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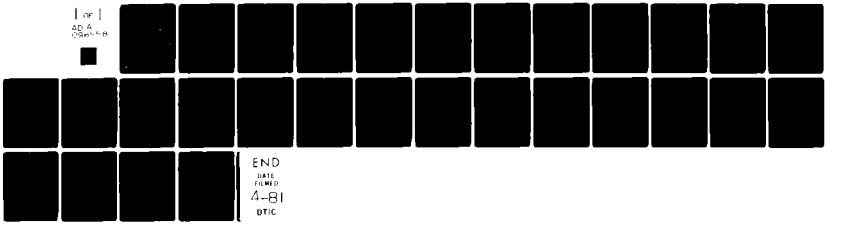

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number):

The major achievements made during this contract period have been summarized in this final report. In addition, the conditions affecting the survival of Trichophyton mentagrophytes arthrospores and their resistance to various physical, chemical and biological agents were described.

The effects of several physical and chemical agents on the survival of Tricho phyton mentagrophytes arthrospores were investigated. Although arthrospores →

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of this dermatophyte were highly resistant to chilling and freezing, they were extremely susceptible to moderate heat (above 50°C) and desiccation. This high susceptibility could be significantly reduced when they were dried in the presence of exogenous proteins. These arthrospores were markedly susceptible to glutaraldehyde. They appeared to be significantly more resistant than their hyphal counterparts to common antimycotics such as clotrimazole, griseofulvin, miconazole nitrate, and nystatin. Clinical and epidemiological implications of these observations are discussed.

FINAL REPORT

ONR Contract # N00014-76-C-0376

Task No. NR204-023

CONTROL OF DERMATOMYCOSES BY PHYSICAL, CHEMICAL AND BIOLOGICAL AGENTS

February 28, 1981

submitted by

M. J. Blumenthal, Ph.D., Professor and Chairman

and

T. Nashimoto, M.D., D. Med. Sci., Professor

Department of Microbiology
Loyola University Stritch School of Medicine
Maywood, Illinois 60153

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Several lines of evidence listed below have led us to believe that the key to the radical cure of dermatomycoses and the control of ringworm infections in communal life is to develop effective methods that kill dermatophytic arthrospores in infected lesions or in our environment.

1. Arthrospores are the only dormant or resistant form of dermatophytes produced in lesions. The presence of abundant arthrospores in active and chronic lesions has been well documented in the literature.

2. Once arthrosporulated, the dermatophyte Trichophyton mentagrophytes cells become extremely resistant to several antifungal agents commonly used in the chemotherapy of dermatomycoses (T. Hashimoto and E. J. Blumenthal, Appl. Environ. Microbiol. 35:273-277, 1978). In other words, most lesions containing arthrospores are highly recalcitrant to chemotherapy.

3. Arthrosporulation of the dermatophyte T. mentagrophytes is in fact significantly stimulated by the presence of sublethal doses of antifungal agents such as clotrimazole, nystatin and griseofulvin (Emyanitoff and Hashimoto, 1979, Can. J. Microbio. 25:362-366, 1979). The use of insufficient doses of antifungal agents rapidly converts susceptible hyphae to resistant arthrospores.

4. Arthrospores are fully capable of germinating into infectious hyphae by the normal skin constituents. In fact, some of the aged arthrospores are quite capable of transforming into infectious hyphae in the presence of water only. (T. Hashimoto and E. J. Blumenthal, Infect. Immuno. 18:479-486).

These observations may account for our common experience that the radical cure of dermatomycoses is difficult to achieve despite the extensive use of various chemotherapeutic agents. We now strongly believe that neither radical cure nor the control of ringworm infections in communal life would be possible unless effective methods for killing arthrospores are made available for general use.

Weighing these factors carefully we have decided to concentrate our research effort on the following four specific areas: (1) testing of various physical, chemical and biological agents on arthrospores of T. mentagrophytes and (2) elucidating the morphological and physiological basis of arthrospore resistance to various agents (3) chemical and immunological characterization of arthrospore walls and (4) treatment of experimentally produced dermatomycoses with glutaraldehyde. The second approach is considered important because such studies may provide useful clues to the development of effective arthrosporocidal agents.

I. Physical, chemical and biological agents with arthrosporocidal activity-potential candidates for antidermatomycotic agents.

We took several different approaches to develop methods that effectively inactivate dermatophytic arthrospores in vitro and in vivo.

1. Topical application of moist and warm temperature prior to and during active chemotherapy.

The rationale behind this approach is based on our observation that dormant and activated arthrospores are able to germinate and transform into hyphae which are highly susceptible to antifungal chemotherapeutic agents. It appears to us that the continuous application of either topical or systemic chemotherapeutic agents to dried (most physicians advise keeping the lesions as dry as possible!) skin lesions is a futile attempt. This approach will be tested in an animal model and an in vivo experiment is now in progress.

2. Topical application of chemicals with arthrosporocidal activity.

The arthrosporocidal activity of dialdehyde (glutaraldehyde) has been thoroughly tested and established in our laboratory (Technical report #2). The therapeutic as well as antiseptic effect of this compound against arthrospore bearing lesions is shown in Table 2 and described in the later section.

3. Topical application of enzymes that actively digest the cell wall of arthrospores.

As described later, one of the cellular factors contributing to the poor penetrability of reagents into arthrospores appears to be the thick wall. It seems reasonable to search for enzymes that digest off the thick wall thus making arthrospores more susceptible to antifungal chemotherapeutics or unable to survive in osmotically unstable environments. Although we indicated this possibility in our earlier report, a more likely applicability of such enzymes in the treatment of dermatomycotic lesions emerged when we elucidated the structure and chemical composition of the wall of T. mentagrophytes arthrospores. The chemical analysis of purified cell wall of T. mentagrophytes revealed that glucans (mostly β1:3 linked) and chitin are the major wall constituents. Interestingly, these glucan-chitin components are directly exposed to the surface of the wall and are readily accessible to the lytic activity of exogenous glucanase and chitinase. Based on this reasoning, we have tested the arthrosporocidal effects of various lytic enzymes and the results of such tests will be described in the following section.

A. Resistance of T. mentagrophytes arthrospores to physical and chemical antifungal agents.

The effects of several physical and chemical agents on the survival of Trichophyton mentagrophytes arthrospores were investigated. Although arthrospores of this dermatophyte were highly resistant to chilling and freezing, they were extremely susceptible to moderate heat (above 50°C) and desiccation. This high susceptibility could be significantly reduced when they were dried in the presence of exogenous proteins. These arthrospores were markedly susceptible to glutaraldehyde. They appeared to be significantly more resistant than their hyphal counterparts to common antimycotics such as clotrimazole, griseofulvin, miconazole nitrate, and nystatin. Clinical and epidemiological implications of these observations are discussed.

Effect of temperature. Essentially all T. mentagrophytes arthrospores were inactivated within 2 min at 60°C and almost 90% became nonviable within 5 min at 50°C (Fig. 1). Evan at 48°C, approximately 50% were killed within 30 min. In contrast to this, the arthrospores were remarkably resistant to chilling and freezing. Those stored in distilled water at 4 or 10°C remained viable (90%) for as long as 2 months. More than 95% of the arthrospores could survive for more than 1 year, and approximately 65% remained viable for 2 years when stored in distilled water at -20°C.

Effect of desiccation. The arthrospores were exceptionally susceptible to desiccation. Both air-dried (25°C) and lyophilized arthrospores lost their viability rapidly during storage, regardless of the storage temperature (Table 1). Their extremely high susceptibility to desiccation was reduced significantly when they were dried in the presence of exogenous proteins (Fig. 2).

Effect of ultraviolet light. The kinetics of killing of arthrospores exposed to ultraviolet light from a common laboratory germicidal lamp is illustrated in Fig. 3. Essentially all were killed within 10 min under our experimental conditions.

Effect of selected disinfectants and chemotherapeutic agents. Resistance to ethanol, phenol, and glutaraldehyde is summarized in Table 2. A remarkably high susceptibility to low concentrations of glutaraldehyde may be worth noting and will be further discussed later. Figure 4 illustrates the survival of arthrospores exposed to various concentrations of antifungal chemotherapeutic agents. It is evident that those exposed for 24 h to the drugs at concentrations usually lethal to the hyphal growth could remain viable and were able to germinate once the drugs were removed. Under our experimental conditions, it is not certain whether the drugs adsorbed to arthrospores were completely removed during the washing process. In any event, the high degree of resistance to T. mentagrophytes arthrospores to these chemotherapeutic agents had not been observed previously.

