Effects of Vinblastine on the Proliferative Capacity of L Cells and Their Progress Through the Division Cycle

N. BRUCHOVSKY, A. A. OWEN, A. J. BECKER, AND J. E. TILL

Department of Medical Biophysics, University of Toronto, and The Ontario Cancer Institute, Toronto, Ontario, Canada

SUMMARY

L cells in the exponential growth phase in cell culture were exposed to vinblastine sulfate (VLB) for various lengths of time and tested for their ability to form macroscopic colonies and to progress through the phases of the cell-division cycle. Concentrations of VLB in the vicinity of $10^{-7}$ gm/ml were found to inhibit the proliferation of L cells and to cause an accumulation of mitotic figures, which reached a maximum after approximately 1 doubling time. The cells in the VLB-treated cultures showed a progressive loss of colony-forming ability; this loss was closely correlated with the accumulation of mitotic figures. When cells were prevented from entering mitosis by the action of a reversible inhibitor of cell proliferation, phenethyl alcohol, they showed no such progressive loss of colony-forming ability in the presence of VLB. Partially synchronized cell populations, in which only cells in late G1 phase retained colony-forming ability, were tested for their response to VLB. It was found that no loss of colony-forming ability occurred until the cells began to attempt cell division in the presence of VLB. Studies of the time relationships between the various phases of the cycle in VLB-treated cells did not reveal any important retarding effects of VLB on the progress of L cells through the phases of the cell cycle other than mitosis. VLB appears to act primarily on cells at or near mitosis and to cause an irreversible loss of colony-forming ability.

Vinblastine sulfate (VLB) is an inhibitor of cellular proliferation, which has been used in the treatment of a number of human cancers (3, 11). Its action at the cellular level has been studied by Palmer et al. (6) with 2 lines of proliferating cells in cell culture. These workers observed an accumulation of metaphases in VLB-treated cultures and concluded that VLB acts by inhibiting spindle formation, resulting in metaphase arrest. A similar conclusion was reached by Cutts (2), who studied the mitotic arrest induced by VLB in vivo. However, these investigations did not establish to what extent the inhibitory effects of VLB are reversible, nor was it clear whether the progress of cells through the phases of the division cycle other than mitosis was affected by the presence of VLB. In the work reported in this paper, the effects of VLB on colony-forming ability of L cells in culture have been examined as a function of the position of the cells in the division cycle. The results obtained indicate that both retardation of progress through the cycle and irreversible loss of colony-forming ability due to the action of VLB occur only in cells at or near mitosis.

MATERIALS AND METHODS

Cells.—A clonally isolated subline, L6OTM, of Earle's L cells was used in all experiments. The growth medium was CMRL 1066 (7), from which thymidine and coenzyme concentrate had been omitted. The medium was supplemented with 10% horse serum (Connaught Medical Research Laboratories, Toronto) and antibiotics. Cells were maintained in the logarithmic growth phase in spinner flasks (0. H. Johns Glass Co., Toronto) at 37°C; under optimal conditions, the cell population doubled every 16–20 hr. The durations of the phases of the cell cycle in VLB-treated cells did not reveal any important retarding effects of VLB on the progress of L cells through the phases of the cell cycle other than mitosis. VLB appears to act primarily on cells at or near mitosis and to cause an irreversible loss of colony-forming ability.

1 This investigation was supported in part by grants from the National Cancer Institute of Canada, and the USPHS (grant CA-06229-02).

2 Research Fellow of the National Cancer Institute of Canada.

The abbreviations used are: VLB, vinblastine sulfate; Tdr-7H, tritiated thymidine; DNA, deoxyribonucleic acid; PEA, phenethyl alcohol; S, DNA synthesis period; G1, pre-S period; G2, post-S period; and M, mitosis.

Received for publication March 10, 1965.

