Two Cases of Cutaneous Protothecosis : Unique Histopathological Findings with Crystal Violet Staining and the Therapeutic Effect of Itraconazole

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Cutaneous protothecosis sometimes poses diagnostic and therapeutic problems. Isolation of the causative organism may not be successful and spores may be mistaken for other skin diseases unless the characteristic sporangia are detected in tissue sections. Because there are few cases, the optimal therapy is still being debated. On Lieb's crystal violet staining we found charateristic purplish dots in *Prototheca* spores; these correspond to the amyloplasts or dense bodies found under electron microscopy. The isolated organisms were inhibited *in vitro* by itraconazole, amphotericin B, ketoconazole, and amorolfine and we were able to successfully treat two patients with itraconazole. (Ann Dermatol 9:(3) 201~207, 1997).

Key Words : Protothecosis, *Prototheca wickerhamii*, Crystal violet, Itraconazole

Protothecosis is a rare chronic disease caused by the species *Prototheca*(P.). It was traditionally included among the mycoses, although species of *Prototheca* are now considered to be achlorophyllous algae.[1,2]

Protothecosis is so rare that most dermatologists do not have enough clinical experience to recognize the disease, but the diagnosis is easily made if the characteristic sporangia are found in tissue sections or biochemical sugar assimilation tests of the isolated organism are carried out.[3,4] Sometimes, though, the pathogens are not isolated,[5,6] or the typical morula-shaped sporangia in tissue sections are not found, making the diagnosis obscure. Davies et al.[7] stated that *P.wickerhamii* might appear in three forms: small single cells; an intermediate form with single or multiple cleavages; or morula with multiple septations and endospores. For uncertain cases, an electron microscopic (EM) examination[3,8,9] or direct immunofluorescence (IF) study using specific antibody on paraffin-embedded tissue sections[9-12] could be carried out.

To date, the treatment of protothecosis has been problematic.[1-5] Amphotericin B has been used successfully, with or without excision. The effects of ketoconazole have varied and the role of newer imidazoles is yet to be determined.[13] We used itraconazole(ICZ) and experienced good responses in our cases. This therapeutic effect was confirmed by determining *in vitro* minimal inhibitory concentration(MIC) of ICZ in two cases.

CASE REPORTS

Case 1.

On January 1994 a 62-year-old female patient visited the dermatology clinic of Seoul City Boramae Hospital with a non-healing skin lesion on right forearm. The lesion had developed after she had lightly scratched that site four months previously. Examination showed erythematous, thick crusted, small pea-sized papules and pustules forming a double walnut-sized plaque on the extensor area of her right forearm(Fig.1). Her past history included

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Table 1. *In vitro* sensitivity test of isolated organisms

Agents	Case 1	Case 2
Amphotericin B	S	S
Flucytosine	R	R
Ketoconazole	S	S
Miconazole	I	S
Econazole	S	S
Nystatin	S	I

* The results of in vitro sensitivity tests are those tested by the Department of Clinical Pathology of Seoul National University.
* S; sensitive R; resistant
I; intermediate

bronchial asthma with intermittent corticosteroid therapy over a period of 10 years. Laboratory evaluation revealed diabetes mellitus. A skin biopsy and fungus culture were carried out. Protothecosis was diagnosed and we prescribed itraconazole 200mg q.d. perorally. The skin lesion cleared within 6 weeks, but some residual atrophic scars remained.

Case 2.

A previously healthy 45-year-old woman visited the dermatology clinic of Seoul City Boramae Hospital on December 1994 complaining of a skin lesion on her left cheek. She had applied topical steroid cream to her face for several years for the treatment of an unidentified skin disease. Several months previously, erythematous papules had developed on her left cheek. In spite of the application of topical steroids, the skin lesion had become worse. She denied any history of trauma, and on examination, an erythematous, 1.5cm x 1.5cm in size, slightly eroded, annular patch was observed on her left cheek. The patient's past history and laboratory findings were not contributory to the diagnosis. On direct examination, numerous spores of different sizes were observed. A skin biopsy and fungus culture were taken. Under the diagnosis of protothecosis we prescribed itraconazole 200mg per day. After 12 weeks of treatment, it had improved only slightly. We discovered that the patient was continuing to apply topical steroid cream and recommended that she should instead apply amorolfine cream 0.25%. After 4 weeks of combined treatment with itraconazole 200mg q.d. po and amorolfine cream 0.25%, her skin lesion cleared up.

