Effect of Digitonin on Cellular Division

IV. Interrelation between nucleus and phragmoplast

L. V. Olah and J. J. Bozzola

Botany Department, Southern Illinois University
Carbondale, Illinois 62901, U.S.A.

Received September 5, 1970

As previously reported, purified digitonin as a mitotic agent selectively dissociates formation of the mitotic spindle from the phragmoplast (Olah 1963, 1965; Underbrink and Olah, 1963, 1965, 1968). Digitonin mitosis is characterized by a selectively suppressed spindle and development of a garbled phragmoplast which produces a geometrically upset, branched cell wall system associated with the restituting tetraploid nucleus. This constant and characteristic association deserves further study. Extensive information concerning the chemical nature of the cell plate and wall system found in this substance was published earlier (Olah 1965).

Mitotic figures in treated cells may be classified into two categories: 1) Cells with arrested metaphases identical to those found during colchicine metaphase, showing randomly scattered chromosomes and no trace of rudimentary spindle substance (Figs. 1-3). At anaphase, only cytoplasmic drifting seems to account for chromatidial movement. 2) Cells showing partial stathmokinetic effect characterized by limited "polarized" movement forming groups of separated chromatids and/or chromosomes (Fig. 4).

The problem of whether both types of cells are able to form nuclei with the associated phragmoplast material was investigated by determining the frequency of both types of cells, and comparing this with the frequency of tetraploid nuclei with and without associated phragmoplast substance. In addition, we: studied development, structure and birefringency of post-mitotic cell wall complexes in treated cells; studied the course of the second and subsequent divisions of cells which underwent one digitonin mitosis; investigated incorporation patterns of tritiated thymidine and uridine in treated cells and controls; studied the role of the nucleus involved with phragmoplast association; and compared the digitonin effect with functionally or structurally related substances as tomatin, digitalin, digitoxin, deoxycholate, and Tween 20 (a non-ionic detergent).

Materials and methods

Roots of *Allium sativum* cloves aseptically grown in tap water or 0.5% agar-water were treated with 0.25 and 0.50% purified digitonin (courtesy of Merck, Sharp, and Dohme, Inc.) for varying times. Rooted cloves were washed, centrifuged at 6,500 rpm, and recovered in sterile tap water or agar-water. Excised root tips were fixed with Helly, Navashin, Zenker, LaCour 2BE, and Farmer fluids.

Staining was with carmine, orcein, and Feulgen method for squashes; methyl green-pyronin, Feulgen, light green, fast green, Flemming triple stain, toluidine blue, methylene blue, aniline blue, PAS-picric acid, hematoxylin, and mercuric bromphenol blue for 10 μ paraffin sections; 70% sulfuric acid and iodine for fresh material. A Leitz polarizing microscope was used to study the disposition of microfibrils. Statistical counts of all divisions were carried out in the same root tip (15 hrs treatment with 0.25% digitonin) to determine the proportion of the two types of mitotic divisions and resultant nuclei.

For autoradiographic studies, root tips were treated with 0.25% purified digitonin in double-distilled water at pH 6.9 for 17 hrs, washed, and recovered in boiled tap water for 24 hrs. Rooted cloves were then placed in ^3H uridine (5 mCi/ml) for 20 min, chased in double-distilled water for 0, 10, 20, 30, 40, 60, 120, 180, 240 min., 5, 6, and 10 hrs. Excised tips were then washed and macerated in 5% pectinase at 33°C for 2–4 hrs. ^3H thymidine (5 mCi/ml) was also used for 30 min, chased in cold thymidine for 0, 1–10 min, $\frac{1}{2}$, 1–6 hr at hourly intervals, 8–24 hrs at 2 hr intervals, 26–30 hrs at hourly intervals, and 32 hrs. After both incorporations, Farmer-fixed root tips were hydrolyzed, methyl green-pyronin and Feulgen-stained, root caps were removed, and the tips squashed. Smear slides were incubated for 2 days at 24°C for drying, coverslips removed and remounted on a slide with smear side up. Fuji or Kodak stripping films were applied to slides and coverslips with exposure times of 15 and 21 days for uridine and thymidine respectively.

Root tips were also treated at various concentrations and times with the glycosidal alkaloid tomatin (crude extract), sodium deoxycholate, Tween 20, digitoxin, and digitalin in order to compare their effects to those of digitonin. Rosette-like crystals found in some digitonin-treated cells were also studied. In all experiments, control material was run concurrently.

