Antimicrobial Properties of Cytochalasins and Their Alteration of Fungal Morphology

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(Accepted for publication 4 February 1972)

SUMMARY

Cytochalasin A inhibited growth of *Bacillus subtilis* and *Escherichia coli* and increased motility of the latter. Both cytochalasins A and D have antifungal properties, inducing branching and, at higher concentrations, swelling of hyphal tips in *Botrytis cinerea*. Cytochalasin B showed neither antibacterial nor antifungal activity. The observed antimicrobial effects of cytochalasins A and D are discussed in relation to those of related antibiotics.

INTRODUCTION

Cytochalasins, a class of mould metabolites with unusual biological activity, have been isolated by Aldridge, Armstrong, Speake & Turner (1967). Cytochalasins A and B have been shown to be identical with 5-dehydrophomin and phomin respectively (Rothweiler & Tamm, 1970) while cytochalasin D is identical (Aldridge & Turner, 1969) with zygosporin A (Hayakawa, Matsushima, Kimura, Minato & Katagiri, 1968; Minato & Matsumoto, 1970).

Carter (1967) has described some of the effects of cytochalasin B on mouse fibroblasts in culture. In varying conditions of concentration and time, the compound inhibited motility, produced extrusion of the nucleus and blocked cytoplasmic cleavage while nuclear division continued with the production of multinucleate cells. It was later found that the contractile machinery of many animal cell types is reversibly inhibited by cytochalasin B (see the review by Wessells *et al.* 1971).

To our knowledge, antimicrobial properties of cytochalasins have not been described in the literature except for two short statements. Hayakawa *et al.* (1968) have reported that zygosporin A did not inhibit the micro-organisms tested, except *Trichomonas vaginalis*, which was inhibited at a relatively low concentration. Cell division, flagellar movement and mating of *Escherichia coli* are reported to be insensitive to cytochalasin B (Wessells *et al.* 1971).

Structurally cytochalasins A and B belong to the class of aglycosidic macrolides of fungal origin. Two other members of the same class, namely cyanein and monorden (= radicicol) have antimicrobial activity. Cyanein, besides showing other biological effects, is an antifungal antibiotic (Betina, Nemeč, Dobias & Baráth, 1962) and monorden also shows a pronounced activity against filamentous fungi (Delmotte & Delmotte-Plaquée, 1953). This paper reports on the antibacterial and antifungal activities of cytochalasin A and cytochalasin D.

METHODS

Organisms. The following strains of micro-organisms were used in our experiments: *Bacillus subtilis* SDPC 1:220, *Escherichia coli* B, *Saccharomyces cerevisiae* Sherry v3, *Candida albicans* Pn 10, *Mucor corymbifer* 24 IP, *Aspergillus fumigatus* K IP, and *Botrytis cinerea* 4-22. They were obtained from the collection of the Department of Technical Microbiology and Biochemistry of the Slovak Polytechnical University, Bratislava, Czechoslovakia.

Culture media. Cultures of *Bacillus subtilis* were grown in a complete medium containing glucose, sodium citrate, tryptone (Oxoid) and mineral salts (Betina & Mičková, 1972). *Escherichia coli* was grown in a meat extract-peptone medium (Imuna, Šarišské Michal'any, Slovakia). Cultures of *Saccharomyces cerevisiae* and *Candida albicans* were grown in the synthetic Vita medium containing glucose, mineral salts and six vitamins (Svobodová & Drobica, 1962). Cultures of *Mucor corymbifer*, *Aspergillus fumigatus* and *Botrytis cinerea* were grown on malt extract agar. When necessary the above media were solidified by adding 2% of agar.

Antibiotics. Cytochalasins A and B were kindly provided by Dr W. B. Turner of the Biochemical Research Department, Pharmaceutical Division, Imperial Chemical Industries, Mereside, Cheshire. Zygosporein A (= cytochalasin D) was a gift from Dr H. Minato, Shionogi Research Laboratory, Shionogi & Co. Ltd, Fukushima-ku, Osaka, Japan.

Activity assays. In qualitative tests sterile 10 mm discs of Whatman no. 3 filter paper were used. Antibiotics, dissolved in ethanol were applied to give a concentration of 200 µg per disc and dried. The discs were put on to agar plates in Petri dishes inoculated with bacteria, yeasts or with suspensions of spores of filamentous fungi. Plates with bacteria and yeasts were incubated for 20 h at 37 °C and 28 °C respectively and examined for zones of inhibition. Filamentous fungi were incubated at 25 °C and plates were examined after 20 h with *Mucor corymbifer*, after 48 h with *Aspergillus fumigatus* and after 64 h with *Botrytis cinerea*.