B. Sporocidal Activity of Cell Wall Lytic Enzymes

METHODS OF EXPERIMENT

Glusulase (snail intestinal juice) was purchased from Endo Laboratories, and β-1:3 glucanase was prepared from an imperfect fungus and provided by Dr. Nagasaki. These enzymes were relatively stable when stored at 4°C. (No significant loss of activity was seen for 6 months). Arthrospores were produced and purified according to the method described earlier (Technical Report #2).

Approximately 10^7 ml of arthrospores were mixed with enzyme solutions and incubated at 30°C for predetermined periods. In some experiments, the enzymes were diluted with 0.05 M citrate phosphate buffer (pH 5.5) to obtain desired concentrations. Aliquots of the mixture were filtered through millipore filter (pore size 0.45 μm) and cells retained on the filter were washed with sterile Sabouraud dextrose broth. Washed cells were incubated in Sabouraud dextrose broth for 15 hourse and the viability of enzyme treated arthrospores was determined microscopically as described earlier (Hashimoto and Blumenthal, Appl. Environment. Microbiol. 35:273-277, 1978). For each sample, a total of 200 spores was examined and the percentage of survived cells was calculated.

RESULTS

Typical survival curves of T. mentagrophytes arthrospores exposed to glusulase and purified β-1:3 glucanase are shown in Fig. 5. The isolated cell walls of T. mentagrophytes arthrospores were highly susceptible to the lytic action of β-1:3 glucanase (Fig. 6) and chitinase (data not shown) which were the two major lytic principles of glusulase.

SIGNIFICANCE

The susceptibility of fungal apores to lytic enzymes is dependent on their chemical composition as well as on their location within the wall. The whole spores may not be lysed by lytic enzymes when the substrate is surrounded by an impervious layer resistant to the enzymes. On the other hand, if the entire spore wall is made of materials sensitive to the lytic action of given enzymes, spores may be lysed rapidly regardless of its thickness. The ultrastructural and chemical studies showed that the majority, if not all, of the arthrospore wall of T. mentagrophytes is made of β-1:3 glucans and chitin, and these components are directly exposed to the surface of the wall suggesting an easy accessibility of glucanase and chitinase to the major cell wall components. Since electron microscopy of glusulase or β-1:3 glucanase killed arthrospores demonstrated that they still retained a considerable amount of the wall materials, it appears that the injuries in the cytoplasmic membrane resulting from the partial disintegration of the wall are the primary cause of death of enzyme treated arthrospores. These observations coupled with the relatively stable nature of the enzymes, make these enzymes prospective therapeutic agents in the topical treatment of dermatomycoses. Very recently, a possibility of systemic and topical use of wall lytic enzymes for the treatment of deep seated and superficial mycotic infections has been proposed by Davies and Pope (Nature 173:235-236, 1978).

II. Structural and physiological properties uniquely associated with arthrosporulation of T. mentagrophytes.

In order to find some unique structural and physiological characters that may be responsible for the resistance of dermatophytic arthrospores, we examined T. mentagrophytes arthrospores by means of electron microscopy and analyzed the chemical composition of certain subcellular components. The results so far obtained indicate that there are at least two structural components that characteristically associated with arthrospores. It may be plausible that the inhibition of the development of these structures or the specific destruction of these cellular components might lead to the rapid killing of arthrospores.

A. Arthrospore wall.

Electron microscopy of T. mentagrophytes clearly revealed that the wall of arthrospores is several times thicker than that of vegetative hyphae. During arthrosporulation, cells synthesize a thick new wall layer immediately beneath the existing hyphal wall. By the time spores became mature most of the outer wall was lost and the cytoplasm was surrounded by a new thick (sometimes it exceeds 1 μm!) wall synthesized de novo during sporulation. During this ultrastructural study, we experienced considerable difficulties in fixing and embedding mature arthrospores. We attributed these difficulties to the thick wall of arthrospores. The penetration of the fixatives and other reagents seemed to be hampered by the impervious wall. This speculation was confirmed by our subsequent observation that the spheroplasts of arthrospores could be readily fixed for thin sectioning. Although further experimentation is needed to draw a more definitive conclusion, the resistance of arthrospores to various antifungal agents may well be related to the presence of the unique spore wall.

B. Lipid and carotenoid granules.

One of the most striking observations made during this study was the demonstration of carotenoid pigments within the lipid granules of T. mentagrophytes arthrospores. In T. mentagrophytes these pigments were shown to occur only in arthrospores. No trace of such pigments was found in either the hyphal or microconidial form of this dermatophyte. Although the significance of these pigments in the resistance of arthrospores is not immediately clear, we believe that these observations are highly significant because carotenoid pigments in plants are considered to be vital for the protection of the photosynthetic apparatus from ultraviolet injury. In view of this possible significance the full technical detail related to this work will be included in this report.

TABLE 1. *Effect of desiccation on viability of T. mentagrophytes arthrospores*

Desiccation conditions	Storage temp (°C)	% Survival after:		
		24 h	48 h	168 h
Air dried on cheesecloth, 25°C	4	52	39	0
	25	34	18	0
	37	29	11	0
Lyophilized[a]	4	21	0	0
	25	11	3	0

[a] Arthrospores suspended in sterile distilled water were lyophilized as described in the text.

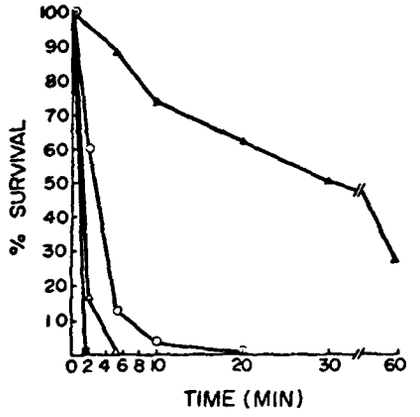


FIG. 1. *Effect of heat on viability of T. mentagrophytes arthrospores. Arthrospores were suspended in distilled water and heated for various periods at 60°C (■), 55°C (△), 50°C (○), or 48°C (▲), after which the viability was determined as described in the text.*

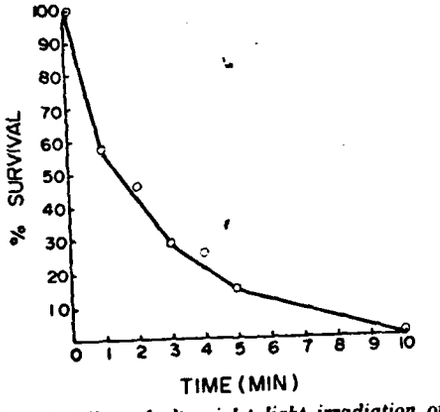


FIG. 3. *Effect of ultraviolet light irradiation on viability of T. mentagrophytes arthrospores.*

TABLE 2. *Arthrosporocidal activities of aqueous ethanol, phenol, and glutaraldehyde at 25°C*

Exposure time (h)	% Survival after exposure to:					
	Ethanol (70%)	Phenol		Glutaraldehyde		
		0.1%	1.0%	0.001%	0.01%	0.1%
0.5	0	93	0	97	7	0
1	0	88	0	90	0	0
3	0	85	0	90	0	0

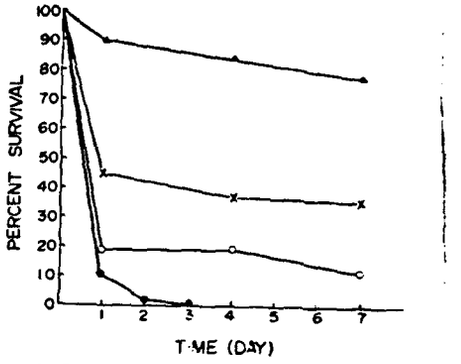


FIG. 2. *Protective effect of proteins on survival of lyophilized T. mentagrophytes arthrospores. Arthrospores were lyophilized in the presence of specified proteins as described in the text and stored at 25°C. Symbols: ▲, powdered skim milk; ×, albumin; □, gelatin; and ●, control (no proteins). All points except for day 1 data for gelatin are significantly different ($P < 0.01$) from those of the controls.*