Results

Up to early anaphase, the effect of digitonin is usually similar to that of colchicine (Figs. 1–3). Any substance which could be considered a remnant of rudimentary spindle is absent. The partial stathmokinetic effect is shown in Fig. 4 with apparent multipolar movement of grouped chromosomes and chromatids. Among 3,224 mitotic configurations counted in a root tip (0.25%, 15 hrs) 201 prophase cells, 1,740 cells showing arrested c-meta- and anaphases, and only 87 cells with multipolar spindles were found. 1,169 telophases revealed a clear association with phragmoplast substance, while only 27 were found without any detectable phragmoplast substance. Besides these, 3,841 completely restituted tetraploid nuclei were found to be associated with phragmoplast and cell wall complex and only 40 tetraploid nuclei did not possess such substance. The number of multinucleate cells with separating wall systems was 352.

Early and late telophase digitonin mitoses are shown in Figs. 5–11. Constant association of phragmoplast substance with restituting chromosomes is apparent. The refractive phragmoplast-wall complex appears at the onset of telophase (Figs. 5, 6 arrows) always among the decondensing chromosomes. The association

Figs. 1-14. Key to abbreviations: Ac=aceto-carmine; cent=centrifugation; c.p.=cell plate; c.w.=cell wall; dig=digitonin; F=Feulgen; PAS=periodic acid-Schiff; %=percent digitonin used (0.25% unless specified); ph=phragmoplast; treatment time in hrs/recovery time in hrs 1-36 squashes, except 12-14. 1-3, 19/0, Ac. Dig. meta-, ana-, and late anaphase, respectively. 4, 17/0, F. Multipolar spindle. Arrow, entire chromosome. 5-11, 17/0, F. All taken from nearly adjacent cells. Dig. telophase and reinitiating nuclei. Arrows, first appearance of ph substance. Note ph substance surrounded by chromosomes. 12-14, 16/15, PAS. Restituted nuclei. Arrows, PAS-positive material associated with nuclei.

Figs. 15–22. 15, 17/cent 5 min/54, F. 16–17, 42/cent 5 min/30, F. Arrow, cell wall material. 18, 17/54, F. Arrows, ph substance associated with two separate nuclei. 19, 17/48, F. Arrow, diploid nuclei which did not divide during treatment. 20–21, 42/30/cent/14 hr rerecovery, F. Arrows, three areas of associated ph substance. 22, 17/48, Ac.

Figs. 23-37. 23, 17/48, Ac. Diploid nuclei which did not divide during treatment. 24-25, 17/68, F. 26, 42/44, F. 27-28, 17/68, F. 29, 42/44, F. 30-36. 17/24, pectinase maceration, methyl green-pyronine stain. Tritiated uridine, 20 min chase in distilled water. 30, 20 min treatment, 0 chase. Arrows, unlabeled c.w. complex. 31, drawing of cells in Fig. 30. Nucleoli, arrows; nuclei, solid lines; two dense bodies lower most. 32-36, all 20 min treatment with chases of 10, 20, 30, 60, and 120 min. 37, multiple spindle, left; c.w. development at later stage. *Sequoia gigantea*.

of this PAS-positive substance persists in all later stages (Figs. 12–14 arrows, 15–29). Rapid removal of digitonin by centrifugation or repeated washing followed by recovery may have a two-fold effect: conspicuous increase of cell wall substances (Figs. 16, 19) and striking elongation of certain cells (Figs. 15, 24). In Figs. 15–17, 21 and 28, the nuclear material completely encircles the associated substance, while in Figs. 19, 25, and 27, a sidewise association is apparent. Fig. 23 shows undivided diploid nuclei unaffected by treatment and Figs. 20, 22, 26, and 29 reveal distorted tetraploid nuclei still adhering to cell wall substances. Fig. 24 shows an elongated cell with a nucleus in which a segment of cell wall is sandwiched.

Bi- and multinucleate cells frequently attempt to separate into compartments by wall segments (Fig. 47). In Fig. 18, two nuclei in a tetranucleate cell are associated with phragmoplast material. The trinucleate cell shown in Fig. 45 contains one second division anaphase nucleus with no trace of phragmoplast material among the chromatids. However, in the same cytoplasm, the nucleus above it is in mid-telophase with several decondensing chromatids and contains sharply delineated, refractive, dumb-bell shaped associated phragmoplast substance (single arrow). A rudimentary wall segment apparently formed during the first digitonin mitosis is indicated by double arrows.

