells to synthesize hemoglobin (*13*). Other reagents that affect sulfhydryl groups can prevent spore germination by covalent modifications (*14*). However, the effects on receptors and spore germination were reversible when the sulfhydryl blocking agent was removed from the cultures (*12–14*), whereas the effects of PCMS on *H. capsulatum* were permanent and irreversible.

The fact that treated yeast can be passaged for many generations and still retain the capacity to transform to viable mycelia, but produce mycelia that die when challenged to return to the yeast phase, implies that PCMS affects a hereditary change in the organism. One possibility is that a plasmid encoding at least one gene required during the switch of mycelia to yeast is cured or inactivated by PCMS treatment. A second is that the switch requires a chromosomal translocation somewhat analogous to the casette shifts that occur during yeast mating (*15*) or the variation of surface antigens in trypanasomes (*16*). The former possibility has received some support in that both ethidium bromide and PCMS can cure plasmids in *Saccharomyces cerevisiae* (*17*). Ethidium bromide also blocked the transition of mycelia of the Downs strain of *H. capsulatum* to yeast, with the subsequent death of the organisms challenged at 37°C (*18*).

A question of long-standing interest is whether the transition of mycelia to yeast is required for the pathogenicity of *H. capsulatum*. PCMS-treated Downs mycelia do not answer this question since pathogenesis is interrupted because the cells die when the phase transition fails. The blockage of pathogenicity is thus an epiphenomenon, but it is a very interesting one if the abortive infection is sufficient to provoke a protective immune response in animals. The results of this study show that infection with lethal doses of a virulent strain of *H. capsulatum* is attenuated and there is a significant increase in survival of mice that had previously received PCMS-treated Downs mycelia as a vaccine.

Functions sensitive to PCMS may be critical for phase transitions in other dimorphic pathogenic fungi (*19*) or even in organisms like trypanosomes and sporulating bacteria. For any infectious organism that changes from one form to another as part of its life cycle and has a pathogenic process as part of a defined progression of gene expression, a new route to vaccination against it may be possible: mutations in a gene involved in the transition beyond the point of commitment, or irreversible blockage of the transition by treatments similar to PCMS, will cause it to die at that point and serve as an effective vaccinating inoculum.

Table 1. Concentrations of cytochromes (cyt) in mitochondria isolated from control and PCMS-treated mycelia at several times after the 25° to 37°C temperature shift. The cytochrome concentrations were calculated from low-temperature difference spectra of dithionite-reduced minus oxidized mitochondria by the method of Williams (*20*), with the use of the extinction coefficients of Lambowitz *et al.* (*4, 21*). Similar results were found in three independent experiments.

Days	Cytochrome concentration (nmol/mg mitochondrial protein)					
	Control cultures			PCMS-treated cultures		
	Cyt aa_3	Cyt b	Cyt c	Cyt aa_3	Cyt b	Cyt c
0	0.15	0.25	0.17	0.16	0.27	0.17
1	0.02	0.11	0.05	0.06	0.14	0.10
2	ND*	0.07	0.05	0.02	0.10	0.07
3	ND	0.06	ND	ND	0.08	ND
4	ND	0.03	ND	ND	0.04	ND
6	ND	0.02	ND	ND	0.04	ND
7	ND	0.04	ND	ND	0.04	ND
14	0.04	0.18	0.12	ND	0.04	ND
17	0.24	0.20	0.16	ND	ND	ND

*ND, not detectable.

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