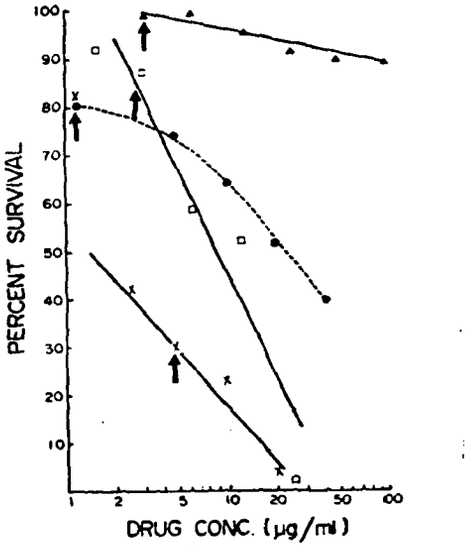


FIG. 4. *Effect of a 24-h exposure (25°C) to various concentrations of antidermatomycotic drugs on survival of T. mentagrophytes arthrospores. Symbols: ●, clotrimazole; ×, nystatin, ▲, griseofulvin; and □, miconazole nitrate. Arrows indicate minimal inhibitory concentration of each drug for hyphal growth.*

Fig. 5

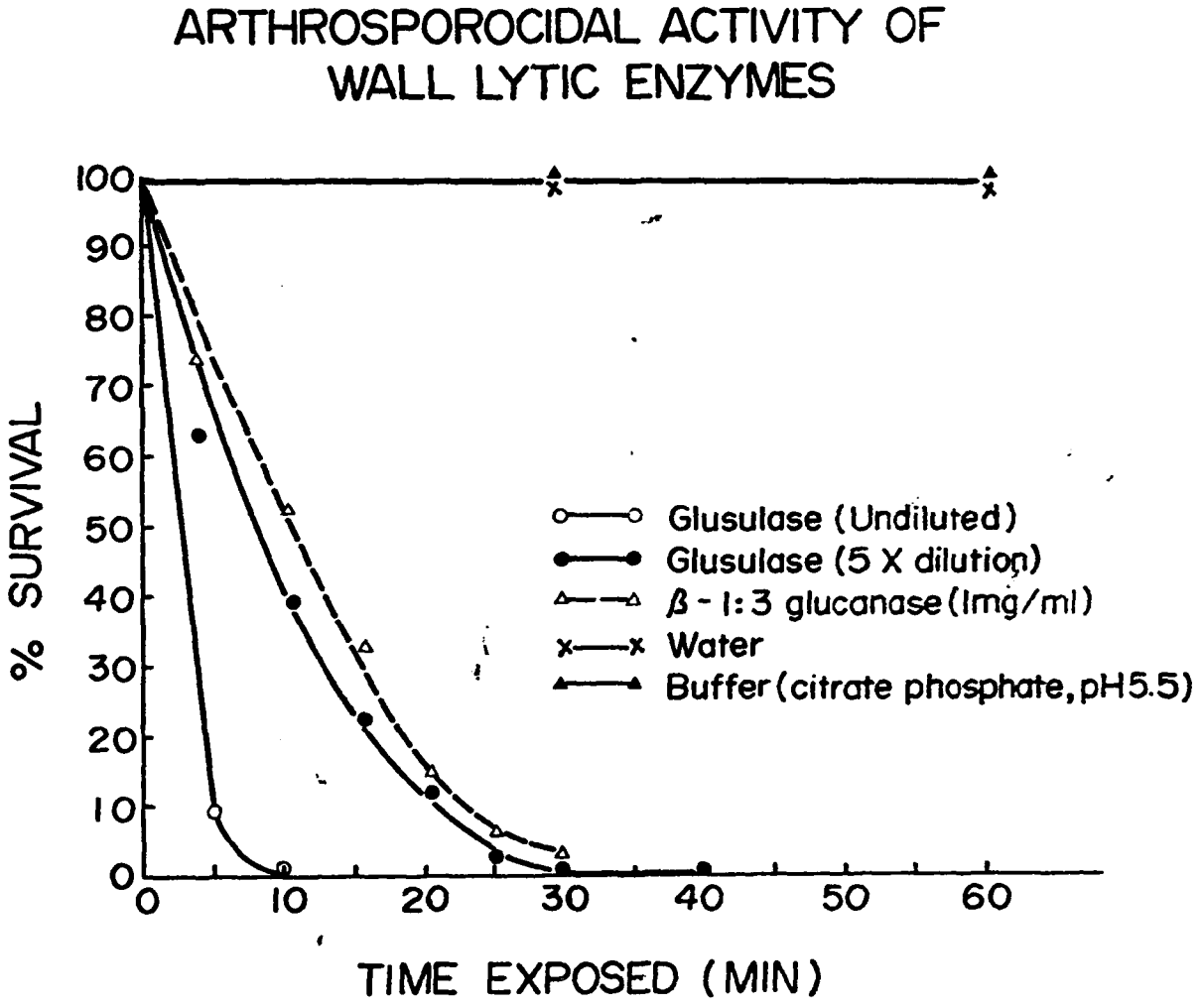
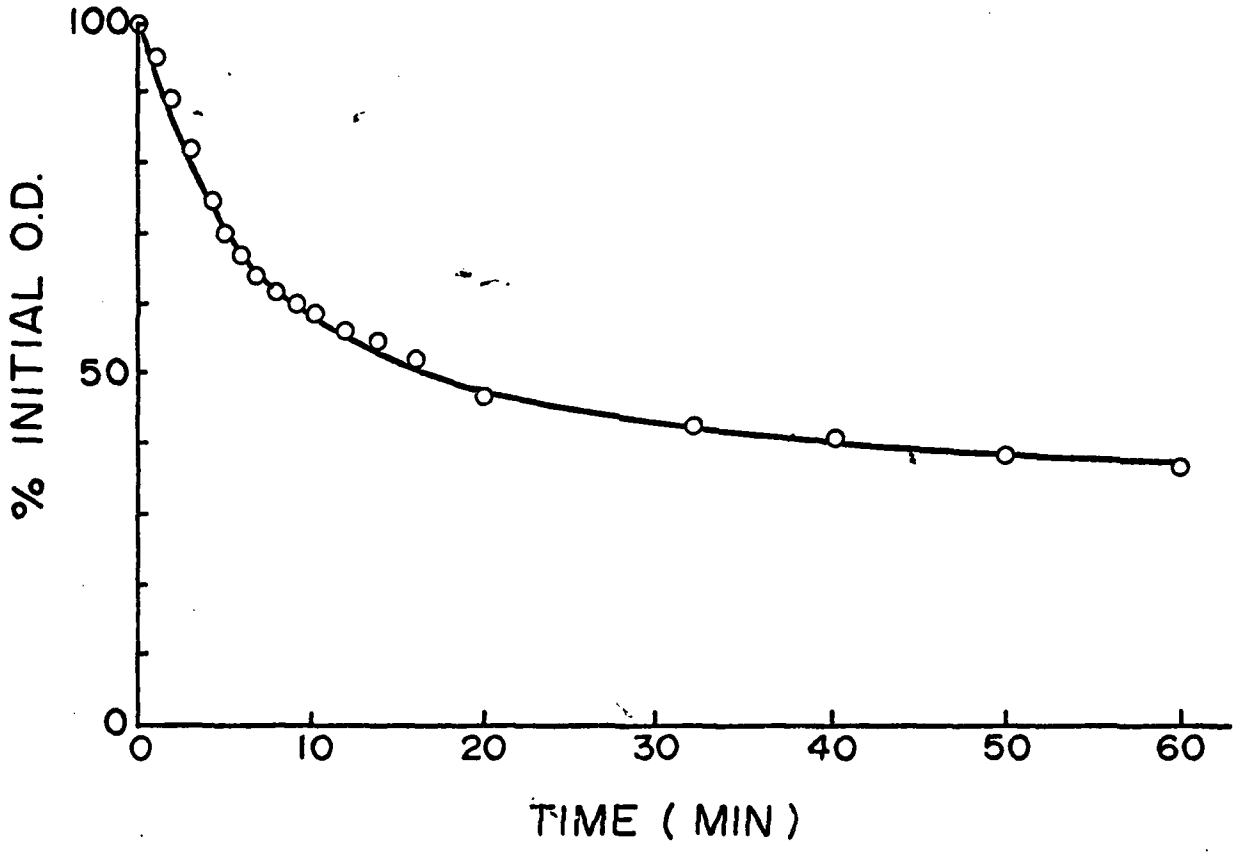

ARTHROSPOROCIDAL ACTIVITY OF
WALL LYTIC ENZYMES
% SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
TIME EXPOSED (MIN)
Glusulase (Undiluted)
Glusulase (5 X dilution)
β - 1:3 glucanase (1mg/ml)
Water
Buffer (citrate phosphate, pH5.5)

Fig. 6

LYSIS OF ARTHROSPORE WALLS BY
β 1:3 GLUCANASE



One of the most logical approaches to the biological control of dermatomycoses is to immunize susceptible individuals with properly prepared vaccine. This prospect has been considerably enhanced in the recent years when it has been clearly demonstrated that the local delayed hypersensitivity is critically important in the resistance to dermatomycoses (Grappel et al. Bacteriol. Rev. 38:222-250, 1970, Poulain et al., J. Invest. Dermatol. 74: 205-209, 1980). Currently, no such vaccine with proven efficacy is available.