SUMMARY OF STUDIES

Skin biopsies were taken and specimens were processed as usual and stained with hematoxylin-eosin(H&E), periodic acid-Schiff(PAS), Gomori's methenamine silver(GMS), and Lieb's crystal violet. Biopsied tissue was minced with a surgical blade and inoculated on Sabouraud dextrose agar(SDA) and incubated at 25°C The sugar assimilation test was performed using two kinds of commercial kits, the API 20C AUX(API 20C, bioM êrieux, France) and the yeast biochemical card (YBC, bioM êrieux, France). We determined the *in vitro* MIC of several antifungals using isolated organisms and compared the results with those obtained by Janssen Research Foundation. We performed transmission EM studies as usual by using five-day-old cultures. Because the antibody for performing the IF studies was not available, we sent our paraffin embedded tissue sections to Dr. Kaufman, Ph.D., at the Centers for Disease Control and Prevention, Atlanta. He performed IF studies with fluorescein-conjugated, species-specific antibodies.

1. Phycological findings

The organisms were isolated on SDA as yeast-like creamy-white colonies which were completely inhibited by cycloheximide(Fig. 2). They assimilated galactose, trehalose, glycerol, and glucose and using API 20C were considerded to be *C.glabrata* in the above two cases, but using YBC to be *P.wickerhamii* in both cases. When API 20C is used, *P.wickerhamii* is automatically regarded as

Table 2. *In vitro* minimal inhibitory concentrations of isolated organisms(g/ml)

Agents	Case 1	Case 2
Amphotericin B	0.32	0.32
Itraconazole	1.6	1.6
Flucytosine	>100	>100
Ketoconazole	1.6	3.2
Miconazole	13	13
Fluconazole	>100	>100
Amorolfine	<0.1	not carried out
Terbinafine	not carried out	>100

* The results of *in vitro* MIC are those tested by the Janssen Research Foundation.

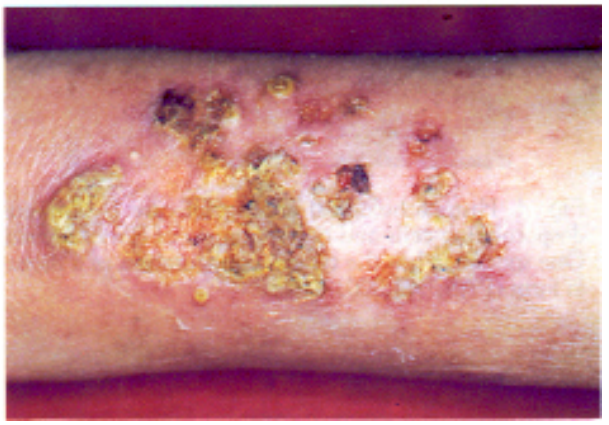

Fig. 1. Erythematous, cruted, double walnut-sized, pustular plaques on the extensor surface of right forearm before treatment (case 1).

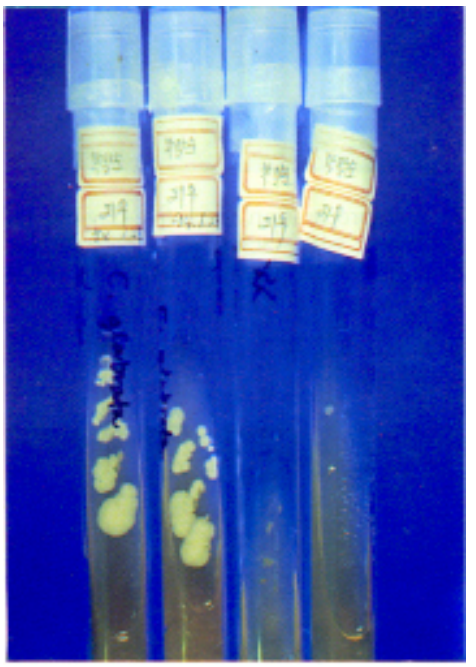

Fig. 2 Creamy-white colonies were completely inhibited by cycloheximide. By using API 20C they were considerded as *C.glabrata*, but using YBC they were identified as *P.wickerhamii* in both cases.