Cells which underwent one digitonin mitosis frequently enter a second normal mitosis if digitonin is removed and recovery started in time. In such cells, the garbled and rudimentary “first generation” cell wall (Figs. 38–40, 42–44 arrows, 41 long arrow) may constitute a physical obstacle. Mitosis in such cells is dislocated and found in the area which is free from the first generation wall complex. This dislocation hampers and disarrays movement of those chromatids approaching the substance; whereas, movement of the chromatids is unhampered at the opposite pole. The second generation cell plate appears in the equatorial plane of the quasi-normal spindle (Figs. 40, 41 short arrow, 43, 44). A joint between the first and second generation wall system was frequently observed (Fig. 44 arrow). More

Figs. 38–50. 38–44, 50 Paraffin sections, 45–48 squashes. 38, 1.0%, 47/20, PAS. Late anaphase of second normal division cells which underwent one dig mitosis. Arrow c.w. complex formed during first division. 39, 1.0%, 20.5/46, same as 38. 40, 26/27, F. Telophase of similar second division forming transverse c.p. Arrow, c.w. material formed during first division associated with upper daughter nucleus. 41, 0.5%, 27/20, PAS. Telophase. First generation complex associated with lower group of chromatids, long arrow. Short arrows, c.p. of second division dislocated. 42, 1.0%, 27/20, PAS. Anaphase of second division of diploid nucleus. Arrow, c.w. material associated with upper group of chromatids. 43, 1.0%, 47/20, PAS. Early telophase. Arrow, first generation wall complex. Note c.p. in normal spindle. 44, same as 43. Arrow, two generations of c.p. fused. 45, 17/45, F. Two asynchronous divisions in same cell. Lower nucleus in anaphase, upper in telophase. Arrow, associated ph substance. Lower left cell in arrested metaphase. Double arrow, rudimentary c.w. material formed during first division. 46, same treatment. Apparently octoploid nuclei. Arrows, two generations of associated ph material filling separate areas among the chromatids. 47, 17/48, Ac. Second dig mitosis. 48, 17/48, Ac. Second dig anaphase. Arrow, ph complex formed during previous division. 49, dig crystals obtained *in vitro* by mixing dig in 70% alcohol with cholesterol. 50, 2.0%, 19/51, PAS-positive rosette-like crystals in treated cells. Magnifications: 30–36, 48=600×; 42–44=900×; 38–41=1200×; 1–4, 12–14, 23–29=1400×; 5–11, 45, 46=1600×; 15–22, 47=1800×.

than 200 such cells were analyzed. Unexpectedly, in several cases it was established that the dividing nucleus was not tetraploid but remained diploid (Fig. 42). No sign of micronuclei or chromosomal elimination was observed in such diploid cells which possessed abortive first generation substance. Fig. 48 shows a second digitonin mitosis at anaphase. Cell wall substance formed during the preceeding division (arrow) apparently will be trapped again by the restituting nucleus. Octoploid nuclei shown in Figs. 21 and 46 possess associated substance separated into several interconnected bodies (arrows). All post-mitotic cell wall complexes show marked difference in molecular structure when compared with maternal walls of the same cells. The former were not found to be birefringent while the latter were always birefringent.

Incorporation of ^3H uridine in treated cells is shown in Figs. 30–36. In cells fixed immediately after 20 min treatment, almost all nucleoli were labeled distinctly (Fig. 30) while the phragmoplast-wall complex was unlabeled (arrow). Fig. 31 outlines both cells and their nuclei with nucleoli marked by arrows. Figs. 32–36 show cells fixed after 10, 20, 30, 60, and 120 min chase. Arrows mark labeled nucleoli. The pattern of labeling is clearly discernable with the nucleoli, then nuclei (Fig. 35), and finally the cytoplasm (Fig. 36) sequentially incorporating label while the phragmoplast complex is unlabeled. However, this same pattern was found in treated cells which underwent mitosis, in cells which did not divide during treatment, and in untreated cells.