In view of the important role played by dermatophytic spores, especially arthrospores, which are produced only during the parasitic phase of their life cycle, in the transmission of the dermatomycotic infections in the communal life, it was reasoned that certain cellular components of arthrospores would be excellent sources of immunogens which might be used in the preparation of dermatophytic vaccine. It was noted earlier by electron microscopy that considerable modification and differentiation of the cytoplasmic and wall structures occur during arthrospore formation. It is likely that arthrospores contain cytoplasmic and wall constituents which are chemically and immunologically quite distinct from those of their hyphal or microconidial counterparts. Since the cell wall of dermatophytic arthrospores had never been characterized chemically or immunologically, we first attempted to isolate and purify them from T. mentagrophytes ATCC26323, a strain originally isolated from a U.S. military personnel in Vietnam and its pathogenicity was confirmed in our laboratory. As described below, various fractions of the purified cell wall of T. mentagrophytes arthrospores have been chemically characterized and their immunological and antigenic properties will be investigated in the future. Arthrospores of T. mentagrophytes ATCC26323 were produced by the method developed in our laboratory during this ONR contract work and the detailed procedure for preparation of arthrospore has been described in our earlier technical report (P. 9, Technical Report #3).

Preparation of arthrospore walls. Arthrospores were broken using a French pressure cell. In addition, arthrospores were also broken in a Mickle cell disintegrator at 4°C using acid-cleaned glass beads (170-180 µm, B. Braun Melsungen Appartebau). Three to four 20 min treatments, with alternating 10 min colling periods achieved 99% breakage. Glass beads and unbroken arthrospores were allowed to settle for 15 min. The broken arthrospore walls removed and washed to remove cytoplasmic debris as described earlier.

Alkali extraction of arthrospore and hyphal walls. Cell walls (50 mg) were extracted with freshly prepared 1N NaOH in capped teflon centrifuged tubes (Malge) which were flushed with nitrogen before closing to retard destruction of sugar polymers. In one experiment the walls were pretreated with sodium borohydride (Fisher) using the procedure of Bardalaye and Nordin to prevent destruction of sugar polymers. However, this pretreatment was ineffective. The alkali extraction (100°C, 1h) was repeated three times. The combined extracts were filtered through a teflon filter (Milipore, 0.45 µm), neutralized with 6 N HCl, dialyzed against distilled water at 4°C for 48 h, and lyophilized.

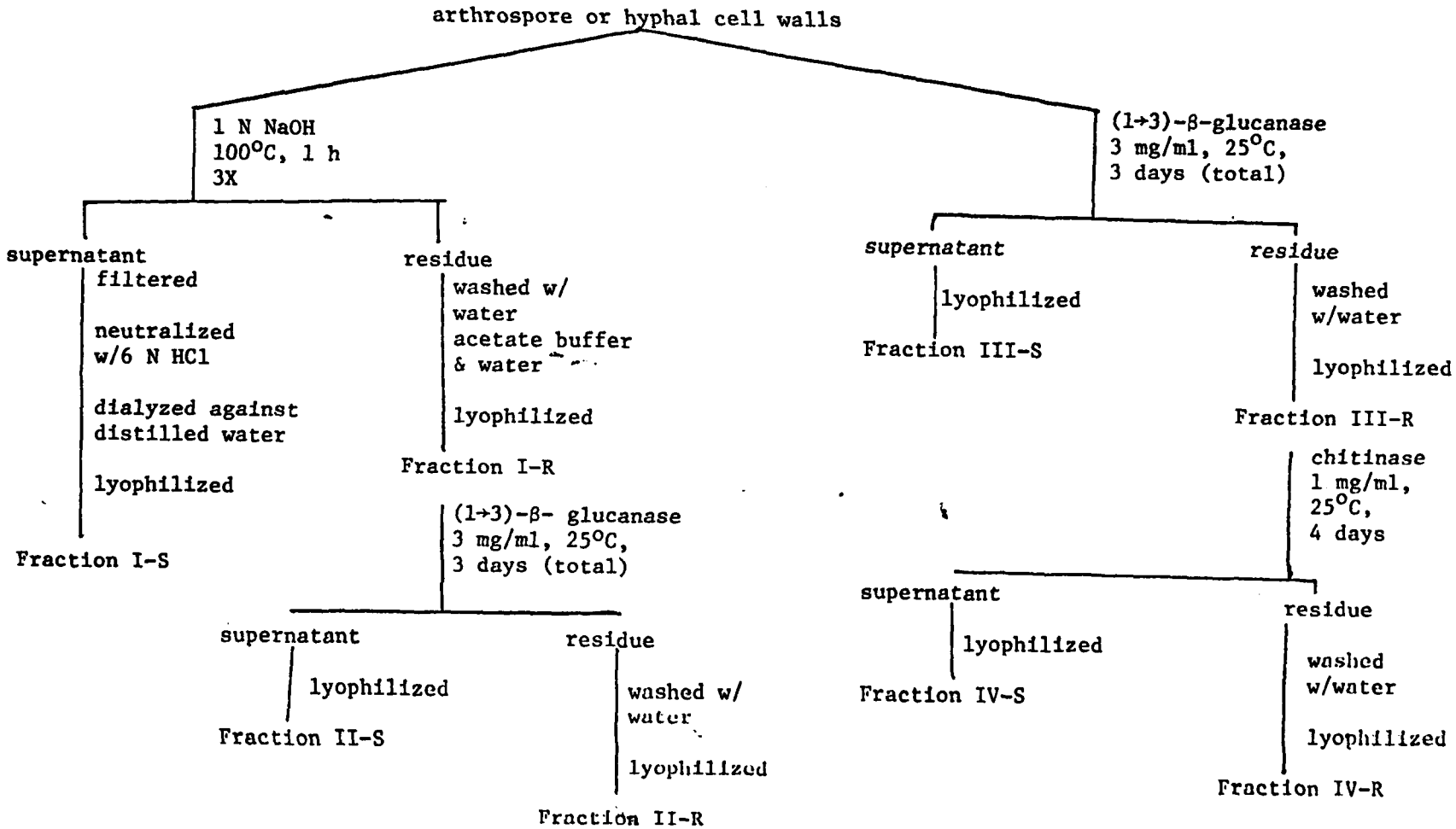
The alkali insoluble fraction washed with distilled water five times until the wash water was neutral. To completely remove any traces of alkali the residue was suspended in a sodium acetate buffer (0.1 M, pH 5.5) overnight at 4°C, washed with the buffer twice more, followed by four washes with distilled water to remove the buffer and lyophilized.