C.glabrata, since this organism also assimilates glucose, glycerol, and trehalose and in API 20C there is no item for *P.wickerhamii*. This problem was easily solved, however, by observing the characteristic sporangia in a wet preparation stained with lactophenol cotton blue(Fig. 3) or by using another yeast identification kit such as YBC.

The *in vitro* MIC of several antifungals was determined for the isolated organisms of both cases; the results of Seoul National University Hospital(Table 1) and those of the Jassen Research Foundation(Table 2) were relatively accordant. The organisms were inhibited by amphotericin B, ketoconazole, amorolfine and itraconazole but were resistant to flucytosine and fluconazole.

2. Histopathologic findings

On H&E slides, granulomatous inflammation with mixed inflammatory cell infiltrates was found. Numerous spores of various sizes(2-15 μm) were clearly seen on GMS and PAS slides, usually in the dermis and sometimes in the epidermis. The most characteristic feature was a mulberry-like sporangium. It contained symmetrically-arranged endospores (Fig.4). On crystal violet staining, purplish dots were clearly observed in the organisms (Fig.5 A,B).

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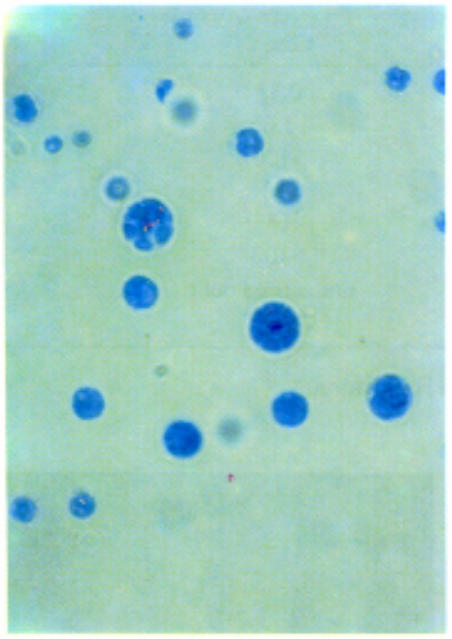


Fig. 3. Wet preparation from five-day-old culture of case 2 stained with lactophenol cotton blue. Cartwheel-like sporangia are evident. (LPCB, ×1,000)

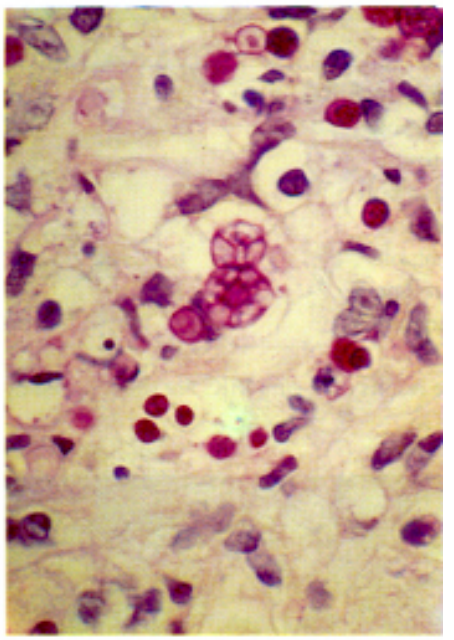
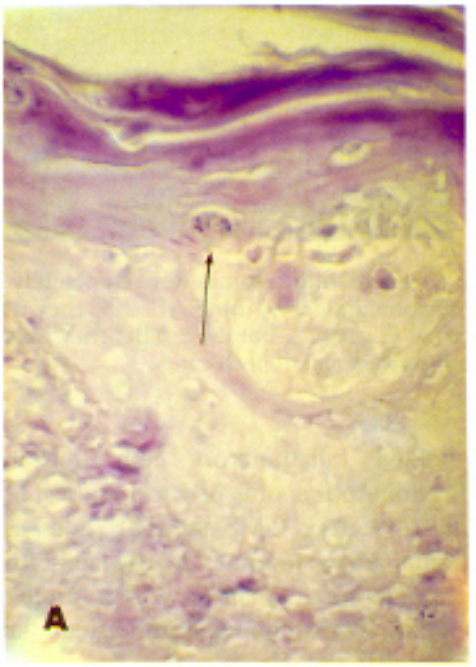