Fig. 49 shows digitonide rosette-like crystals obtained *in vitro* by mixing cholesterol with digitonin in 70% alcohol. One occasionally finds similar rosette-like crystals in treated cells (Fig. 50) but our attempts to dissolve *in vivo* crystals indicated that their behavior is different than *in vitro* crystals. Solvents which readily dissolve digitonide, as hot xylene, pyridine, acetic acid or acetic anhydride failed to extract any crystals from cells. Furthermore, digitonide crystals embedded in gelatin will not be stained with PAS, crystal violet, or methylene blue, whereas cytoplasmic crystals stained red after PAS (Fig. 50) and had a blue tint after crystal violet or methylene blue. Tomatin, digitoxin, digitalin, deoxycholate, and Tween 20 did not reveal any distinct cytological effect when applied on *Allium sativum*.

Discussion

Attempts to establish the nature of the rosette-like crystals found in treated cells failed to substantiate the occurrence of the Windaus reaction *in vivo*. Although digitonin and tomatin are both used for quantitative determination of certain sterols via the Windaus reaction, only digitonin had a distinct cytological effect in dividing cells.

Centrifugation of treated cells influenced the shape of the nucleus and location of the rudimentary transverse wall system. However, its main effect seemed to be the thorough removal of digitonin. Subsequent recovery may produce an unusual amount of cell wall material (Figs. 16, 19). Cellulose in maturing garbled complexes was established with hydrocellulose reagents on fresh sections (Olah 1965). Ultrastructural studies have shown that regardless of location, shape

or size, the amorphous cell wall complex is entirely coated by plasmalemma (Underbrink and Olah 1965). Birefringency studies on fixed and sectioned material indicated the absence of parallel orientation of cellulose microfibrils. Our studies related to cellular elongation and orientation of cortical microtubules found to be associated with the garbled transverse wall system will be discussed separately.

Cells which underwent two digitonin mitoses may result in octoploid nuclei associated with two generations of phragmoplast-wall complex. If a second division similar to Fig. 48 produces a second generation substance during the second telophase (which will be trapped by the restituting nucleus), the result will be octoploid nuclei containing two generations of cell wall complex. Such nuclei are shown in Figs. 21, 46. However, in cells recovered between the first and second division, both wall complexes will lie in the cytoplasm (Figs. 40, 41, 43, 44). The fact that these complexes may fuse together indicates that differences are architectural rather than chemical. Since the first division produces a tetraploid nucleus, the second division should display 32 chromatids in each daughter nuclei. However, in several cells only 16 chromatids were found (Fig. 42). The only explanation for this unique phenomenon would be Mole-Bajer's, that phragmoplast formation in chloral-hydrate treated cells may occur before nuclear disintegration at prophase (1967, 1969). Following this unusual line of reasoning, it may be assumed that cells which did not divide during treatment divide first during recovery. During this single division at prophase, phragmoplast substance develops before nuclear breakdown. During the same mitosis at telophase, cell plate forms again in the area of the spindle. However, any theory which assumes that the sequential order of metabolic steps can be reset and/or interchanged is difficult to accept; the idea that steps involved in cytokinesis may precede steps essential for nuclear division is improbable.

The *in vivo* observations initiated by Wada and exploited by Bajer and Mole-Bajer have indeed contributed much to understanding problems related to spindle and phragmoplast. These results were extensively reviewed by Wada (1966) and Bajer (1971). Bajer and Mole-Bajer (1961) observed *in vivo* that developing phragmoplast may dislocate and indent restituting chromatin. Thus, it is apparent that nuclear shape depends upon the size and location of the developing phragmoplast and vice versa. It is pertinent to note that regardless of the shape of the restituted nucleus, the entire nuclear body was always covered by nuclear envelope which separated the two associated components (Underbrink and Olah 1965).

It is true that relatively small meristematic cells with large nuclei must produce phragmoplast associated with the tetraploid nucleus due to crowded spatial conditions. However, we believe that crowding is not the only factor involved since the same association was observed in larger differentiated cells. Furthermore, in bi- and multinucleate cells, more than one nucleus may be associated with such substance (Fig. 18). In such cells, it seems that each nucleus possesses centers of initial synthesis or assembly. In multinucleate cells, however, the smaller nuclei may have lost the ability to induce the formation of such substance. It is especially instructive to note the behavior of two nuclei in the cell shown in Fig. 45. One nucleus is in digitonin anaphase, the second (above it) in telophase, and the third

in interphase. Thus, the first digitonin mitosis produced a trinucleate cell. However, only a small amount of rudimentary cell wall material has been formed (Fig. 45 double arrow) and the cell was not able to separate the nuclei. Digitonin apparently was not removed since all dividing nuclei are undergoing digitonin mitosis (note lower left cell in arrested metaphase). Since no trace of phragmoplast material is observable among the anaphase chromatids, the initial steps of formation apparently start later—probably concomitant with catachromasis. In the upper nucleus, at telophase, the dumb-bell shaped phragmoplast substance (long arrow) among the decondensing chromosomes indicates that this nucleus was now able to “induce” phragmoplast substance.

