Cytological and Autoradiographic Studies of the Effects of Vincalukoblastine (VLB) on the Root Meristem Cells of *Vicia faba*

Sharda Srivastava, S. R. V. Rao, and V. C. Shah

Department of Zoology, University of Delhi, Delhi-7, India

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The effects of Vincalukoblastine (VLB) have been studied on the tissue culture cells, ascites tumor cells and normal bone marrow cells (Palmer *et al.* 1960, Cardinali *et al.* 1961, Cutts 1961). In these studies, it has been shown that VLB acts like colchicine as it produces mitotic arrest resulting in accumulation of metaphases. We have studied the effects of VLB on the lateral root meristem cells of *Vicia faba* and report the drug's effect on mitosis and macromolecular synthesis.

Material and methods

Growth conditions: Seeds of *Vicia faba* (A. L. Castle Inc.) were soaked and washed in running tap water for 24 hours. Seed coats were removed and primary roots were allowed to grow at 21°C. Only the secondary roots measuring 2-3 cm were utilized. The following VLB concentrations 10 µgm, 15 µgm, 20 µgm and 25 µgm/ml at pH 6.4 were used. All experiments were carried out under controlled temperature of $21 \pm 2^\circ\text{C}$.

Continuous treatment: Four seedlings with a large number of roots were immersed in each concentration of drug and four seedlings each were maintained in distilled water as controls. At various intervals ranging from 6 to 24 hours, 4 root tips were excised from the above seedlings and fixed in acetic alcohol. In another experiment, the roots were continuously treated with 25 µgm/ml VLB for 12 hours and fixed every hour. Roots were stained with Feulgen and permanent squash preparations were made.

Brief treatment and recovery: From the above studies, we found that 25 µgm/ml dose was most effective and was used in all the subsequent experiments. Roots were exposed for 6 hours and transferred to distilled water after a thorough washing. The distilled water was changed once in 24 hours. At various intervals of 6, 12, 18, 24 and 48 hours, root tips were fixed in acetic alcohol.

VLB in combination with aminoacids: Glutamic acid ($3.3 \times 10^{-3}\text{M}$) and cysteine solution (10^{-3}M) were used in combination with the drug. Adequate number of roots were fixed at 6, 12 and 24 hours after each treatment. Both the treated and their respective control roots were fixed in acetic alcohol and stained with Feulgen and permanent squash preparations were made.

Autoradiography: The effects of VLB on the synthesis of DNA, RNA and protein were studied by exposing roots to H^3 -thymidine 3 µc/ml (Sp. Act. 6.0 C/mM),

H³-uridine 5 μ C/ml (Sp. Act. 1.1 C/mM), H³-lysine 3 μ C/ml (Sp. Act. 20.5 mC/mM) and H³-arginine 3 μ C/ml (Sp. Act. 247 mC/mM) as suitable precursors for 30 minutes. The radioactive precursors were obtained from Bhabha Atomic Research Centre, Trombay, Bombay. Samples were fixed immediately after treatment and were processed for autoradiography. Squash preparations were dipped in Kodak NTB-3 emulsion. H³-thymidine and H³-uridine slides were exposed for 4 weeks, H³-arginine for 10 days and H³-lysine for 5 days. All slides were developed for 3 minutes in Kodak 19B developer and fixed in acid fixer. The percentage of labelled interphase cells was determined from a total of 3,000 cells. Grain counts from H³-thymidine slides were made from 100 whole nuclei, both from control and treated roots. Grain counts from H³-uridine, H³-lysine and H³-arginine slides were made through a reticule encircling a constant area of 7.85 μ^2 from nucleus and/or cytoplasm. Quantitative estimation of DNA per nucleus was made by a cytophotometer as described by Swift and Rasch (1956) using wavelength 570 m μ .

Observations

Continuous treatment: The effects of various concentrations of VLB on the mitotic index is shown in figure 1. The continuous treatment of VLB for 6 hours had little or no effect on mitotic index at 10 μ gm/ml concentration while at 15 μ gm/ml and 20 μ gm/ml showed slight effect. However, 25 μ gm/ml for similar hours reduced the mitotic index by nearly 50 per cent. A 12 hour treatment resulted in a further decrease in the mitotic index. At 15 μ gm/ml and 20 μ gm/ml the mitotic index was reduced by 31.08 and 45.58 per cent, respectively and at 25 μ gm/ml it was reduced by 88.72 per cent. On prolonging the treatment to 24 hours, 15 μ gm/ml and 20 μ gm/ml dosage decreased the mitotic index by 38.95 and 65.12 per cent respectively. The treatment with 25 μ gm/ml showed more or less complete inhibition (98.45 per cent).