Enzymatic digestion of the cell walls. The enzymes used in this study included chitinase (Sigma Chemical Co.), (1→3)-β-glucanase (provided by S. Nagasaki, Japan), and Glusulase (a mixture of various hydolytic enzymes, including chitinase and (1→3)-β-glucanase, Endo Laboratories). Chitinase, which was used without further purification, was found to be slightly contaminated with (1→3)-β-glucanase and possibly an α glucanase, as paper chromatographs of concentrated chitinase lysates of laminaran [(1→3)-β-glucan] and of the lyophilized alkali extract of the arthrospore wall (which does not contain β-glucan) both revealed glucose spots. The glucanase was shown to be completely free of all chitinase activity. Cell walls were digested with (1→3)-β-glucanase (3 mg/ml of distilled water) for 24 h. The glucanase was replaced with fresh enzyme and the digestion was continued for 48 h more. The residue was collected by centrifugation (5,000 X g for 15 min) and washed 5 times with distilled water. The residue was further digested with chitinase (1 mg/ml of distilled water) for 48 h at room temperature. The chitinase was replaced with fresh enzyme and the digestion was continued for 48 h more. The residue was collected and washed as previously and then lyophilized. All enzymes were filtered (0.45 µm pore size, Millipore) prior to use, and one or two drops of toluene were added to prevent bacterial growth. In order to ascertain that the enzymatic digestions were complete, a small amount of wall residue was suspended in fresh enzyme solution in a colorimeter tube (absorbance between .200 to .300) and the absorbance monitored for a 6 h period. If there was no drop in absorbance during this period the digestion was considered complete. The enzyme hydrolysates were checked for the absence of bacterial contamination and lyophilized.

An outline of the chemical and enzymatic digestions are shown in figure 7.

The chemical composition of the arthrospore cell wall is very similar to the composition of the hyphal wall from which it derives. Both contain about the same neutral sugars, glucose, mannose and galactose in approximately the same quantities (50%, 10%, and 5%) as determined by dry weight measurement (Table 3). Both arthrospore and hyphal walls contain approximately the same percent hexosamines (18.6% and 17.7%). Both values were calculated without considering whether the hexosamines are aceylated. The differences in wall composition seem to be in minor components. The hyphal walls contain about three times more protein (11.2% to 4.1%) and twice as much lipid (1.8% to 0.7%). Both wall types contain negligible amounts of phosphorus (0.2% and 0.1%).

Figure 7. Fractionation procedure of arthrospore and hyphal cell walls.



In order to determine if any quantitative differences in wall components exist, the walls were treated with hot alkali (1 N NaOH, 100°C, 3 h). This treatment solubilizes most wall polymers with the exception of chitin and β glucans. The hot alkali removed essentially all the wall protein, mannose, galactose from both wall types (Table 1). A further 67% of the wall glucan was removed from the hyphal walls and 74% of the glucan from the arthrospore wall. Small amounts of glucosamine (0.9%) and galactosamine (2.1%) are also removed. Hydrolysis of the residue, followed by paper chromatography of the hydrolysates, reveals the presence of only two components, glucose and glucosamine. There seems to be significant differences between the hyphal and arthrospore walls in this residue. The hyphal wall contains more glucose (52.1% to 39.4%) which would indicate that the hyphal wall contains more β glucan than the arthrospore wall. On the other hand the arthrospore walls contain almost twice as much glucosamine (42.4% to 22.7%) as does the hyphal walls. This would seem to indicate that the arthrospore wall has almost twice the amount of chitin found in the hyphal wall.

In order to further elucidate the nature of the alkali resistant polymers the arthrospore walls were further digested with enzymes. Digestion of Fraction I-R (Fig 3) with (1→3)-β-glucanase removes 94% of the glucose from that residue (Table 4). Paper chromatography of the enzyme hydrolysates reveal the presence of glucose and glucose oligomers, supposedly gentiobiose [β(1→6) linked glucose] and laminaribiose and laminaritriose [β(1→3)] linked glucose. This result would indicate the β-glucan is most probably linked 1→3 with side chains linked 1→3 and 1→6.

Digestion of the arthrospore wall with glucanase removes about 63% of the intact wall as determined by dry weight (Table 4). This figure is much greater than the percent of (1→3)-β-glucan in the wall (16%, Table 1). As the glucanase showed no activity on the alkali soluble Fraction (I-S, Fig. 2), it is possible that this might indicate some type of linkage between the β-glucan and the alkali soluble polymers. The (1→3)-β-glucanase was also shown to release 1.0% N-acetyl glucosamine from the arthrospore wall, as measured by the method of Reissig (16). Since the glucanase was free of chitinase, the acetylated glucosamine is not part of the chitin polymer.

Under the phase contrast microscope no visible change can be observed in the arthrospore wall appearance following digestion by alkali or glucanase either individually or sequentially. However, following digestion with chitinase there is an observable thinning of the wall. Nevertheless, extensive digestion with chitinase does not seem to destroy the cell wall shape. There also appears to be a much higher percentage of septa in the residue as compared with preparations of intact walls. Electron micrographs of this fraction (IV-R) show complete dissolution of the chitin fibrils in both walls and septa (T. Nashimoto, J. E. Pollack, and H. J. Blumenthal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, J 23, p. 92).

This fraction is 13.4% of dry weight of the intact wall. Chemically, it contains mainly glucosamine (66.2%) with only a small amount of neutral sugars (4.7%), (Table 5). It is not known whether this glucosamine polymer is acetylated. The chitinase does remove 47% of Fraction III-R in the form of N-acetyl glucosamine (Table 5) which is equivalent to 17.5% of the arthrospore wall. Therefore at least 17.5% of the arthrospore wall is chitin.

In order to determine the nature of Fraction IV-R, it was subjected to various chemical and enzymatic treatments. It was not solubilized by hot 1N acetic acid, hot 1 N HCl, or HNO_2, treatments known to dissolve chitosan (deacetylated chitin). On the other hand it dissolved in cold 6 N HCl and 30% NaOH conditions at which chitin is resistant. The residue is resistant to lysozyme and proteases followed by chitinase (Table 6).

Amino acid analysis of the arthrospore walls show aspartate threonine, serine, glutamine, proline, and glycine to constitute 66.8% of all amino acids. In Fraction I-R lysine and histidine are the major amino acids (54.3% and 15.2% in the arthrospore wall fraction and 41.3% and 10.3% in the hyphal wall fraction, Table 7).

We made some limited attempts to use glutaraldehyde, which has been shown to have fungicidal activity against all forms of dermatophytes (microconidia, macroconidia, arthrospores and vegetative hyphae) at relatively low concentrations, in the treatment of experimental dermatomycoses produced in guinea pigs. Our earlier data (Technical report I and II) clearly show that T. mentagrophytes parasitically growing or arthrosporulating in isolated human nail and hair are rapidly killed by a brief exposure to 1% alkaline glutaraldehyde solution. However, all attempts to prove the therapeutic efficacy of such glutaraldehyde treatment in experimentally produced dermatomycoses have been severely hampered by the rapid spontaneous cure of the experimentally produced lesions in guinea pigs. Such rapid spontaneous cure of the infection makes an accurate assessment of the drug effect extremely difficult. The dermatophytes lesions produced by T. mentagrophytes arthrospores (approximately 10^7 spore/cm^2) were treated topically with 1% alkaline glutaraldehyde (stored in sealed glass ampules or plastic envelopes) twice daily. However, these dermal lesions completely cured by day 21 regardless of such glutaraldehyde treatment. With the present guinea pig model, it was not possible to prolong the course of infection beyond three weeks. It is apparent that a new animal model which more closely mimics chronic human dermatomycoses is urgently needed for more accurately assessing the efficacy of this prospective antidermatomycotic agent. As noted recently by Gorman et al. (J. Appl. Bacteriol. 48: 161-190, 1980) relatively low toxicity of glutaraldehyde ensure that it is an important disinfectant and chemosterilizer with a potentially wide field of microbiological and possibly therapeutical uses.

Table 3. Chemical composition of the hyphal and arthrospore walls before and after alkali extraction.