1232
Calif.) containing the growth medium described above, but supplemented with 20% horse serum. Cultures containing VLB were diluted at least 5000-fold before plating. All dishes were incubated at 37°C in an incubator continuously flushed with 10% CO₂ in air, with 90–95% relative humidity, for 10–14 days. The medium was then removed from the plates, and the colonies were stained with a saturated solution of methylene blue in water and counted. The plating efficiency of L cells under these conditions was 60–100%.

For determinations of mitotic indices, cells were diluted by treatment with hypotonic saline, then fixed with 3:1 ethanol-acetic acid and stained with acetic orcein. At least 1000 cells were scored for each point.

**Labeling of cells with tritiated thymidine.**—Tritiated thymidine (Tdr-³H), with a specific activity of 6.7 c/mmole, was obtained from the New England Nuclear Co., Boston. Two types of labeling experiments were done. In the first (“continuous labeling”), the cells were exposed continuously to a Tdr-³H concentration of 0.1 μc/ml of medium for 30 hr. In the second (“pulse labeling”), cells were exposed to a Tdr-³H concentration of 1.0 μc/ml for 30 min, after which sufficient unlabeled thymidine was added to bring the concentration of thymidine to 10 μg/ml. The resulting reduction in specific activity of the Tdr-³H was sufficient to yield negligible further labeling of the cells over the time intervals used in these experiments. Labeling of cells was detected by means of autoradiography, with methods described previously (9). The exposure time of the autoradiographs was 3–5 days.

**Partial synchronization of cell multiplication.**—Partially synchronized cell populations were obtained by the procedure of Whitmore et al. (12). Tdr-³H (6.7 c/mmole, 2 μc/ml) was added to the cultures for a period of 7 hr. This amount of Tdr-³H is sufficient to destroy colony-forming ability of cells entering, or passing through, the S phase during this interval. Since the combined duration of the G₂, M, and G₁ phases for the cells used in these experiments was 10–13 hr, the only cells that retained colony-forming ability at the end of the 7 hr exposure to Tdr-³H were those which had not yet entered the S phase. These consisted of cells in a “window” of a duration of 3–6 hr, situated in the G₁ phase immediately prior to S.

**Phenethyl alcohol (PEA).**—In 1 experiment, phenethyl alcohol (Matheson, Coleman and Bell, Cincinnati) was used, at a final concentration of 0.1% by volume. This compound is a reversible inhibitor of deoxyribonucleic acid (DNA) synthesis in bacteria (1), and, at this concentration, has been found (N. Bruchovsky and J. E. Till, unpublished data) to inhibit reversibly both DNA synthesis and colony-forming ability in the strain of L cells used in this study.

**RESULTS**

**Inhibition of cell multiplication by VLB.**—Chart 1 shows the cell count as a function of time in a control culture and in cultures treated with 3 different concentrations of VLB, 2 × 10⁻³, 10⁻⁷, and 5 × 10⁻⁸ gm/ml. Whereas the cells in the control culture increased in number with a doubling time of 18 hr, no detectable change in cell number occurred in the VLB-treated cultures over an interval of 24 hr.

Chart 2 shows the mitotic index of VLB-treated cultures as a function of time in the presence of VLB. An accumulation of mitotic figures was observed over a 20-hr period, reaching a maximum of 70–75%. Similar results were obtained for each of the 3 concentrations of VLB tested. The data shown in Chart 2 include cells exhibiting abnormal nuclei (mainly micronuclei), which were assumed to be derived from cells arrested in metaphase by VLB. The relative contribution of such cells to the total counts is shown in Table 1. It may be seen from the data of Table 1, Column 2, that the frequency of metaphases reached a maximum shortly before the maximum in the total index (Table 1, Column 4; Chart 2), and that the time at which the maximum in frequency of metaphases was seen, 17.5 hr, is in good agreement with the measured doubling time of 18 hr, obtained from Chart 1.