Growth curves. To study the effects of the active antibiotics on growth of bacteria and yeasts the following procedures were used. Culture medium (10 ml) in an L-shaped tube with a volume of 20 ml was inoculated with one loopful from a slant culture of the micro-organism and incubated with shaking at 30 °C (bacteria) or 28 °C (yeasts) for 16 h. The tubes were adapted for direct measurements of extinctions with a PREMA Spectrophotometer. A portion of fresh medium was then inoculated with the growing culture to give an extinction of about 0.05 at 650 nm and distributed in amounts of 5 ml into L-shaped tubes containing 0.05 ml of antibiotic solutions in dimethyl sulphoxide (DMSO) or DMSO alone (controls). The final concentration of DMSO being 1% was not inhibitory to the cultures studied. The cultures of bacteria and yeasts in L-shaped tubes were then incubated with vigorous shaking in water baths at 37 °C and 28 °C respectively. Extinctions of duplicate sets of tubes were measured at 650 nm at intervals.

Botrytis cinerea was selected as a representative of the filamentous fungi used in the paper-disc tests. The active antibiotics in DMSO, or DMSO alone in the controls, were added to Petri dishes immediately before pouring malt extract agar to obtain desired concentrations of inhibitors, the final concentration of DMSO being 1%. The solidified plates were inoculated in the centre with a loopful of a spore suspension or with vegetative inocula as follows. Sterile 5 mm discs of Whatman no. 1 paper were situated around a growing colony of *B. cinerea* on an agar plate without antibiotics and further incubated at 25 °C until the marginal hyphae of the colony grew just over the discs. Individual discs were then transferred aseptically on to agar plates containing antibiotics. Duplicate sets of agar plates inoculated with

Fig. 1. Inhibition of *Escherichia coli* by cytochalasin A added at the beginning (a) and during logarithmic phase (b) of growth in meat extract-peptone medium at 37 °C with shaking in a water bath. The concentrations of cytochalasin A are given in $\mu\text{g/ml}$.

either of the above inocula were incubated at 25 °C and the diameters of growing colonies were measured at intervals and the fungi examined for morphological changes.

Test for morphogenic effects. A disc with growing hyphae of *Botrytis cinerea* obtained as above was located in the centre of an agar plate which was then incubated at 25 °C for 48 h. The disc was removed to limit growth of aerial mycelium and three 5 mm holes were cut at a distance of about 10 mm from the colony margins. A drop of melted malt extract agar was added to each hole. After solidification 0.1 ml of antibiotic solutions in DMSO (or DMSO alone in the controls) were pipetted into the holes. The plates were re-incubated at 25 °C and the morphology of marginal hyphae was examined microscopically *in situ* at intervals. Photomicrographs were also taken *in situ*. When necessary hyphae were coloured with 0.5 % cotton blue in lactophenol.

RESULTS

Under the experimental conditions with paper discs containing 200 μg of the antibiotics tested, cytochalasin A inhibited all bacteria and fungi used. Cytochalasin D showed only antifungal activity and cytochalasin B was inactive against all micro-organisms used.

Of the micro-organisms used in preliminary tests, *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Botrytis cinerea* were further used for more detailed studies with cytochalasins A and D.

Effects of various concentrations of cytochalasin A on the growth of *Escherichia coli* are presented in Fig. 1(a). The antibiotic at 25 $\mu\text{g/ml}$ completely inhibited growth when added at the beginning of the lag phase. At lower concentrations the lag phase was significantly prolonged. When cytochalasin A was added to logarithmically growing bacteria, a decrease of extinctions indicating relatively rapid lysis was observed at the concentrations of 50 and 25 $\mu\text{g/ml}$ (Fig. 1b). Cytochalasin A added to growing *E. coli* induced increased motility of cells, as observed microscopically, which was followed by gradual disintegration of cells.

Growth of *Bacillus subtilis* was prevented by cytochalasin A at 50 $\mu\text{g/ml}$ when added at the beginning of lag phase. The same concentration of the antibiotic added to exponentially growing cells caused their lysis but not as rapidly as with *Escherichia coli*.

Growth of *Saccharomyces cerevisiae* in the synthetic Vita medium was inhibited by both

Fig. 2. Inhibition of *Saccharomyces cerevisiae* by cytochalasin A (a) and cytochalasin D (b). The strain was grown in the Vita medium at 28 °C with shaking in a water bath. Antibiotics were added at the beginning or during logarithmic growth (indicated by arrows) and concentrations are given in $\mu\text{g/ml}$.