Component	Composition (1%) Intact Wall H[a]	Intact Wall A[b]	I-S[d] H (44.4)[c]	I-S[d] A (57.0)[c]	I-R[d] H (55.6)[c]	I-R[d] A (43.0)[c]
Neutral Sugars[e]	71.0	66.4	60.2(92.8)[f]	61.2(100.3)[f]	52.1	39.4
glucose	56.7	52.1	38.8(59.8)[f]	42.0(68.8)[f]	52.1	39.4
mannose	8.3	10.1	14.7(22.7)[f]	13.4(22.0)[f]		
galactose	6.0	4.2	6.7(10.3)[f]	5.8(9.5)[f]		
Hexosamines[e]	17.7 (15.9)[gi]	18.6 (18.5)[gi]	1.0	1.0	27.0[gj] (24.0)	44.3 (35.0)[gj]
glucosamine	16.6 [gi]	17.5 [gi]	0.3[h]	0.3[h]	22.7[gj]	42.4 [gj]
galactosamine	1.1 [gi]	1.0 [gi]	0.7	0.7	4.3[gj]	1.9 [gj]
Protein[e]	11.2	4.1 (3.7)[gj]	19.4(20.3)[f]	7.6(11.3)[f]	---- (0.4)[gj]	---- (0.4)[gj]
Lipid[e]	1.8	0.7	NT	NT	NT	NT
Phosphorus[e]	0.2	0.1	NT	NT	NT	NT
Ash	NT[k]	NT	NT	NT	NT	NT
Total	101.9	89.9	80.6(114.1)	69.8(112.6)	79.5	84.1

a Hyphal wall.
b Arthrospore wall of 8-9 day old arthrospores. Similar values were obtained with 4 day old arthrospore walls.
c Figures in parentheses represent percent of dry weight of intact walls in each fraction. Data based on four to six experiments.
d See Fig. 1 for fraction designation.
e See Materials and methods for method of determination.
f Figure not in parentheses was determined by analyzing the alkali extract directly with first dialyzing and lyophilyzing sample. Figure in parentheses was determined by analyzing the lyophilyzed sample. The large differences are indicative of destruction of sugar polymers by alkali.
g Figures in parentheses represent analyses done using the automatic amino acid analyzer.
h Determined by the method of Wagner.
i Analysis following 6 h hydrolysis with 6 N HCl.
j Analysis following 22 h hydrolysis with 6 N HCl.
k Not tested.

Table 4. Carbohydrate composition of the arthrospore wall following enzymatic degradation.

Component	Composition (1%)		
	II-R[a] (28.7)[b]	III-R[a] (37.3)[b]	IV-R[a] (13.4)[b]
Neutral sugars[g]	4.0	9.4	4.7
Hexosamines[c]	53.8[d]	42.3[d]	66.2[e] (59.1)[f] (46.9)[d]

a See Fig. 2 for fraction designation.
b Figures in parentheses represent percent of dry weight of intact arthrospore walls in each fraction.
c Values are calculated as if all hexosamines are non-acetylated. If the hexosamines are 100% acetylated all values would be increased by a factor of 1.23.
d Determined using Winzler's modification of the Elson-Morgan method. Analysis done on hydrolysates which were dried under vacuum in order to remove HCl.
e Determined by the method of Johnson.
f Determined by Winzler's modification. Analysis was done on neutralized sample using a standard glucosamine solution prepared in an identical fashion.
g Measured using glucose as the standard.

Table 5. Percent N-acetyl glucosamine in arthrospore wall fractions.

Composition (%)[a]	
III-S[r]	IV-S[b]
1.0	17.5 (47.0)[c]

a Values calculated as percent (w/w) of the intact arthrospore wall, using Reissig's method.
b See Fig. 1 for fraction designation.
c Percent (w/w) of Fraction III-R.

Table 6. Effect of selected chemicals and enzymes on Fraction IV-R of the arthrospore wall.

Treatment	Effect[a]
1 N HCl, 100°C, 1 h	None
3 N HCl, 25°C, 1 h	None
3 N HCl, 100°C, 2 h	Partial hydrolysis, wall fragments still visible
6 N HCl, 25°C	Immediate dissolution
1 N NaOH, 100°C, 3 h	None
10% NaOH, 100°C, 3 h	None
20% NaOH, 125°C, 3 h	None
30% NaOH, 25°C, 1 h	Gradual dissolution
3.9M HNO_2 25°C, 3 h	None
1 N Acetic acid, 100°C, 2 h	None
0.025M $NaIO_4$, 25°C	None
followed by chitinase 6 h	None
Lysozyme (1 mg/ml) 24 h	None
Pronase (1 mg/ml), pH 7.8, 24 h	None
Alkaline protease (1 mg/ml), 24 h	None
Trypsin (1 mg/ml), 24 h	None
The above proteases followed with chitinase (1 mg/ml) 24 h	None

a Determined by phase contrast microscopy.

Table 7. Amino acid composition of hyphal and arthrospore walls before and after alkali extraction.

Amino Acid	mol %			
	Intact walls		I-R[a]	
	H[b]	A[c]	H	A
lysine	5.2[d]	3.3	41.3	54.3
histidine	trace	0.6	10.3	15.2
arginine	4.5	2.2	-	-
aspartate	12.6	8.4	6.9	4.8
threonine	7.3	10.5	1.4	1.0
serine	8.4	11.9	2.8	4.8
glutamine	11.1	12.2	2.1	4.8
proline	6.4	10.2	-	-
glycine	8.3	13.6	6.2	7.6
alanine	8.8	5.5	4.1	1.9
valine	6.2	7.1	1.4	-
methionine	1.3	3.1	-	-
isoleucine	4.7	4.4	6.9	1.0
leucine	7.9	3.8	2.1	1.9
tyrosine	3.0	1.1	5.5	-
phenylalanine	3.5	2.1	9.0	2.9

a See Fig. 7 for fraction designation.

b Hyphal walls.

c Arthrospore walls from 8-9 day old cells. Similar results were obtained with 4 day old walls.

d Data cited in C.D.R. Wu., Ph.D. Dissertation, Loyola University, 1977.

Summary of all research accomplished:

A method has been developed which enabled the preparation of large quantities of microscopically pure, viable micro- and arthrospores of a virulent strain of Trichophyton mentagrophytes. Under optimal conditions the microconidia of T. mentagrophytes underwent complete and synchronous germination via phase darkening, swelling and germ tube development within a span of a few hours in tryptone or peptone. This is the first report that germinating fungal spores undergo phase darkening prior to germtube development and allows comparison to be made between fungal and bacterial spore systems.

A search for the effective compounds in peptone which triggered germination of T. mentagrophytes resulted in the identification of 7 amino acids (leucine, isoleucine, valine, tryptophan, methionine, alanine, and glycine) which initiated the germination of the microspores as rapidly as that which occurred in peptone; either alone or in combination with yeast nitrogen base. A number of carbohydrates, including glucose were incapable of inducing germination and had no appreciable effect upon the germination (phase darkening) of the microspores induced by amino acids. The chemically defined germination medium proved to be highly useful for the assessment of inhibitors of germination and post-germinative growth of the dermatophytes and has greatly facilitated the screening of agents which might be useful for the sterilization of materials contaminated with infectious spores or hyphae of the pathogenic dermatophytes.

Several derivatives of leucine or valine (n-valeric acid, δ-amino-N-valeric acid, n-isovaleric acid, and isopentyl alcohol) were effective inhibitors of germination of T. mentagrophytes microconidia at concentrations less than 0.1%. Fatty acids ranging from C_6-C_{12} were also highly inhibitory for the T. mentagrophytes microconidial germination at the same range of concentration. It was also found that phenylmethylsulfonyl fluoride, a known proteinase inhibitor, completely inhibited the microconidial germination at 10^{-3}M. In contrast to these, griseofulvin, a common antiermatophytic antibiotic, had no significant inhibitory effect against microconidial germination although it was a highly effective inhibitor for the postgerminative hyphal growth.

The complete elucidation of the nutritional requirements for the germination of microconidia and the hyphal growth of T. mentagrophytes resulted in the elaboration of a chemically defined medium which is adequate for enumerating accurate microconidial counts. The medium consist of 1% glucose, 0.2% asparagine, 0.1% L-leucine, 0.05% KH_2PO_4, 0.05% $MgSO_4$, 0.2 mg/liter of $FeCl_2$ (pH 6.5). The omission of leucine from this medium only allowed the growth of vegetative hyphae of the fungus. This chemically defined medium is believed to be useful in assessing the antitrichophytic activity of various compounds in vitro.

Inhibition of growth of Trichophyton mentagrophytes ATCC 26323 was demonstrated when the conc. of both glucose and phosphate in the synthetic and complex media were increased. This inhibition of growth was enhanced when relatively high conc. of glucose (0.2 M) and phosphate (0.1 M) were incorporated in the media and autoclaved together. This inhibition was significantly alleviated when glucose and phosphate were autoclaved separately. Several other strains of Trichophyton and Epidermophyton were similarly sensitive to high conc. of glucose and phosphate whereas 2 species of Microsporum and other fungi such as Saccharomyces cerevisiae and Aspergillus niger were not. A partial characterization of the inhibitory substance(s) indicated that it was absorbed to activated charcoal, and eluted from the charcoal with cold ethanol.

