FORMATION OF FUNGAL MULTINUCLEAR GIANT CELLS BY TUNICAMYCIN¹

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In the presence of sublethal concentration of tunicamycin, conidia of fungi belonging to the genera *Penicillium* and *Aspergillus* could not germinate and changed to multinuclear giant cells of 10–100 μm in diameter. Some of these giant cells could grow and divide. Number of nuclei per cell increased to over 50. When tunicamycin was added to the germinating conidia, the hyphal tips became swollen at first, and then the whole hyphae were swollen showing a “beaded appearance.” After removal of tunicamycin, the giant cells germinated and converted to a mycelial form.

Tunicamycin is a glucosamine-containing antibiotic active against viruses, gram-positive bacteria, yeasts, and fungi (1). It preferentially inhibits the biosynthesis of membrane glycoproteins (2), and induces morphological changes in bacteria and yeasts (3). We have investigated the effect of this antibiotic on fungi belonging to the genera *Penicillium* and *Aspergillus*, and found that characteristic morphological changes were induced in liquid media which contained a sublethal concentration of tunicamycin; the conidia converted to multinuclear giant cells, some of which had more than 50 nuclei per cell, and hyphae showed a “beaded appearance.”

MATERIALS AND METHODS

Measurement of fungal growth. A culture tube containing 1 ml of a liquid medium was inoculated with a suspension of conidia of a strain belonging to the genus *Penicillium* or *Aspergillus*. After shaking at 30° for 4 days, the cells were

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collected by centrifugation, dried at 110° for 4 hr, and weighed. Effect of tunicamycin on fungal growth was judged from the dry cell weight.

Culture media. Medium A contained (g per liter) glucose, 50; Polypepton, 5; KH_2PO_4 , 0.5; K_2HPO_4 , 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4. Medium B contained glucose, 50; NaNO_3 , 5; KH_2PO_4 , 27; KCl , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1. Medium B-P contained KH_2PO_4 , 0.5 and K_2HPO_4 , 0.5 in place of KH_2PO_4 , 27 of medium B. Medium C contained glucose, 15; glycerol, 10 (ml); yeast extract, 1; malt extract, 1; casamino acid, 5. Medium K contained sucrose, 30; NaNO_3 , 2; KCl , 44.7; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; yeast extract, 0.5; malt extract, 0.5; casamino acid, 0.5. pH of each medium was adjusted to 5.5.

Optic microscopy. For optic microscopy, the Olympus microscope FHF Tr-II was used, and photographs were taken by the use of a phase-contrast objective, $\times 40$.

Giemsa staining. Nuclei were examined by the Giemsa staining method of HARTWELL (4).

Chemicals. Tunicamycin was prepared according to the method reported previously (1), and the same lot of the antibiotic was used throughout all the experiments reported in this paper.

RESULTS

Minimum inhibitory concentration of Tunicamycin

The effect of tunicamycin on the growth of fungi was examined with 11 strains of *Penicillium* and 11 strains of *Aspergillus*. Conidia of *P. citrinum* (THOM 1131) in medium B were more sensitive to tunicamycin (minimum inhibitory concentra-

Fig. 1. Effect of tunicamycin on the growth of *P. citrinum* (THOM 1131) in various media.

○ medium A, ● medium B, △ medium C, ▲ medium K, × medium B-P

tion (MIC), 5 $\mu\text{g/ml}$) than those in other media (MIC, 10–50 $\mu\text{g/ml}$) (Fig. 1). The sensitivity to this antibiotic in medium B varied from species to species and even from strain to strain in the genus *Penicillium* (Fig. 2). On the other hand, conidia of *A. niger* (AHU 7117) in medium A were more sensitive to tunicamycin (MIC, 20 $\mu\text{g/ml}$) than those in other media including medium B (MIC, more than 200 $\mu\text{g/ml}$) (Fig. 3). Conidia of *A. oryzae* (var. No. 13) were rather resistant to this antibiotic (MIC, more than 200 $\mu\text{g/ml}$) in all the media examined (Fig. 4).

Fig. 2. Effect of tunicamycin on the growth of various strains of *Penicillium* in medium B.

○ *P. citrinum* (NRRL 1841), ● *P. citrinum* (PW-4P), △ *P. citrinum* (NRRL 805), × *P. citrinum* (THOM 1131)

Fig. 3. Effect of tunicamycin on the growth of *A. niger* (AHU 7117) in various media.

Symbols are the same as in Fig. 1.

Fig. 4. Effect of tunicamycin on the growth of *A. oryzae* (var. No. 13) in various media.

Symbols are the same as in Fig. 1.

