PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 155, 239-242 (1977)

Comparison of Cytochalasins (A, B, D, and E) in Chick Explant Teratogenicity and Tissue Culture Systems[1] (39781)

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Introduction. Cytochalasins are produced by a variety of mold species identified as pathogens or contaminants of crops and foods. The reports of their interference with cell motility, contractility, and adhesion (1-4) led us to speculate upon their possible teratogenicity resulting in anencephaly or meningomyelocele by interruption of neurulation. Inhibition of neural tube closure with cytochalasin B in an explanted chick embryo system has been demonstrated (5, 6).

Despite their related chemical structure (7-11), reports of the biologic effects and potency of the thus-far-described cytochalasins have been contradictory in different test systems (12). In this paper we report a comparison of four of these compounds in tissue culture and explanted chick embryo systems.

The commercially available and relatively stable cytochalasins A, B, D, and E, along with chloroform extracts of known cytochalasin-producing molds, were selected. Variation in ability to cause binucleation of cells in culture through interference with cytokinesis and the resistance of inactivation of this effect by heat were evaluated. Their relative strengths in this bioassay were then compared to their abilities to inhibit neural tube closure in the explanted chick embryo.

Material and methods. Binucleation assay (bioassay). An established mouse embryo cell line designated "Rose," obtained from Dr. Stanley Gartler, Department of Genetics, University of Washington, was maintained in M199 supplemented with 15% fetal calf serum (v/v) (Grand Island Biological Co.) plus 50 μg of streptomycin and 100 U of penicillin/ml. Ten thousand cells (by Coulter counter Model ZBI) dissociated with 0.25% trypsin (National Biochemical) were seeded in 1 ml of the above media in each well of four-chamber Lab-Tek tissue culture chamber slides (Lab-Tek, Naperville, Ill.) and incubated at 37° with 5% CO_2 for 48 hr before the addition of the test material or dimethyl sulfoxide (DMSO) control in fresh media. After an additional 24 hr, they were formalin fixed and stained with Giemsa (Harlico). To evaluate binucleation, at least 200 cells were counted at 500× magnification in an area of nonconfluent growth, and those cells in which there were two nuclei of equal size both containing nucleoli were tabulated, excluding from the count overstained or rounded-up cells and cells with lobulated nuclei or nuclear debris.

Cytochalasin stock. Stock solutions, stored at room temperature, obtained through the courtesy of Dr. S. B. Carter, Imperial Industries, Ltd., England, were made up in DMSO such that the final concentrations per milliliter of medium could be obtained with a volume not to exceed 10 μl. Except for cytochalasin A these solutions stored at 22-24° were shown to retain the same assayed tissue culture activity for up to 3 months.

Fungal stock. Dried chloroform extracts from 1.5-liter cultures of *Rosellinia necatrix* (cytochalasin E),[3] Drechslera (previously *Helminthosporium*) *dematoidea* (cytochalasins A, B, and F), and *Metarrhizium anisopliae* (cytochalasins C and D) were produced by Dr. Joseph Kuc, Department of Plant Pathology, University of Kentucky, redissolved in 1 ml of DMSO, and bioas-

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[1] Supported by the National Institute of Child Health and Human Development Grant HD08554.

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[3] Type of cytochalasin produced as listed in American Type Culture Collection (13).

ISSN 0037-9727

sayed at concentrations from 0.02 to 2.0% of the original culture volume. One milligram of cytochalasin B was added to a crude culture filtrate of *Helminthosporium carbonum* before concentration and extraction to test recovery by the extraction method.

Chick embryo explant. Cold fertile hen eggs incubated 26 to 29 hr at 37° to stage 6 to 7 of Hamburger–Hamilton (14) were explanted by New's technique (15), treated as previously described (5) with appropriate concentrations of cytochalasins A, B, D, or E dispersed in thin albumin diluted 1:4 with water, and compared to embryos treated with equal concentrations of DMSO. For cytochalasin E, chick embryos were also explanted by Spratt's technique as modified by DeHaan (16). The explants by either technique were incubated an additional 26–30 hr to Hamburger–Hamilton's stage 11 to 13 (12 to 20 somites) and those with beating hearts were observed under a dissecting microscope for state of closure of the cephalic portion of the neural tube.