The frequencies of prophases were also affected. The effect was both dose and duration dependent (Fig. 2a). However, 25 μ gm/ml was found to be most effective as it virtually inhibited almost all prophases by 12 hours (99 per cent).

The percentage of metaphases was observed to increase at 6 hours with all the concentrations and especially, 25 μ gm/ml dosage. However, at 12 hours a gradural decline was seen (Fig. 2b)

During the continuous treatment with 25 μ gm/ml VLB the effect was seen as early as 2 hours after treatment. The percentage of prophases showed a sharp decline and at 10 hours no prophases could be observed. There was an increase in the percentage of metaphases. The latter started declining after 8 hours of treatment and was below control levels at 12 hours (Fig. 3).

The post metaphasic stages showed reduction at all concentrations. The reduction in the number of anaphases indicated an inhibition of spindle mechanism.

Recovery: When the treated roots were recovered in distilled water the mitotic index initially decreased and reached to 1.5 per cent during the first 6 hours of recovery i.e. at 12 hours (Fig. 4). But at 12 hours there was a sudden burst of mitosis resulting in a rapid rise of mitotic index to 10 per cent. In general, all

the mitotic stages showed recovery (Fig. 4). The recovery of prophase was not complete since the percentage of prophase was always below control level.

Glutamic acid ($3.39 \times 10^{-3}M$) and cystein ($10^{-3}M$) prevented mitotic arrest and prophase inhibition to varying degrees. Glutamic acid reversed the action of

Fig. 1. Effects of various concentrations of VLB on mitosis in lateral roots of *Vicia faba*. Solid circle—untreated control; hollow triangle—VLB treatment $10 \mu\text{gm/ml}$; solid triangle—VLB treatment $15 \mu\text{gm/ml}$; solid square—VLB treatment $20 \mu\text{gm/ml}$; open circle—VLB treatment $25 \mu\text{gm/ml}$.

VLB on mitotic index (Fig. 5). It prevented prophase inhibition at 6 hours as the percentage of prophase was 6.39% as in controls (5.51%). Glutamic acid also reversed the metaphase arrest. Cystein was more effective in preventing the metaphase arrest but did not prevent prophase inhibition.

Morphological effects: With $25 \mu\text{gm/ml}$ concentration for 12 hours, most of the chromosomes were highly condensed and sticky. Spindle disruption was ap-

Fig. 2. Effects of various concentrations of VLB on different stages of mitosis. 2a—prophase; 2b—metaphase. Legend same as in Fig. 1.

Fig. 3. Effects of 25 µg/ml VLB on prophase and metaphases, 1) Solid circle—untreated control; 2) hollow circle—VLB treated (25 µg/ml).

Fig. 4. Mitotic recovery of *Vicia faba* cells in distilled water (open circle with dotted lines). Arrow indicates transfer of roots after 6 hours treatment to recovery medium.

Fig. 5. Effect of VLB (25 $\mu\text{gm/ml}$) in combination with glutamic acid on mitosis. Solid circle—distilled water control; hollow triangle—VLB plus glutamic acid; solid triangle—glutamic acid control; open circle—VLB in distilled water.

parent as no post metaphasic stages were observed and large number of micro-nuclei were seen.

Effect of VLB on macromolecular synthesis: Feulgen cytophotometric measurements indicated a reduction in DNA content in the VLB treated nuclei. The mean DNA content was 789.14 in arbitrary units in treated nuclei as compared to 1058.31 in controls. Autoradiography data (Table 1) also suggested DNA inhibition (Fig. 6a, b) as the incorporation of label in individual nuclei showed a mean grain count of 116.20 ± 2.62 in controls as compared to 75.91 ± 2.77 in the treated nuclei. However, the percentage of labelled cells was about the same in treated (56.94) as well as in controls (59.79).

Fig. 6. Autoradiographs showing the effects of VLB on the incorporation of labelled precursor into DNA (a, b) and RNA (c, d). a) H^3 -thymidine incorporation in control cells. $\times 800$; b) H^3 -thymidine incorporation in treated cells. $\times 800$; c) H^3 -uridine incorporation in control cells. $\times 2,000$; d) H^3 -uridine incorporation in treated cells. $\times 2,000$.

The mean grain count in H^3 -uridine slides over an unit area of nucleus was 25.00 ± 0.76 in controls as against 10.00 ± 0.43 in treated roots (Table 1) suggesting a significant inhibition of RNA synthesis (Fig. 6c, d).