Both microconidia and vegetative hyphae of T. mentagrophytes completely killed by moist heat treatment which is commonly employed for pasteurization. They are both equally sensitive to ultraviolet irradiation.

Both microconidia and hyphae of downy mutants derived from T. mentagrophytes SF306A/68 (granular strain) were more susceptible to alkaline glutaraldehyde than those of the parental strain; they were irreversibly inactivated (>99%) within 10 min of exposure to 0.01% glutaraldehyde at pH 7.5. To achieve the same effect for the granular strain, 0.1% glutaraldehyde was required. The antidermatophytic activity of glutaraldehyde appeared to be most critically influenced by both pH and the presence of organic materials, especially protein(s) and peptide(s) in the test environment. The previous exposure of keratinous tissues to glutaraldehyde did not enhance their resistance to the subsequent infection or parasitization by T. mentagrophytes. Under optimal conditions, human hair exposed to 0.1% alkaline glutaraldehyde for 30 minutes appeared to be less readily parasitized by T. mentagrophytes infected with hyphae or spores in the presence of an appropriate germinant.

Glutaraldehyde was also shown to be an effective fungicidal agent in experimental dermatomycotic infections of the nails (onychomycosis), a form of dermatomycosis most recalcitrant to conventional chemotherapy. When human nail clippings collected from healthy individuals were infected with T. mentagrophytes and incubated under high humidity and limited oxygen tension, the infecting fungus partially penetrated the nail surface and formed abundant arthrospores within several days. These arthrospores were able to survive at least for several months under most storage conditions tested. However, when exposed to 3 to 5% alkaline glutaraldehyde solutions (pH 7.5, 0.1 M Na phosphate buffer) for 30 to 60 minutes, they were rendered completely non-viable as determined by the loss of their germinability under the most favorable conditions. The duration required for the complete inactivation of arthrospores appeared to depend on the size of the nail clippings and the concentration of glutaraldehyde used. These results clearly suggest that glutaraldehyde may be effectively used for the treatment of tinea unguium.

By modifying the method of Knight (J. Invest. Dermatol. 59:427-431, 1973) we were able to grow T. mentagrophytes and M. gypseum in human stratum corneum cultured in vitro that mimicked the in vivo growth condition of the dermatophytes. Arthrospores inoculated on stratum corneum stripped off human skin germinated and invaded the keratinous tissue without addition of any exogenous nutrients. This culture provided an excellent semi-in vivo system suitable for testing the antidermatophytic activity of glutaraldehyde against the parasitic form of the dermatophytes. It was shown that 0.01% glutaraldehyde (pH 7.5) completely and irreversibly inactivated the germination ability of T. mentagrophytes and M. gypseum arthrospores. Since the arthrospores represent the most predominant parasitic form of the dermatophytes in chronic lesions of tenacious and recurrent dermatomycoses, such a high degree of susceptibility of the arthrospores to low concentrations of alkaline glutaraldehyde has further enhanced the applicability of this antidermatophytic agent in the treatment of ring worm infections.

Glutaraldehyde, by its intrinsic nature, tends to undergo polymerization and oxidation during storage, especially at an alkaline pH where its antidermatomycotic activity is most pronounced. In order to minimize such loss of antidermatophytic activity during storage, due to oxidation and polymerization, we tested a number of conditions that caused a minimum deterioration of glutaraldehyde. The results showed that the storage at 4 C under nitrogen gas provided the best protection against deterioration. A 10% aqueous solution of glutaraldehyde could be stored for as long as 2 years without any sign of oxidation or polymerization when sealed under nitrogen gas and stored at 4 C. Simple freezing of glutaraldehyde solution (10-50% aqueous solution) provided little protection against deterioration. However, it should be emphasized that a partial loss of antidermatophytic activity due to oxidation and polymerization does not critically affect the usefulness of the drug since glutaraldehyde is so effectively fungicidal at very low concentration provided that the drug is stored undiluted.

When glutaraldehyde (1-50%) was stored in sealed plastic containers or in sealed glass ampules, it retained antidermatophytic activity (at 0.1%) for more than one year. However, glutaraldehyde stored either in alkaline or acidic solutions underwent partial polymerization resulting in the reduction of its antidermatophytic activity regardless of the storage temperature (-10, 4, and 25 C) tested. The polymerized glutaraldehyde formed a white precipitate upon dilution with distilled water or buffers. Aside from its toxic effects on the skin and the mucous membrane of the host, some stabilizing device which minimizes the process of polymerization and simultaneously prevents oxidation appears essential if we are to adopt glutaraldehyde as an effective antidermatophytic agent for both prophylactic and therapeutic purposes.

Attempts to test the therapeutic efficacy of glutaraldehyde for dermatomycoses have been hampered by the lack of a suitable animal model in which one can produce chronic dermatomycotic infections similar to those seen in human cases.

Enzymes contained in the intestinal juice of snails were found to lyse and kill the hyphae, arthrospores and microconidia of T. mentagrophytes. The lysis of these dermatophytic cells occurred at room temperature at a pH range of 4.5-7.5. The enzymes were relatively stable at room temperature. The lethal action of the enzymes was particularly rapid on microconidia; 99.9% of a microconidial population were rendered non-viable within a couple of minutes at room temperature when 1:4 dilution of the enzymes were employed. The arthrospores, dormant form of the dermatophyte in vivo, were also rapidly killed by the action of the enzymes. Unlike many other fungi and their dormant forms, the hyphae and spores of the dermatophytes appear to have a cell wall which is extremely susceptible to the snail intestinal enzymes, possibly because its susceptible layer is directly exposed to the exterior environment.

The active principles of the snail intestinal juice that rapidly lysed and killed the hyphae and spores (arthrospores as well as microconidia) of T. mentagrophytes and M. gypseum were found to be (1→3)-β-glucanase and chitinase. The snail intestinal enzymes were relatively stable if stored at refrigeration temperature. The snail intestinal juice stored at 4 C for more than two years lysed and killed the hyphae and spores of T. mentagrophytes in less than 30 seconds. (1→3)-β-glucanase isolated from an unidentified species of fungi imperfecti and chitinase isolated from Aspergillus oryzae, showed similar lytic effects on the hyphae and spores of all dermatophytes tested.

The sporolytic activity of the enzyme was further enhanced when combined with chitinase (from Streptomyces griseus). Since these enzymes are abundant in nature and relatively stable, requiring no co-factors for their lytic activities, their therapeutic value may merit further investigation.

Arthrosporulation of T. mentagrophytes was found to be significantly stimulated when the fungus was incubated at 37°C under slightly reduced oxygen tension. No stimulation of arthrosporulation occurred with carbon dioxide. Under limited humidity, most arthrospores underwent rapid disarticulation forming either single- or short-chained arthrospores.

Single celled arthrospores were isolated, purified and used in the subsequent experiments. Arthrospores of T. mentagrophytes were extremely susceptible to moderate heat (55-60°C for 5 minutes) and desiccation. Once thoroughly desiccated, approximately 90% of arthrospores lost their viability within 12 hours and more than 98% became non viable in 24 hours at room temperature. Although conditions affecting such a high susceptibility of arthrospores to heat and desiccation needs more scrupulous analyses, the use of these simple physical agents for sterilization of dermatophytic arthrospores may merit further investigation. Although the hyphal growth of T. mentagrophytes was effectively inhibited by normal therapeutic doses of several clinically adopted drugs (griseofulvin, clotrimazole, and triacetin), arthrospores of the fungus were totally insensitive to these agents at concentrations 10 times higher than their minimal inhibitory concentrations. Arthrospores became susceptible to

these agents only after they underwent germination. Since dermatophytes in most chronic lesions are transformed into arthrospores, prolonged oral administration of griseofulvin appears to need reevaluation, particularly in view of a recent report (Cripps et al.: J. Invest. Dermatol. 68: 82-87, 1977) that griseofulvin causes porphyria in mice and leads to the development of hepatomas. Some common disinfectants such as lysol and hypochlorite were also ineffective against arthrospores at the recommended concentrations. Glutaraldehyde (0.01-0.001%) was one of a few agents that inactivated arthrospores as well as hyphae under conditions reported earlier.