Morphological changes

In the presence of 1–2 $\mu\text{g/ml}$ of tunicamycin in medium A, conidia of *P. citrinum* (THOM 1131) could germinate, but the germ tubes were swollen (Fig. 5-1). In the presence of more than 10 $\mu\text{g/ml}$ of the antibiotic in medium A, the conidia could not germinate, and increased cell mass to form giant cells (Fig. 5-2, 3). This morphological change was also observed in medium B supplemented with more than 5 $\mu\text{g/ml}$ of tunicamycin (Fig. 5-4). Some of these giant cells were observed to grow and divide (Fig. 5-3, 5, 6).

When the giant cells of *P. citrinum* (THOM 1131), which had been formed from the conidia during their incubation with 50 $\mu\text{g/ml}$ of tunicamycin in medium A for 24 hr, were transferred to fresh medium A (without the antibiotic), some of the cells started to germinate (Fig. 5-7) and finally normal mycelia were formed.

When 50 $\mu\text{g/ml}$ of tunicamycin was added to the germinating conidia of *P. citrinum* (THOM 1131), the hyphal tips became swollen after overnight shaking (Fig. 5-8). After 3 days of incubation, most of whole hyphae were observed to be swollen showing a “beaded appearance” (Fig. 5-9).

In the case of *A. niger* (AHU 7117) and *A. oryzae* (var. No. 13), giant cells formed in the presence of tunicamycin were very large. The diameter was up to 100 μm (Table 1 and Fig. 5-10, 11).

Table 1. Comparison of the effects of tunicamycin on various fungal strains.

Strain	Minimum inhibitory concentrations ($\mu\text{g/ml}$)		Diameter of		
			Giant cells (μm)		Conidia (μm)
Medium	A	B	A	B	
<i>P. citrinum</i> (THOM 1131)	20	5	6–30	5–12	2–3
<i>A. niger</i> (AHU 7117)	50	≥ 200	8–120	5–10	3–5
<i>A. oryzae</i> (var. No. 13)	≥ 200	> 200	8–50	6–30	6–8

These giant cells induced by tunicamycin were not osmotically labile, because the cells were formed in the medium without a stabilizer such as 20% sucrose and 0.6 M NaCl, and these cells were not ruptured in water.

Giemsa staining

Cells of normal hyphae germinated from the conidia of *P. citrinum* (THOM 1131) in the absence of tunicamycin had one nucleus per cell (Fig. 5-12). Many cells of slightly swollen germ tubes germinating from the conidia of *P. citrinum* (THOM 1131) in the presence of 2 $\mu\text{g/ml}$ of the antibiotic in medium A contained two nuclei per cell (Fig. 5-13). Cells of swollen germ tubes and giant cells formed from the conidia of *P. citrinum* (THOM 1131) in the presence of 2 $\mu\text{g/ml}$ of tunicamycin in medium A had more than 3 nuclei per cell (Fig. 5-13-18). Giant cells with diameter of more than 20 μm which were formed from conidia of *P. citrinum* (THOM 1131) or *A. niger* (AHU 7117) in the presence of more than 10 $\mu\text{g/ml}$ (more than 2 $\mu\text{g/ml}$ in the case of *P. citrinum*) of tunicamycin in medium A had more than 50 nuclei per cell (Fig. 5-16, 17, 19, 20, 23-27). In the presence of tunicamycin at concentrations higher than MIC (20 $\mu\text{g/ml}$), population of giant cells lacking distinct nuclei increased in *P. citrinum* (THOM 1131) (Fig. 5-21, 22).

DISCUSSION

Tunicamycin was observed to induce osmotically stable multinuclear giant cells and a "beaded" filament from conidia and germ tubes of *P. citrinum* and *A. niger*. The multinuclear cell formation by antibiotics was only reported in mammalian cells (L cell (5)) and SV40-transformed mouse cell (6) treated with cytochalasin-B. In the latter case, number of nuclei per cell increased up to 10. On the other hand, some of the fungal multinuclear giant cells were confirmed to contain more than 50 nuclei per cell. Probably such a large number of nuclei have not been observed in any giant cells so far reported. Furthermore, as shown in Table 2, ratio of cell volume to the number of nuclei in the giant cell was nearly equal to that in normal hyphal cell or conidium. This means that the increase in the number of nuclei paralleled the increase of cell mass. These observations indicate that tunicamycin

Table 2. Cell volume and numbers of nuclei of cells of normal hyphae, conidia and giant cells of *P. citrinum* (THOM 1131).

	Normal		Giant cell		
	Hyphal cell	Conidium	A	B	C
Cell size (μm)	$1.2\phi \times 15$	3ϕ	10ϕ	20ϕ	30ϕ
Volume (μm^3) ^a	17	14	98	785	2650
No. of nuclei	1	1	≈ 8	≥ 40	≥ 100
Volume/No. of nuclei	17	14	≈ 12.3	≤ 19.6	≤ 26.5

^a Volume of hyphal cell, conidium, and giant cell was calculated on the assumptions that a hyphal cell is cylindrical and a conidium and a giant cell are spherical.