Using H^3 -lysine and H^3 -arginine the incorporation of these into protein was studied. VLB treatment had an inhibitory effect on protein synthesis (Figs. 7ab, cd). H^3 -lysine incorporation was inhibited considerably at 6 hours. The mean

Table 1. Effect of VLB (25 $\mu\text{gm/ml}$, 6 hrs.) on the incorporation of H^3 -thymidine, H^3 -uridine, H^3 -arginine and H^3 -lysine in the lateral root meristem cells of *V. faba*

Labelled Compound*		Control	Treated	% Inhibition
H^3 -thymidine	Nucleus	116.20 ± 2.62	75.91 ± 2.77	34.69
H^3 -uridine	Nucleus	25.00 ± 0.76	10.00 ± 0.43	60.00
H^3 -arginine	Nucleus	36.10 ± 1.25	13.09 ± 0.44	63.88
	Cytoplasm	24.0 ± 0.88	19.0 ± 0.70	20.83
H^3 -lysine	Nucleus	42.0 ± 1.1	19.0 ± 0.55	54.76
	Cytoplasm	61.0 ± 1.5	31.0 ± 0.88	49.18

* 30 minutes label.

Note: Grain count determinations were made on 100 interphase nuclei over an unit area of cytoplasm and nucleus. In case of H^3 -thymidine the grain count was made on 100 interphase nuclei only. Fig. 7. Autoradiographs showing the effects of VLB on the incorporation of H^3 -arginine (a, b) and H^3 -lysine (c, d) into protein. a, c controls $\times 800$. b, d treated $\times 800$.

grain count in treated cells was 31 ± 0.88 in cytoplasm and 19.0 ± 0.55 in nucleus as compared to 61 ± 1.5 and 42 ± 1.1 of controls. In case of H^3 -arginine the mean grain count per unit area over cytoplasm was 19.0 ± 0.70 and in nucleus it was 13.09 ± 0.44 in treated cells, whereas it was 24 ± 0.88 and 36.10 ± 1.25 grains, respectively, in controls (Table I).

Discussion

Earlier studies (Palmer *et al.* 1960, Cardinali *et al.* 1961, Cutts 1961) indicated that VLB interfered with cell division in animal tissues resulting in an increase in the mitotic index due to accumulation of metaphases. The present study has shown that in addition to its effect on metaphases, VLB has some effect on pro-phases as well as post-metaphasic stages. This results in the reduction in mitotic index (Fig. 1). A similar effect was also seen by Deysson *et al.* (1963) in onion roots treated by VLB.

Our experiments of continuous treatment show that metaphase block begins at 2 hours after treatment and continues up to 8 hours resulting in the accumulation of metaphases. However, after 2 hours the frequency of pro-phases is seen to reduce (Fig. 3). This fall in pro-phases appears to be due to the inhibition at interphase (G_2). This interphase block was investigated in detail in recovery experiments.

It was seen that most of the treated roots recover in distilled water after a brief treatment. The frequencies of pro-phases, post metaphasic stages and the mitotic index, in general, reestablished more nearly as in controls (10.6%). Since the treated cells recovered within 6 hours it seemed that recovery may be due to the release of the block in G_2 which is approximately 4 to 6 hours (Sharda *et al.*, unpublished).

The inhibition of G_2 may suggest that synthesis of some proteins or RNA necessary for mitosis was blocked. Analysis of autoradiograms clearly indicate significant inhibition of both RNA and protein synthesis. It is interesting to note that Richards *et al.* (1966) and Creasy *et al.* (1965) also found inhibition of RNA in thymus cell suspensions and Ehrlich ascites cells, respectively, after VLB treatment.

Treated cells can however, overcome the effects of VLB when they are exposed to glutamic acid and cystein prior to treatment or simultaneously along with the drug. Although the mechanism of glutamic acid in reversal remains obscure, nevertheless Creasy (1965) envisages that glutamic acid in some way regulates protein synthesis through RNA synthesis. There is considerable evidence that SH groups containing proteins play an important role in spindle formation (Mazia *et al.* 1958). Our experiments showed that the addition of cystein, an SH containing amino acid, along with VLB considerably reverses the inhibition. Thus the reduction of inhibition due to the above mentioned amino acid is understandable.

Besides affecting the synthesis of RNA and proteins, VLB was found to affect DNA synthesis as seen from cytophotometry and autoradiography data. But

the interference concerns only with the rate of DNA synthesis rather than the inhibition of DNA *per se*. The percentage of labelled interphase cells remains more or less the same in both the treated (56.94) and control (59.79) although the photometric and grain count data (Table 1) indicates slight reduction in the amount of DNA. However, Richards *et al.* (1966) seem to have observed a significant inhibition of DNA in VLB treated thymus cells.

The morphological effects like chromosome condensation and C-mitosis are most likely due to the secondary effects of prolonged metaphases rather than the direct action of VLB on chromosomes.

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