The requirement for germination of T. mentagrophytes arthrospores has been completely elucidated. Exposure of dormant arthrospores to water rapidly conditioned spores to germinate. Such activated arthrospores could initiate germ tube formation in the total absence of any exogenous nutrients as long as sufficient humidity was provided. However, germination of arthrospores was significantly facilitated by trace amounts of several amino acids (glycine, leucine, valine, alanine, serine and threonine), peptides and salts, all of which occurred naturally in human skin. It is apparent from this observation that unless arthrospores of dermatophytes are eliminated from chronic lesions, complete cure of dermatomycoses can hardly be expected. We strongly feel that the eradication of arthrospores both in the patients' skin and the surrounding environment is by far the most important element in the successful control of dermatomycoses in communal life.

Germination of dormant arthrospores occurred only in rich complex media such as Sabouraud dextrose broth or vitamin-free Casamino Acids. However, once activated, arthrospores were able to germinate under wide ranges of pH (5.5 to 8.0, optimal 6.5) and temperature (20 to 39°C) in the presence of certain single amino acids or oligopeptides known to be present in the human cutaneous tissues. Dormant arthrospores could be activated by incubation in distilled water 25°C for 24 h or by brief exposure to sublethal doses of heat (45°C for 10 to 20 min). Approximately 20% of activated arthrospores underwent spontaneous germination at 37°C during an additional 18 h of incubation in distilled water. All monosaccharides, purines, pyrimidines, and nucleosides tested failed to induce germination of T. mentagrophytes arthrospores. Germination rate was affected by the concentration of germination inducers as well as that of arthrospores. The germination process of T. mentagrophytes arthrospores was found to be oxygen dependent and was relatively tolerant to NaCl.

Although arthrospores of this dermatophyte were highly resistant to chilling and freezing, they were extremely susceptible to moderate heat (above 50°C) and desiccation. This high susceptibility could be significantly reduced when they were dried in the presence of exogenous proteins. This may imply that dermatophytic arthrospores in fallen hairs and loosened

squames from infected individuals are quite able to survive under desiccated conditions for some time, posing a serious epidemiological problem. These arthrospores were markedly susceptible to glutaraldehyde. They appeared to be significantly more resistant than their hyphal counterparts to common antimycotics, such as clotrimazole, griseofulvin, miconazole nitrate and nystatin. On the basis of these observations, it was proposed that assessment of antidermatomycotic activities of prospective drugs for the treatment of ringworm infections or for the sterilization of materials contaiminated with dermatophytes should take into consideration their effect upon arthrospores as well as upon vegetative hyphae.

Publications

Full papers

(a) Hashimoto, T., C. D. Wu, and H. J. Blumenthal (1972), "Characterization of L-leucine induced germination of Trichophyton mentagrophytes microconidia." J. Bacteriol. 112, 967-976.

(b) Wu, C. D. (1973), "Germination of Trichophyton mentagrophytes microconidia." M. S. Thesis, Loyola University.

(c) Hashimoto, T., C. D. Wu-Yuan, and H. J. Blumenthal (1976). Isolation and characterization of the rodlet layer of Trichophyton mentagrophytes microconidial walls. J. Bacteriol. 127:1543-1549.

(d) C. D. Wu-Yuan and T. Hashimoto (1977). Architecture and chemistry of microconidial walls of Trichophyton mentagrophytes. J. Bacteriol. 129: 1584-1592.

(e) Hashimoto, T. and H. J. Blumenthal (1977), "Factors affecting germination of Trichophyton mentagrophytes arthrospores," Infection and Immunity, 18: 479-486.

(f) Hashimoto, T. and H. J. Blumenthal (1978), "Survival and resistance of Trichophyton mentagrophytes arthrospores," Appl. Environ. Microbiol. 35: 274-277.

(g) Hashimoto, T., J. H. Pollack, and H. J. Blumenthal (1978), "Carotenogenesis associated with arthrosporulation of Trichophyton mentagrophytes," J. Bacteriol. 136: 1120-1126.

(h) Emyanitoff, R. G., and T. Hashimoto (1979), "The effects of temperature, incubation atmosphere, and medium composition on arthrospore formation in the fungus Trichophyton mentagrophytes." Can. J. Microbiol. 25: 362-366.

Abstracts

(a) Hashimoto, T., C. D. Wu, and H. J. Blumenthal (1972), "Characterization of the amino acid induced germination of Trichophyton mentagrophytes microspores." Abstracts of the annual meeting of the American Society for Microbiology, 1972, p. 56.

(b) Hashimoto, T. and H. J. Blumenthal (1973), "Nutritional requirements for germination and postgerminative growth of Trichophyton mentagrophytes microconidia." p. 139. Abst. of the annual meeting of the American Society for Microbiology 1973.

(c) Hashimoto, T and H. J. Blumenthal (1974), Stimulation of the arthrospores formation of Trichophyton mentagrophytes by certain amino acids and peptides. p. 142 Abst. of the annual meeting of the American Society for Microbiology 1974.

(d) Greenberg, M. L., H. J. Blumenthal, and T. Hashimoto (1975), Inhibition of Trichophyton mentagrophytes growth by high concentrations of glucose and phosphate. p. 38. Abst. of the annual meeting of the American Society for Microbiology 1975.

(e) Wu, C. D. R., T. Hashimoto, and W. A. Samsonoff (1975), The fine structure and chemical composition of Trichophyton mentagrophytes microconidial walls. p. 180 Abst. of the annual meeting of the American Society for Microbiology 1975.

(f) Hashimoto, T., C. D. Wu-Yuan, and H. J. Blumenthal (1976), Isolation and characterization of the rodlet layer of the microconidial wall of Trichophyton mentagrophytes. Abst. of the Annual meeting of the American Society for Microbiology 1976.

(g) Emyanitoff, R. G., H. J. Blumenthal and T. Hashimoto (1977), Effects of temperature and oxygen on arthrosporulation in Trichophyton mentagrophytes. Abst. of the annual meeting of the American Society for Microbiology 1977.

(h) Hashimoto, T., Pollack, J. H., and Blumenthal, H. J. (1978), "Carotenogenesis associated with arthrosporulation of Trichophyton mentagrophytes," J21, Abst. annu. meet. Amer. Soc. Microbiol., 1978.

(i) Emyanitoff, R., Mock, R., Hashimoto, T., and Samsonoff, W. (1978), "Ultrastructure of arthrospore formation in Trichophyton mentagrophytes, J11, Abst. annu. meet. Amer. Soc. Microbiol. 1978.

(J) Hashimoto, T., J. H. Pollack, and H. J. Blumenthal (1979), "Ultrastructure and chemical composition of Trichophyton mentagrophytes arthrospore walls and septa." Abstracts of the annual meeting of the American Society for Microbiology. p. 92, 1979.

(k) Mock, R., and T. Hashimoto. Effect of visible light on carotenogenesis of Trichophyton mentagrophytes. I. 26, p 88, Abstracts of the annual meeting of the American Society for Microbiology, 1980.

List of major accomplishments:

a. Development of an in vitro method to produce a large quantity of pure arthrospores of Trichophyton mentagrophytes which are normally seen only in infected tissues. This has made the systematic and detailed characterization of the parasiti form of dermatophyte spore possible.

b. Complete elucidation of physiological and nutritional requirements for the formation and germination of T. mentagrophytes microconidia and arthrospores. This has partially clarified the nutritional basis of their pathogenesis and promoted significantly, our understanding of the pathophysiology of dermatophytes.

c. Clarification of the physical conditions necessary for inactivation of infectious hyphae and spores of T. mentagrophytes thus providing the information essential for sterilization of materials (clothes, socks and bath room floors) contaminated with dermatophytic spores and hyphae.

d. Successful application of alkaline glutaraldehyde to the sterilization of materials contaminated with dermatophytic spores and hyphae.

e. Discovery of the extremely high resistance of T. mentagrophytes arthrospores to commonly used therapeutic drugs such as clotrimazole and griseofulvin thus clarifying the reason why chronic dermatomycoses are so difficult to treat by the conventional therapeutic methods. The structural and physiological basis of the resistance was partially elucidated.

f. Elucidation of chemical constituents of the walls of T. mentagrophytes hyphae, microconidia and arthrospores providing the fundamental information essential for the development of an effective dermatophytic vaccine.