The influence of carbohydrases on the growth of fungal pathogens *in vitro* and *in vivo*

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Summary

Mixtures of mycolytic enzymes from various sources release protoplasts from living fungal tissue under suitable conditions. Such enzyme mixtures obtained from *Coprinus comatus* (mycolase I), *Physarum polycephalum* (mycolase II) and *Lycoperdon pyriforme* (mycolase III) are of low toxicity in mammals when given parenterally and are able to cure experimental systemic fungal infections in mice when administered alone or in conjunction with normally ineffective levels of conventional antimycotic drugs such as amphotericin B. The effect is believed to be due to enzymic degradation of the fungal cell wall either killing the fungus directly or enhancing activity of existing antifungal agents by increasing access to the cell interior.

Introduction

Fungal infections are a major cause of morbidity and mortality throughout the world (Ajello, 1971; Mycoses, 1975) and patients under induced immunosuppression for organ transplantation or receiving chemotherapy for cancer are susceptible to disease caused by 'opportunistic' organisms such as *Aspergillus* and *Candida* spp. (Bodey, 1977; Mason *et al.*, 1976). In spite of increasing awareness of the importance of fungal infections, there is a shortage of effective antimycotic agents and those that are available have drawbacks that limit their usefulness. Amphotericin B has been the drug of choice for treatment of systemic fungal disease, but it is nephrotoxic and has other undesirable side effects (Bennett, 1974). Flucytosine is less toxic than amphotericin but emergent fungal resistance to this drug is a potentially serious problem (Utz, 1977). Newer antifungal agents such as miconazole and clotrimazole are still being evaluated.

The essence of effective chemotherapy is to exploit differences between host and pathogen so that the host is unaffected by the treatment. In fungal infections, exploitable differences are few because both host and pathogen are eukaryotes. However, an obvious difference between fungal and mammalian cells that has not been exploited is the presence in fungi of a polysaccharide cell wall that frequently contains chitin (Bartnicki-Garcia, 1968). The cell

wall can also be seen as a barrier to effective chemotherapy. Certainly, Gale *et al.* (1975) have shown that protoplasts of *C. albicans* are more sensitive to amphotericin B than are intact cells. The novel approach to antimycotic therapy described in this communication exploits the cell wall by making chitin and other polysaccharides the target for enzymatic attack. Damage to or removal of the cell wall should either kill the fungus directly or, at least, enhance the activity of existing drugs by increasing their access to the cell interior. Experiments intended to test this hypothesis are described below.

Materials and methods

Organisms and media

A. fumigatus and *C. albicans* were obtained from the Searle culture collection. *Physarum polycephalum* (strain i x a7029) was obtained from Dr M. J. Carlile, Department of Biochemistry, Imperial College of Science and Technology, Prince Consort Road, London, S.W.7. Specimens of *Coprinus comatus* and *Lycoperdon pyriforme* were collected in the Chiltern beechwoods around High Wycombe and stored at -20°C until required.

A. fumigatus and *Candida albicans* were grown on glucose peptone medium containing (per litre) glucose 10 g, peptone 2 g, KH_2PO_4 0.5 g, MgSO_4 0.5 g. Universal bottles containing 10 ml of this medium were inoculated with a spore suspension of *A. fumigatus* and incubated for 20 hr at 24°C on a Luckham Rolamix blender. *P. polycephalum* was grown on the medium described by Carlile (1971).

Enzyme preparation

Enzyme mixtures were obtained from fresh or frozen specimens of *Coprinus comatus* and *L. pyriforme* either by allowing the fruit bodies to autolyse at 4°C in sterile containers for 24-48 h or by homogenizing fruit bodies in a Waring Blender for 5 min at 4°C . The homogenates obtained were expressed through muslin and the remaining solid discarded. The fluid was then centrifuged in sterile containers at 3000 g for 15 min and the pellet discarded. The supernatant contained the crude enzyme mixture which was then lyophilized.

A 'purified' extract of *L. pyriforme* was prepared by fractionating the crude enzyme mixture on Bio-Gel P200 (Bio-Rad Laboratories, Richmond, California) and recombining the retarded fractions.

A mixture of mycolytic enzymes was obtained from culture supernatants of *P. polycephalum* as follows: Cellular material was removed by centrifugation at 1600 *g* for 15 min at 4°C and viscous polysaccharide precipitated from the supernatant by adding one volume of cold (−20°C) 95% ethanol and centrifuging at 10 000 *g* for 30 min at 4°C to sediment the resultant precipitate. The clear supernatant was dialysed against water to remove the ethanol and lyophilized.

Chitinase (E.C. 3.2.11.4.) was obtained from Koch Light Laboratories, Colnbrook, Buckinghamshire, and laminarinase (β-1,3(4) glucan glucanohydrolase E.C. 3.2.1.6.) was obtained from Calbiochem, San Diego, California.

Protoplast assay

This was carried out according to the method of Bartnicki-Garcia and Lippman (1966) using molar MgSO₄ as an osmotic stabilizer. *A. fumigatus* was the target organism and all enzymes were at a final concentration of 240 mg/l.

Experimental infection

Groups of 5 or 10 female BALB/c mice weighing 22–25 g were infected by intravenous injection with either 5 × 10⁶ spores of *A. fumigatus* or 25 × 10⁴ blastospores of *C. albicans* in saline. On one or more of the days following infection, animals were treated intraperitoneally with enzymes, conventional antimycotic drugs or combinations of the two. Treatments were given in 0.2 ml of 5% dextrose or water for injection.

TABLE 1. Production of protoplasts from *Aspergillus fumigatus*

Enzyme treatment	Protoplast release
Chitinase	+
β-1,3-glucanase	—
Chitinase + β-1,3-glucanase	++
<i>Coprinus</i> extract – mycolase I	+++
<i>Physarum</i> culture SN – mycolase II	++++
<i>Lycoperdon</i> extract – mycolase III	++++

+ Protoplasts from hyphal tips only.