Chart 3 shows the survival curve obtained for colony-forming ability of cells exposed to VLB for various periods of time. A progressive loss of colony-forming ability with time was observed, to a value of 5–10% after 25 hr for each of the 3 concentrations of VLB tested. The dashed line shown without points in Chart 3 was obtained from the mitotic index data of Chart 2 by subtracting each of the values in Chart 2 from 100%. Thus, the dashed curve represents the proportion of cells in the culture not yet accumulated in metaphase. It may be seen that there is a rather close correlation, at least for the first 20 hr, between the rate of loss of colony-forming ability by
cells in the VLB-treated cultures and the rate of accumulation of cells in mitosis.

The results given in Charts 1 and 2 show that concentrations of VLB in the vicinity of \(10^{-1}\) gm/ml inhibit the proliferation of L cells and cause an accumulation of mitotic figures similar to that reported by others (2, 6). The rather close correlation between loss of colony-forming ability and accumulation of mitotic figures (Chart 3) over an interval equivalent to a doubling time for the control culture suggests that the entry of cells into mitosis in the presence of VLB results in an irreversible loss of proliferative capacity. This close correlation appears to break down for times greater than a doubling time (see “Discussion”).

**Effect of VLB on partially synchronized cells.**—Though the results presented in Charts 1–3 indicate that an irreversible effect of VLB on colony-forming ability of L cells may occur at or near mitosis, the cell populations studied were distributed at random among the different phases of the cell cycle. It was possible that VLB was in fact inhibiting colony-forming ability of cells in other phases besides mitosis and that the correlation between the loss of colony-forming ability and accumulation of mitoses in the presence of VLB was coincidental. To test this possibility, a partially synchronized population was obtained in which the majority of cells retaining colony-forming ability were in the G1 phase of the cycle (see “Materials and Methods”). These cells were permitted to pass through the S and G2 phases in the presence of \(10^{-7}\) gm/ml of VLB. As the partially synchronized cell population proceeded around the cycle in the presence of VLB, samples of cells were removed from the culture and tested for colony-forming ability. The results of 2 experiments of this type are shown in Chart 4. In both experiments, following partial synchronization, the survival of colony-forming ability in the control cultures remained constant at close to 100% of the initial value for approximately 11 hr. The VLB-treated cultures behaved in the same way. Since this interval corresponds to the expected time required for cells to pass from the end of the G1 phase through the S and G2 phases (7 plus 4 hr), these results indicate that VLB had no detectable effect on colony-forming ability of cells in the late G1, S, or G2 periods. Chart 4 also shows that, after 11 hr, colony-forming ability of the VLB-treated cells decreased sharply, whereas that of the control culture increased by approximately 2-fold during the same interval. These results indicate that, at the time that the cell population in the control culture began to divide, the VLB-treated population began to lose colony-forming ability. The results shown in Chart 4 thus support the view that irreversible loss of colony-forming ability occurs only in those cells which enter mitosis in the presence of VLB and that VLB does not affect the proliferative capacity of cells in the S, G2, or late G1 phases.

**Protective effect of phenethyl alcohol.**—If it is correct that

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Metaphases (%)</th>
<th>Cells with abnormal nuclei (%)</th>
<th>Total mitotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.4</td>
<td>1.7</td>
<td>2.1</td>
</tr>
<tr>
<td>4.5</td>
<td>6.9</td>
<td>2.1</td>
<td>9.0</td>
</tr>
<tr>
<td>7.0</td>
<td>16.6</td>
<td>1.6</td>
<td>18.2</td>
</tr>
<tr>
<td>9.0</td>
<td>29.1</td>
<td>1.5</td>
<td>30.6</td>
</tr>
<tr>
<td>14.0</td>
<td>47.7</td>
<td>9.8</td>
<td>57.5</td>
</tr>
<tr>
<td>17.5</td>
<td>51.0</td>
<td>16.2</td>
<td>67.2</td>
</tr>
<tr>
<td>20.0</td>
<td>47.2</td>
<td>24.3</td>
<td>71.5</td>
</tr>
<tr>
<td>24.0</td>
<td>37.5</td>
<td>25.3</td>
<td>62.8</td>
</tr>
</tbody>
</table>