Results. Comparison of the four cytochalasins assayed for their ability to cause binucleation in an established embryo cell line is shown in Fig. 1. Concentrations above those yielding about 60% binucleation were cytotoxic in this system. Cytochalasin E at 0.05 μg/ml achieved a 50% binucleating rate compared to 0.3 μg/ml of cytochalasin A or D and 1.5 μg/ml of cytochalasin B. A flatter dose-response curve was observed with cytochalasin A. The binucleating activity of the four cytochalasins was not reduced by autoclaving at 120° for 15 min.

Binucleating activity was demonstrated in the chloroform extract of mycelial mat (30 g) and culture filtrate of *R. necatrix* at a rate estimated to represent approximately 0.25 mg of cytochalasin E in the original culture. Activity was not demonstrated from *D. dematoidea* or *M. anisopliae* but 100% binucleating activity was recovered when 1 mg of cytochalasin B was added to a crude culture filtrate of *H. carbonum* before concentration and chloroform extraction.

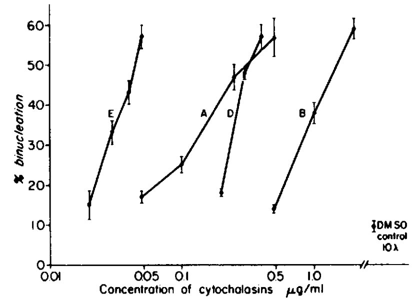

FIG. 1. Comparison of mouse embryo cell binucleation rate when cultured for 24 hr in the presence of varying concentrations of cytochalasins A, B, D, or E. The brackets include plus or minus one standard error.

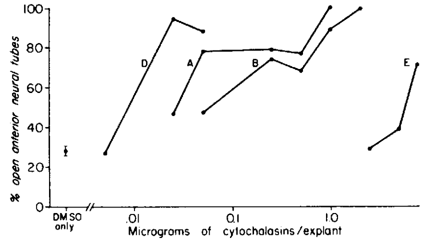

FIG. 2. Comparison of the percentage of open anterior neural tubes when chick embryo explants were cultured for 20–24 hr in the presence of varying concentrations of cytochalasins A, B, D, or E.

The ordering of response of chick embryo explants to the cytochalasins was quite different from that of mouse embryo cells in culture (see Fig. 2 and Table I). As shown, cytochalasin E activity, which was greatest in the cell culture system, has the least interference with neural tube closure. A significant number ($P < 0.05$) of open neural tubes was observed with cytochalasin D treatment at a dose 300-fold lower than that needed with E, the reverse of the response in cell culture. Cytochalasins A and B were of comparable intermediate activity, and again, as in the cell culture, the slope of the cytochalasin A response curve was flatter than those of the other cytochalasins.

Discussion. The wide distribution of cytochalasin-producing fungi, some of which are pathogens of crops or contaminants of foods suggests the need to study their activity in biologic systems beyond the cellular or subcellular level. The five foods reported to contain cytochalasin-producing molds include potatoes (13, 18), tomatoes (19), rice (20), millet (21), and pecans (11) (Table II). The resistance of the cytochalasins to inactivation by heat, reported here, increases the potential for human exposure from cooked or canned foods.

The lack of correlation of response in the

TABLE I. OPEN NEURAL TUBES PER VIABLE EMBRYOS TREATED WITH CYTOCHALASINS A, B, D, OR E.[a]

	Cytochalasin							
Dose (μg/explant)	A	P^b	B	P^b	D	P^b	E	P^b
0.005					4/15			
0.025	7/15	0.7			16/17	0.001		
0.05			8/17	0.1	14/16	0.001		
0.25	15/19	0.05	13/17	0.01				
0.5	14/21	0.01	15/22	0.001				
1.0	18/18		8/9	0.001				
2.0	7/7		3/3	0.05				
2.5							5/17	NS
5.0							7/18	NS
7.5							17/24	0.02
Control DMSO only	6/26		15/66		8/28		5/18 16/54	

[a] Open anterior neural tubes in chick embryos explanted at stage 6 to 7 of Hamburger-Hamilton (14) and cultured for 24 hr in the presence of cytochalasin. All explants were made using New's method (15), except for the three concentrations of cytochalasin E where Spratt's technique (16) was used. No dose response was demonstrated with New's technique at subtoxic levels of cytochalasin E.