Fig. 47 shows a fully recovered tri-nucleate cell dividing synchronously due to incomplete wall separation. Fig. 48 shows a second digitonin division with an arrow marking cell wall complex formed at the first division. The position of the chromatids indicates that after the first division this cell possessed a single hollow tetraploid nucleus associated with the complex. It is also apparent that the resulting octoploid nucleus will trap the first generation phragmoplast. Furthermore, if phragmoplast material again appears among the chromatids in such a cell during the second telophase, the resulting octoploid nucleus may contain two generations of phragmoplast-cell wall complex. Such nuclei are shown in Fig. 46. It is clear that any explanation which implies only a crowded spatial condition is not satisfactory for the above configurations. The tentative explanation for these unique configurations is the assumption that phragmoplast substance formation is initiated by a message released from the restituting chromatids. Although we are not able to present direct evidence to substantiate our assumption, we speculate that during normal mitosis this message is released from the chromatids of both restituting daughter nuclei resulting, via interaction, in the formation of the microtubular phragmoplast and equidistant cell plate. In digitonin-treated cells, collapse of geometrical conditions results in the initiation of the above substance among the chromosomes. The nature of this hypothetical message remains speculative. It is probably enzymatic in nature and may trigger steps essential to the synthesis of phragmoplast microtubules.

Our tritiated thymidine and uridine investigations did not support the assumption that digitonin interferes with DNA or RNA synthesis. The incorporation of thymidine into chromosomes and nuclei was recognizable but since many silver grains appeared over the cytoplasm, our results were similar to Fussell's, who also used *Allium* (1968). The incorporation of uridine was satisfactory and distinct, but treated and control material showed the same pattern of incorporation.

Since we provide evidence that a complete dissociation between phragmoplast and spindle may occur, it is pertinent to note that such dissociation exists in at least three coenocytial systems of higher plants (Olah 1969). In certain PMC's, coenocytial embryo, and non-cellular or nuclear endosperm (Fig. 37: Bucholz 1939) the dissociation is spatial and chronological since phragmoplast spindle and cell plate formation is delayed by one or more complete mitotic cycles. In the multinucleate coenocytia, several phragmoplast spindles may radiate out from one nucleus connecting it with sister and mostly non-sister nuclei (Fig. 37 left). We

cannot escape the logic that if there are any "organizing centers" for this multi-spindle system, these centers which emanate messages should be located in the nuclei. In coenocytial endosperm, the multipolar spindle system is formed in interphase. The tentative hypothesis, that a message is released by interphase chromosomes (centromeres?), would explain the unique mutual nuclear interactions which result in the formation of a multi-spindle system. It also explains the phenomenon of the persistent association of phragmoplast substances with the chromosomes in treated cells with upset geometry. Since microtubules seem to represent a cytoskeletal system for maintenance of normal geometry, we established the effect of digitonin on microtubules (Olah and Hanzely 1969). A separate paper will deal with this selective effect.

Summary

A study of nuclear and phragmoplast association observed in digitonin-treated *Allium sativum* cells led to a tentative hypothesis that initiation of formation of the phragmoplast-cell wall complex is triggered by a message released by chromosomes of restituting nuclei. A similar theory is applied in an attempt to explain the multiple spindle and phragmoplast systems present in certain PMC's, coenocytic embryos, and non-cellular or nuclear endosperm. Tritiated thymidine and uridine incorporations suggest that digitonin does not interfere with DNA and RNA synthesis. Compounds structurally and functionally related to digitonin did not induce digitonin mitosis.

Acknowledgements: The authors gratefully thank the S.I.U. Office of Research and Projects for financial support and the Merck, Sharp, and Dohme Research Laboratories for purification of digitonin. We also express our sincere gratitude to Drs. Tadashi Hirano and Akio Kushanagi for the valuable assistance granted the senior author during his stay at the Tokyo Metropolitan Isotope Center.

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