---

**Chart 1**—Metaphases, cells with abnormal nuclei, and total mitotic index as a function of time in the presence of VLB, expressed as percent of an untreated control. \(\Delta\), \(2 \times 10^{-4}\) gm/ml; \(\circ\), \(10^{-7}\) gm/ml; \(\square\), \(2 \times 10^{-7}\) gm/ml.
VLB has an irreversible effect only on cells at or near mitosis, then the obstruction of the progress of cells into mitosis would be expected to prevent loss of colony-forming ability due to the action of VLB. This expectation was tested by exposing cells simultaneously to VLB and to an inhibitor capable of preventing, reversibly, the entry of cells into mitosis. The inhibitor used for this purpose was phenethyl alcohol (PEA), which has been shown to be an inhibitor of DNA synthesis in bacteria (1) and in mammalian cells (4). Recent work in our laboratory (N. Bruchovsky and J. E. Till, unpublished data) has shown that a concentration of PEA of 0.1% by volume will inhibit both DNA synthesis and the entry of cells into mitosis within 2 hr after the addition of PEA to the culture. Further, for the strain of L cells used in this study, this inhibition is almost completely reversible, in that colony-forming ability of PEA-treated cultures is similar to that of an untreated control.

Table 2 gives the results of an experiment carried out to test the action of $10^{-7}$ gm/ml of VLB on cells treated simultaneously with 0.1% PEA. It may be seen from Columns 2 and 4 of this table that PEA alone had little effect on colony-forming ability of the cells, while the results in Column 3 of the table show that the usual progressive loss of colony-forming ability occurred in the presence of VLB alone. However, when both PEA and VLB were present (Column 5), no progressive loss of colony-forming ability occurred. After an initial decrease, the survival of colony-forming ability remained at approximately 80% of that of the culture treated with PEA alone. The small initial decrease is probably the result of the entry of a limited number of cells into mitosis during the first 2 hr after the addition of PEA. These cells would still be susceptible to the toxic effects of VLB.

The results presented in Table 2 support the view that irreversible effects of VLB on cell proliferation are seen only if progress of cells into mitosis occurs in the presence of VLB.

Progress of cells through the cell cycle.—The results presented above indicated that VLB has no detectable irreversible effects on cells except on those at or near mitosis. However, it was possible that VLB was retarding the progress of cells through the other phases of the cell cycle, so that the time relationships between the various phases of the cycle were being affected by VLB. To test this possibility, VLB-treated cultures and control cultures were exposed for various times to Tdr-$^3$H and the percentage of labeled cells as a function of time was determined autoradiographically.

Chart 5A shows the results obtained when cultures were incubated continuously in the presence of Tdr-$^3$H with and without VLB. VLB and Tdr-$^3$H were added simultaneously. The accumulation of labeled cells was detected by means of autoradiography. Initially, 30% of the cells were labeled in both cultures. For the next 8 hr, labeled cells accumulated at a similar rate in both cultures, indicating that the rate of movement of cells from the G1 into the S phase is not seriously affected by VLB. A maximum of 80% of labeled cells was observed in the VLB-treated culture at times later than 10 hr, compared with 95% in the control culture. This is the result that would be expected if 15–20% of the population were in the G2 phase at the time of addition of VLB and Tdr-$^3$H and if these cells were prevented from entering G1 owing to irreversible arrest at mitosis.

These results indicate that VLB does not seriously affect the rate of progress of cells through the G1 phase and into the S phase. They do not provide information about possible effects on the progress of cells through S and G2 in the presence of VLB. Such information was obtained by pulse-labeling a culture for 30 min with Tdr-$^3$H.