Fig. 4. Histopathological findings of case 1. Non-endosporulating cells and two sporangia of *P.wickerhamii*. One sporangia has the typical configuration of a morula form (PAS, ×1,000).



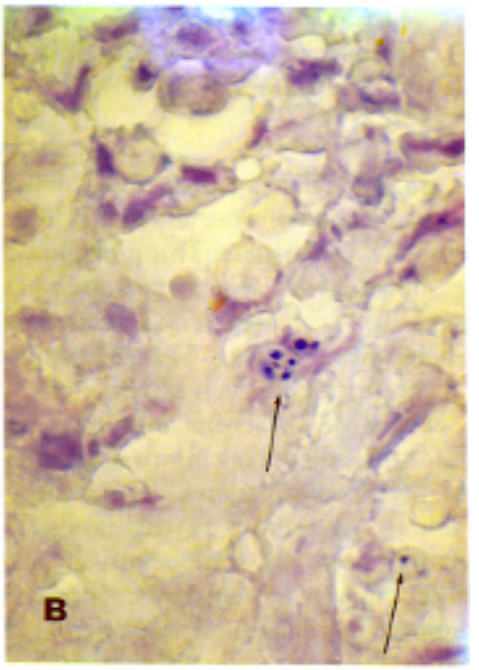


Fig. 5A, B. Small bluish dots are observed in the organisms(arrows) by crystal violet staining. Organisms are located in the epidermis or in the dermis (Crystal violet staining, ×1,000).

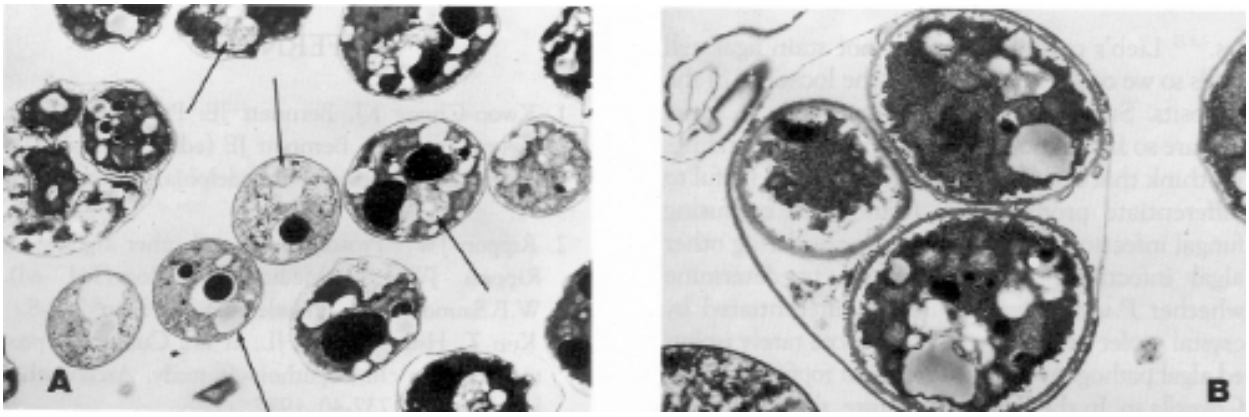


Fig. 6. Ultrastructures of the cultured organisms of case 2.
A. There are dark electron-dense bodies and clear ellisoidal structures (arrors) known as amyloplasts in the spores (×11,500).
B. Sporangia containing endospores are clearly seen (×20,700).