++ Protoplasts from tips and intercalary compartments.

+++ Many protoplasts released throughout mycelium.

++++ Total lysis of mycelium.

Results

Results for protoplast release from *A. fumigatus* are given in Table 1. Chitinase released protoplasts

only from hyphal tips, whereas β-1, 3-glucanase did not liberate any protoplasts. A mixture (1 : 1 by weight) of the 2 enzymes was more effective than either component alone. Mycolases I, II and III (from *Coprinus comatus*, *P. polycephalum* and *L. pyriforme* respectively) were more effective than the commercial enzyme mixture, and treatment with mycolase II resulted in complete lysis of the target mycelium. Similar results have been obtained with *C. albicans* and a range of microfungi (Pope and Davies, unpublished data).

Results in Table 2 show that infected, untreated mice had a mean survival time of 13.5 days (Table 2 a). Under the conditions of the test, a single treatment with the maximal sub-toxic dose of amphotericin B or nystatin significantly prolonged survival, but did not cure the animals (Table 2 k, m). Animals were cured by mycolases alone (Table 2 d, j) but not by the commercial enzyme mixture (Table 2 b). Infections were also cured by the synergistic effect of mycolases and conventional antifungal agents at doses of both components that were ineffective when administered alone (Table 2 o, p, q, r, s). Mice that had been immunosuppressed with rabbit anti-mouse thymocyte globulin (ATG) were also cured by combined therapy, suggesting that a full immune capacity is not an essential prerequisite for the effect. Similar results have been obtained in experimental candidiasis and in the treatment of dermatophyte infection in guinea-pigs (Pope and Davies, unpublished data).

Acute toxicity tests with mycolases given intravenously or intraperitoneally in mice gave an LD₅₀ of 15–20 mg/mouse (600–700 mg/kg).

Discussion

The fungal cell wall has been recognized by other workers as a potential target for therapeutic attack. Specific inhibitors of chitin synthetase, the polyoxins, have been developed (Endo, Kakiki and Misato, 1970), but although these compounds are effective *in vitro*, they have not proved useful clinically. Chitinases occur widely in nature (Stirling, Cook and Pope, 1979) and, although their effects on fungal cell walls *in vitro*, alone and in conjunction with other enzymes are well known, as far as the authors are aware there have been no *in vivo* studies. Miura (1954) suggested that chitinase might be used for treating dermatophyte infections, but did not demonstrate mycoid activity. The results show that chitinase alone is only slightly mycolytic and requires the presence of other enzymes before its full potential can be realized. The increased mycolytic activity of mycolases compared with the commercial enzyme mixture is almost certainly attributable to the wider range of carbohydrases they contain. Enzyme profiles for mycolases will be published elsewhere.

TABLE 2. Fate of BALB/c mice infected systemically with *Aspergillus fumigatus*

Treatment	Days after challenge	Dose	Mean survival (days)
a. None (controls)	—	—	13.5
b. Chitinase + β -glucanase	1, 2, 3	3 $\times$ 1.5 mg	26.3
c. Mycolase I (crude)	1	1.5 mg	21.0
d. " "	1, 2, 3	3 $\times$ 1.5 mg	All survived*
e. " "	12	4.5 mg	35.6
f. Mycolase II (crude)	1	100 μ g	16.2
g. " "	1	10 μ g	14.4
h. Mycolase III (crude)	1	1.5 mg	11.2
i. " (purified)	1	100 μ g	15.8
j. " "	1, 2, 3	3 $\times$ 100 μ g	All survived*
k. Amphotericin B	1	5.0 μ g	23.2
l. " "	1	0.6 μ g	16.0
m. Nystatin	1	100 μ g	30.6
n. " "	1	12.5 μ g	14.6
o. Mycolase I – amphotericin (c–l)	1–2	1.5 mg–0.6 μ g	All survived*
p. Mycolase I – nystatin (c–n)	1–2	1.5 mg–12.5 μ g	All survived*
q. Mycolase II – amphotericin (f–l)	1–2	100 μ g–0.6 μ g	All survived†
r. Mycolase II – amphotericin (g–l)	1–2	10 μ g–0.6 μ g	42.0
s. Mycolase III – amphotericin (h–l)	1	1.5 mg + 0.6 μ g	All survived†
t. ATG‡ before challenge	–1	300 μ l	8.6
u. ATG‡ (t) – mycolase I – amphotericin (o)	–1, 1(C), 2(A) 3(C), 4(A)	2 $\times$ 1.5 mg– 2 $\times$ 0.6 μ g	All survived*

* Significantly different from a at the 1% level.

† Significantly different from a, f and j at the 1% level. (Logrank χ^2 analysis, Peto and Pike, 1973).

‡ ATG is rabbit anti-mouse thymocyte globulin. 300 μ l, injected subcutaneously, was the amount able to prolong the survival of allogeneic skin grafts from 8–10 days to 25–35 days.

Mycolases have been shown to be effective in both immune competent and immunosuppressed animals alone and in combination with existing antimycotic drugs (Davies and Pope, 1978). The latter approach allows reduction in drug dosage and associated toxicity. *In vitro* tests suggest that addition of mycolase gives a 5- to 10-fold reduction in the MIC of a range of antifungal drugs. This is probably due to the increased access to the cell interior through the enzyme-damaged walls.

Mycolases have a wide spectrum of antifungal activity, are of low toxicity and there would seem to be only a remote chance of fungi developing resistance to their attack. The authors believe that mycolases hold considerable promise as an alternative to existing therapy for both systemic and superficial fungal infections.

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