### Table 2

**Colony-forming Ability as a Function of Time for L Cells Treated with both Vinblastine Sulfate (VLB) and Phenethyl Alcohol (PEA)**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Plating efficiency, untreated control (%)</th>
<th>Plating efficiency, $10^{-7}$ gm/ml VLB alone (%)</th>
<th>Plating efficiency, 0.1% PEA alone (%)</th>
<th>Plating efficiency, VLB plus PEA (%)</th>
<th>Survival (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>83</td>
<td>105</td>
<td>89</td>
<td>63</td>
<td>71</td>
</tr>
<tr>
<td>5.5</td>
<td>85</td>
<td>94</td>
<td>76</td>
<td>81</td>
<td>81</td>
</tr>
<tr>
<td>7.5</td>
<td>85</td>
<td>55</td>
<td>104</td>
<td>80</td>
<td>77</td>
</tr>
<tr>
<td>9.5</td>
<td>72</td>
<td>44</td>
<td>76</td>
<td>68</td>
<td>89</td>
</tr>
<tr>
<td>13.5</td>
<td>106</td>
<td>67</td>
<td>60</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>18.0</td>
<td>85</td>
<td>24</td>
<td>93</td>
<td>80</td>
<td>86</td>
</tr>
<tr>
<td>19.5</td>
<td>95</td>
<td>13</td>
<td>66</td>
<td>58</td>
<td>88</td>
</tr>
<tr>
<td>21.0</td>
<td>78</td>
<td>13</td>
<td>67</td>
<td>49</td>
<td>73</td>
</tr>
<tr>
<td>29.0</td>
<td>81</td>
<td>13</td>
<td>67</td>
<td>49</td>
<td>73</td>
</tr>
</tbody>
</table>

a Plating efficiency: number of colonies formed per 100 cells plated.

b Obtained by dividing data of Column 5 by those of Column 4.
exposure to Tdr-3H; VLB and Tdr-3H were added simultaneously. 

 Pulse-labeled for 30 min with Tdr-3H immediately before addition 

 Percentage of labeled mitoses as a function of time in cultures 

 U, Control (no VLB); O, treated with 10^-3 gm/ml VLB. B, 

 Percentage of labeled mitoses as a function of time in cultures 

 pulse-labeled for 30 min with Tdr-3H immediately before addition 

 of VLB. ■, Control (no VLB); O, treated with 10^-7 gm/ml VLB. Mitoses accumulated in the VLB-treated culture (cf. Chart 2).

 The data shown in Chart 5B for the percentage of labeled metaphases as a function of time were scored for the percentage of labeled metaphases as a function of time in cultures pulse-labeled for 30 min with Tdr-3H immediately before addition of VLB. ■, Control (no VLB); O, treated with 10^-7 gm/ml VLB. Mitoses accumulated in the VLB-treated culture (cf. Chart 2).

 The results obtained are depicted in Chart 5B. The data for the control culture are similar to those obtained by others (5) for cultured cells and yield median values of 4 hr for the duration of G2, 7 hr for the duration of S, and 17 hr for the generation time of this particular culture. These values are in good agreement with previous results (9, 10).