Fig. 3. Growth inhibition of *Botrytis cinerea* by cytochalasin A on malt extract agar at 25 °C. Growth of colonies from spore inoculum (a) or from vegetative form (b). The concentrations of the antibiotic are given in $\mu\text{g/ml}$.

Fig. 4. Growth inhibition of *Botrytis cinerea* by cytochalasin D. Conditions as in Fig. 3.

Figs. 5–8. Growth inhibition and morphological changes of *Botrytis cinerea* on malt extract agar caused by cytochalasin D ($25 \mu\text{g}/\text{hole}$). Photomicrographs were prepared directly from the margins of growing colonies and the scale marker represents $100 \mu\text{m}$.

Fig. 5. Inhibition of growing colony by adding cytochalasin D. About $\times \frac{2}{3}$.

Fig. 6. Marginal hyphae in control without antibiotic added. (Stained directly on the agar with cotton blue in lactophenol.) $\times 360$.

Fig. 7. Marginal hyphae 24 h after cytochalasin D was added. $\times 360$.

Fig. 8. Marginal hyphae 39 h after cytochalasin D was added. $\times 360$.

Fig. 9. Structures of cytochalasins A (*Ia*), B (*Ib*) and D (*II*).

cytochalasins A and D but the latter was less effective (Fig. 2*a, b*). Cytochalasin A at 50 µg/ml prevented growth when added at the beginning of growth curve. When added to exponentially growing cultures it decreased the growth rate for about 2 h and then stopped growth almost completely.

Botrytis cinerea was also more sensitive to cytochalasin A than to cytochalasin D. Growth of the fungus was much more inhibited by the former antibiotic when a vegetative inoculum was used instead of spores (see Fig. 3(*a*) and (*b*) for comparison). The less active cytochalasin D revealed a similar difference in its activity towards the fungus growing from a spore inoculum or from a vegetative inoculum (Fig. 4*a, b*).

Both cytochalasins induced morphological changes in growing hyphae of *Botrytis cinerea* at concentrations which partially inhibited growth. Intensive branching of hyphae at the margins of colonies was observed. Fig. 5 to 8 illustrate effects of cytochalasin D on hyphal morphology. Cytochalasin A had similar effects.

DISCUSSION

Cytochalasins A and B have almost identical structures, the only difference being that the former is a ketone and the latter the corresponding secondary alcohol. Cytochalasin D is not a macrolide but the non-macrocyclic part of its molecule is identical with that of the above cytochalasins. Both cytochalasins A and D have a ketone group in comparable positions in their macrocyclic rings (Fig. 9) which might be essential for the antimicrobial effects which they show in addition to other biological activities shared with cytochalasin B. It would be of interest to test in our systems antimicrobial properties of zygosporins D, E, F and G which are structurally related to cytochalasin D (Minato & Katayama, 1970).

According to Carter (1967) cytochalasins A, B, C and D show essentially similar activity in animal cells but differ in potency. Our findings show differences in their antimicrobial properties: cytochalasin B is inactive, cytochalasin D has antifungal and cytochalasin A both antifungal and antibacterial activity. Besides inhibition of growth of *Escherichia coli* by cytochalasin A a pronounced increase of motility was observed under phase-contrast microscopy. Perhaps a more detailed study might reveal possible effects of the antibiotic on flagellar functions in bacteria. Few effects of cytochalasin B upon metabolic functions in animal cells have been found (Wessells *et al.* 1971). It is possible that *E. coli* would be a simpler model for such studies with cytochalasin A.

Cytochalasins A and D show similar effects on the growth and morphology of *Botrytis cinerea*. An intensive branching of growing hyphae of the same fungus can be induced by

cyanein (Betina, Betinová & Kutková, 1966) and by monorden (V. Betina, D. Mičková & P. Nemeč, in preparation), two other members of the family of fungal aglycosidic macrolide antibiotics. At higher concentrations of cytochalasins branching is accompanied by bulging of hyphal tips similar to that induced by aspergillilic acid and flavofungin (Baráthová, Betina & Nemeč, 1969). A more detailed report on these effects of the cytochalasins and related fungal macrolides will be published elsewhere.

Our results suggest that cytochalasins A and D might become useful tools in studying morphogenesis in fungi just as cytochalasin B is widely used in studies of developmental processes of animal cells and tissues.

We thank Mr W. Vollek for assistance with the photomicrographs.

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