Fig. 5. Bars 6 μ m. Swollen germ tubes (1) formed from conidia of *P. citrinum* (THOM 1131) in the presence of 2 μ g/ml of tunicamycin in medium A. Giant cells formed from conidia of *P. citrinum* (THOM 1131) after incubation at 30° for 17 hr (2) or 48 hr (3) in medium A, or 24 hr in medium B (4) in the presence of 10 μ g/ml of tunicamycin. Growing and dividing giant cells of *P. citrinum* (THOM 1131) in the presence of 10 μ g/ml of tunicamycin in medium A at 30° (5, 6). Germinating giant cells of *P. citrinum* (THOM 1131) after removal of tunicamycin in medium A (7). Swollen hyphal tips of *P. citrinum* (THOM 1131) after addition of 50 μ g/ml of tunicamycin in medium A (8). "Beaded" filaments formed from normal hyphae after incubation at 30° for 48 hr in the presence of 50 μ g/ml of tunicamycin in medium A (9). Giant cells of *A. niger* (AHU 7117) formed

from conidia after incubation at 37° for 2 days in medium A in the presence of 10 $\mu\text{g/ml}$ of tunicamycin (10). Giant cells of *A. oryzae* (var. No. 11) formed from conidia after incubation at 20° for 7 days in the presence of 100 $\mu\text{g/ml}$ of tunicamycin in medium A (11). Giemsa staining of normal hyphae (12), or swollen germ tubes (13, 14) formed from conidia of *P. citrinum* (THOM 1131) in the presence of 2 $\mu\text{g/ml}$ of tunicamycin in medium A. Giemsa staining of giant cells formed from conidia of *P. citrinum* (THOM 1131) in the presence of 2 $\mu\text{g/ml}$ (15–17), 10 $\mu\text{g/ml}$ (18–20), 50 $\mu\text{g/ml}$ (21) or 200 $\mu\text{g/ml}$ (22, 23) of tunicamycin in medium A. Giemsa staining of giant cells of *A. niger* (AHU 7117) formed from conidia in the presence of 10 $\mu\text{g/ml}$ of tunicamycin in medium A (24–27).

Fig. 5. (continued)

Fig. 5. (continued)

Fig. 5. (continued)

camycin inhibits neither increase of cell mass nor chromosome replication but selectively inhibits the synthesis of cell surface in both *P. citrinum* and *A. niger*.

There are many reports related to antifungal substances which induce some morphological changes in fungi (7). Giant cell formation was reported in *Cochliobolus miyabeanus* (8) and *Mucor rouxii* (9) treated with polyoxin D, and in *Schizophyllum commune* and other strains treated with a thermostable protein isolated from *B. cereus* (10), and "beaded" filament formation was reported in *A. nidulans* treated with cytochalasin B (11). Unlike tunicamycin-induced giant cells, however, the giant cells induced by polyoxin D were reported to be osmotically labile (8, 9). Furthermore, none of the above-mentioned giant cells and "beaded" filaments were reported to contain many nuclei, while tunicamycin-induced giant cells were multinuclear cells. Therefore, the unique action of tunicamycin on fungi would be distinguished clearly from the action of polyoxin D, cytochalasin B, or the thermostable protein of *B. cereus* in mechanism.

Mechanism of the action of tunicamycin on viruses, animal cells, bacteria, and yeasts has been studied in detail. Both synthesis and function of cell membranes of Newcastle disease virus and chick embryo fibroblasts were suggested to be affected by the antibiotic (2, 12). Morphological changes of *B. subtilis* cells induced by tunicamycin with little effect on the biosyntheses of protein, RNA, and DNA, and with strong inhibition of the incorporation of glucosamine into the acid-insoluble macromolecular fraction suggest that inhibition of cell wall synthesis takes place in microorganisms as in animal cells (3). Further studies on the action mechanism of tunicamycin on chick embryo cells indicate that the target point of the antibiotic in the synthesis of animal cell membrane is the step of the formation of a lipid intermediate, polyisoprenyl-GlcNAc pyrophosphate, in the process of glycosidation of glycoprotein (13). Studies using particulate enzyme preparation from *M. lysodeikticus* indicate that the target point of tunicamycin in the synthesis of bacterial cell wall is also in the step of the formation of lipid intermediate, MurNAc-(pentapeptide)-p-p lipid, in the process of peptide glycan synthesis (14). KUO and LAMPEN (15) reported that tunicamycin selectively blocked the syntheses of wall mannan-protein complex and mannan-containing enzymes in the yeast.

The general conclusion to be drawn from the data presented in this paper, in combination with the above results, is that tunicamycin selectively inhibits the synthesis of cell surface irrespective of the kind of a cell.

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