3. Ultrastructural findings

EM examinations of five-day-old cultures of two cases revealed thick-walled spores containing dense bodies and clear ellipsoidal structures known as amyloplasts (Fig. 6A). Sporangia containing endospores were also clearly seen (Fig. 6B).

4. Immunofluorescnece study findings

IF studies with fluorescein-conjugated, species-specific antibodies were performed on paraffin-embedded tissues of above cases. The tissue sections were all positive for *P.wickerhamii*.

DISCUSSION

Kwon-Chung has confirmed in her recent phylogenetic studies based on ribosomal RNA sequences that *Prototheca* species, as has been previously postulated, are closer to blue-green algae and plants than to fungi.[14] Though protothecosis would still be dealt by medical mycologists because of its morphological and *in vivo* staining characteristics.[14] Protothecosis has been described worldwide. In Asia, it has been identified in Japan, Thailand, China, Hong Kong, Vietnam and Taiwan.[9,15,16]

The pathogenesis of protothecosis is generally believed to be due to traumatic inoculation of the algae.[1,2,15] In our cases, trauma history was identified in case 1 and denied in case 2. There was no history of exposure to swimming pools, fish aquaria, soil, lake water and so on in both cases; diabetes mellitus and systemic steroid administration might be an underlying factor in case 1. In case 2, the patient had applied potent topical steroid cream for a long time, and this might have precipitated the development of protothecosis. Protothecosis is a rare disease but a confirmative diagnosis could be easily made due to the presence of the characteristic sporangia in tissue sections and through biochemical sugar assimilation tests of the isolated organism.[1-4] The pathogens are sometimes not isolated, however[5,6], and sometimes the typical morula shaped sporangia in tissue are not found.[11,12] The organisms stain with PAS, GMS, and Gridly fungus stain(GF),[11,12,15,16] and mucicarmine may stain lightly.[3,15,16] All of these stains stain fungal or algal cell walls so these are unable, however, to differentiate *Prototheca* spp. from other agents such as *Blastomyces dermatitis*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis* and so on without the characteristic endosporulation. To confirm the diagnosis, EM studies[3,8,9] or direct IF studies using a specific antibody on paraffin-embedded tissue sections[9-12] should then be performed; but these studies are expensive and require specific facilities or special antibodies. Easier and cheaper methods are therefore preferable.

We performed Lieb's crystal violet staining and found small purplish dots in the cytoplasm of organisms. Because amyloid or starch deposits stain purplish by crystal violet, we think these dots might be starch deposits such as amyloplast and dense bodies found in *P.wickerhamii* by EM stud-

ies.[3,8,12] Lieb's crystal violet did not stain agal cell walls so we could clearly identify the locations of the deposits. Starch deposits are found only in algae and are so far unknown in fungi. From these findings we think that Lieb's crystal violet might be useful to differentiate protothecosis from other confusing fungal infections. However when considering other algal infections, we were unable to determine whether *P.wickerhamii* could be differentiated by crystal violet staining from other more rarely isolated algal pathogens such as *Prototheca zopfi(P.zopfi)* or *Chlorella* sp. In the English literature, there has so far been only one documented case of *P.zopfi*[7] and one of *Chlorella* sp.[17] *Chlorella* sp. has numerous starch granules so that we guess these starch granules in *Chlorella* sp. would stain more strongly than *P.wickerhamii* by crystal violet staining. *Chlorella* sp. could also be differentiated from *Prototheca* by characteristic chloroplasts observed by EM study[18]. But specific identification may be achieved only by direct IF study.[9-12,18,19]

Protothecosis has so far been difficult to treat[1-5,9] and the scarcity of cases has prevented the development of well defined therapeutic protocols.[15] We tried itraconazole 200mg/day and both patients were cured without recurrence. In the literature we found other cases treated with ICZ,[4,15,20] but clinical successes with this medication were not supported by the *in vitro* MIC test. We would like to propose that if patients are not immunocompromised and there are no contraindications for ICZ then this might be the oral drug of first choice. If a patient is severely immunocompromised and there is a possibility of dissemination, or a liver function test reveals abnormalities, then amphotericin B should be tried first.

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