[b] χ^2 values, after Edwards (17) with Yates corrections for continuity, were computed by comparison with concurrent controls.

TABLE II. CYTOCHALASINS RECOVERED FROM FOOD.

Food	Cytochalasin	Mold contaminant	Reference
Pecan	H	*Phomopsis* sp.	11
Potato	A, B	*Phoma exigua*	13, 18
Tomato	B	*Hormiscium sp.*	19
Cooked rice	E	*Aspergillus clavatus*	20
Kodo millet	D	*Phomopsis paspalli*	21

two systems reported here emphasizes the complexity of the action of the cytochalasins and may indicate that different mechanisms are being monitored. Microfilament disruption probably plays a major role in the block of cytokinesis which leads to binucleation (22). Microfilaments were implicated by Schroeder (22) in bringing about apposition of neural plate cells in neural tube closure in Xenopus. Karfunkel (6) used cytochalasin B to interrupt neurulation in the chick embryo and showed the concurrent loss of microfilaments.

Our comparison of four cytochalasins in two systems showed cytochalasin E most active in interferring with cytokinesis, yet least active in inhibiting neural tube closure. There are several possible explanations for this. In order to determine whether species specificity could account for the differences, chick fibroblasts were used in the binucleation assay. The ordered response was similar to that in the mouse cell system. This does not, however, rule out differences in transport or metabolism of cytochalasins in the cell culture as compared to the whole embryo culture systems.

As indicated above, these two systems may have selective sensitivity to different aspects of the action of cytochalasins. Although interference with microfilaments may be primary, the cytochalasins have also been shown to interfere with hexose transport (23, 24) and to have marked cell surface effects that are reflected in changes in microvilli, mobility, ruffling, and adhesion (1, 4, 25). Further study at the ultrastructural level comparing the actions of different cytochalasins on different cell types, as suggested by Croop and Holtzer (26), is necessary to elucidate the differences implied by the variation in binding sites for ^{3}H-labeled cytochalasins B or D reported by Mayhew *et al.* (27) and Tannenbaum *et al.* (28).

Our aim has been to investigate the teratogenic potential of the cytochalasins and the foods with which they are associated. Despite the lack of specific quantitative correlation of binucleation with neurulation inhibition, the cell bioassay is sensitive and can be quantitated. The ability to demonstrate binucleating activity in only one of the three reported cytochalasin-producing molds may reflect strain variation in production as indicated by Aldrich *et al.* (10) or a suboptimal extraction process of natural cytochalasin.

The cytochalasins may also have varying stability under native conditions (concentration, pH, temperature, etc.). The flatter slope of the response curve for cytochalasin A may reflect its instability with storage or under conditions of culture. The curve steepened and shifted to the left with freshly prepared stocks but too few experiments were made to determine the exact slope.

Although we had not been able to show cytochalasin B to be teratogenic in the mouse, both systems, but particularly the chick, indicated other cytochalasin molecules might be more active. In fact a significant malformation increase has been produced by both oral and peritoneal administration of cytochalasin D in pregnant mice (manuscript in preparation).

Summary. The four cytochalasins studied were found to have quantitative biologic activity at low concentrations, but elicited marked variation in ordering of response when compared in two *in vitro* systems. All four cytochalasins were teratogenic *in vitro*. Cytochalasin D was teratogenic at the lowest concentration, while cytochalasin E was the strongest inhibitor of cytokinesis. Since these compounds have been recovered from food and are stable to heat, their biologic activity in man should be studied.

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Received December 2, 1976. P.S.E.B.M. 1977, Vol. 155.