 The data shown in Chart 5B for the percentage of labeled metaphases as a function of time in the presence of VLB are different from those obtained in the control culture, because of the accumulation of metaphases that occurred in the VLB-treated culture but not in the control. However, the results for the VLB-treated culture agree with the values that would be expected if VLB had little effect on the durations of the G2 and S phases. This may be seen by comparing the times at which abrupt changes in the percentage of labeled metaphases occurred in the control culture with the times at which similar changes occurred in the VLB-treated culture. Initially, for the first 3 hr after the cultures were pulse-labeled with Tdr-3H, only unlabeled metaphases were seen in both cultures. This was because the cells entering mitosis during this interval were in G2 at the time of labeling and were not capable of incorporating Tdr-3H into DNA. From 3 to 5 hr, when an abrupt increase to 100% of labeled metaphases occurred in the control culture owing to the arrival of labeled S-phase cells in mitosis, labeled metaphases began to accumulate in the presence of VLB. However, the rate of increase was not nearly so rapid in the VLB-treated culture as in the control, because of the continuing presence of unlabeled G2 cells detainted at metaphase by the action of VLB, which diluted the newly arrived labeled metaphases. As labeled metaphases continued to arrive in mitosis, the percentage of labeled metaphases in the control remained at 100%, and the percentage of labeled metaphases accumulated in the VLB-treated culture continued to increase. The percentage of labeled metaphases in the treated culture did not reach 100%, again because the unlabeled original G2 cells continued to be held in metaphase by the action of VLB. The peak of labeled metaphases in the VLB-treated culture at 10 hr coincided with the termination of the arrival of labeled S-phase cells in mitosis, and the percentage of labeled metaphases in both the treated and the control cultures decreased as unlabeled cells from the G1 phase reached mitosis in increasing numbers. After a time equivalent to the generation time (17 hr), essentially all the G2 cells had reached metaphase, and, in the VLB-treated culture, the percentage of labeled metaphases approached a constant value. In the control culture, the cycle began to repeat itself, as labeled cells began to enter mitosis a 2nd time. The results shown in Chart 5A indicate that VLB does not affect the progress of cells from G1 into S, and the results shown in Chart 5B indicate no effect of VLB on the progress of cells through S and G2. Taken together, these data support the view that VLB has no important effect on the progress of cells through the phases of the cell cycle other than mitosis. This is in agreement with findings recently reported by Puck (8).

 DISCUSSION

 The results presented in this paper show that concentrations of vinblastine in the vicinity of 10^-7 gm/ml cause an irreversible loss of colony-forming ability of L cells undergoing exponential multiplication in vitro. They also indicate that this loss of colony-forming capacity occurs at or near the time when the cells enter mitosis. This is indicated by the close correlation between the loss of colony-forming ability and the accumulation of cells in metaphase for randomly dividing cultures treated with VLB (Chart 3) and by the close correlation between the initiation of loss of colony-forming ability and the onset of cell division in partially synchronized cultures treated with VLB (Chart 4). However, the possibility that VLB also acts irreversibly on cells near mitosis, either in late G2 or in early G1, cannot be completely ruled out. The correlation between loss of colony-forming ability and accumulation of metaphases (Chart 3) breaks down for times in VLB greater than the doubling times, since the accumulation of metaphases does not exceed a maximum value of 75% after this time, whereas the loss of colony-forming ability continues. The basis for this discrepancy is not known, but could be due to an inhibitory effect of VLB on cells in late G2 or early G1. The precision of the data of both Chart 3 and Chart 4 is such as to permit an uncertainty of about 2 hr in the time of action of VLB in relation to mitosis. In order to eliminate this uncertainty, it would be necessary to study the effects of VLB on
populations of cells fully synchronized with respect to their position in the cell cycle. Such populations are not available at the present.

In any event, it is clear from the results presented in this paper that it is principally cells at or near mitosis that suffer an irreversible loss of colony-forming ability in the presence of VLB. For cells in the remainder of the cell cycle, neither colony-forming ability nor the rate of progress through the cell cycle is detectably affected by the action of VLB. These properties make VLB very suitable as an agent for studying the cell cycles of proliferating cell populations both in vitro and in vivo. The time required to suppress the proliferative capacity of cells randomly distributed around the cell cycle would be expected to provide an estimate of the generation time of the cells. Also, cells that are not in a state of cell cycle would not be expected to be susceptible to the toxic effects of VLB. The results of recent studies (W. R. Bruce, F. A. Valeriote, and B. E. Meeker, Ontario Cancer Institute, personal communication) on the effects of VLB on the proliferation of normal hematopoietic precursor cells and lymphoma cells in vivo are in agreement with this latter prediction.

REFERENCES

Effects of Vinblastine on the Proliferative Capacity of L Cells and Their Progress Through the Division Cycle


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/25/8/1232

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.