A MODEL FOR THE ACTION OF VINBLASTINE IN VIVO

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ABSTRACT A model for the action of vinblastine (VLB) on cells multiplying exponentially in vivo with a generation time, T_G, has been derived. It is based on the assumption that cells attempting to pass through mitosis in the presence of VLB lose their proliferative capacity and that this lethal effect occurs only when the cells are exposed to a concentration of VLB which is above a critical value, C_k. The model leads to two predictions. First, that the percentage of cells surviving at any time after exposure to a dose, D, of VLB is 100% if $D < D_k$ and decreases to 0% after a time, T_G, following a dose $D \geq D_k \cdot 2^{T_G/T_{1/2}}$, where D_k represents the dose of VLB required to produce the concentration C_k, and $T_{1/2}$ is the half-life of the VLB in vivo. Second, that the time, T_G, at which the percentage of cells surviving an exposure to VLB, at doses greater than $D_k \cdot 2^{T_G/T_{1/2}}$, decreases to zero should be equal to the generation time of the cells. Both of these predictions were confirmed experimentally which indicates that the model adequately explains the action of VLB in vivo.

INTRODUCTION

Although the leukopenic and tumorlytic activity of vinblastine (VLB) and the general structure of this dimeric alkaloid have been known for some time (Johnson et al., 1963), there has been some uncertainty about the mechanism of action of this drug. Recent experiments have shown that mouse L-cells exposed to VLB in vitro accumulate in mitosis and concomitantly lose their proliferative capacity (Bruchovsky et al., 1965). When such cells are exposed to VLB for one generation time, most of the cells lose their ability to produce colonies. These experiments have also indicated that VLB acts only upon cells entering into mitosis, causing them to lose their proliferative capacity. Cells that have not entered this stage of the cell cycle do not lose proliferative capacity although exposed to VLB.

It is reasonable to suppose that the action of VLB in vivo is similar to its action in vitro. VLB is known to lead to the accumulation of mitotic figures in animals injected with the drug (Cardinali et al., 1964) and the leukopenic and tumorlytic actions of the drug may well be a result of the loss of proliferative capacity of dividing marrow and tumor cells, respectively. An analysis of the action of VLB in vivo

is hampered, however, by the limited accuracy of conventional assays for cell viability in vivo and by the fact that the concentration of VLB cannot be as easily controlled in vivo as it can in vitro. It is possible that proliferating cells may be exposed to effective levels of VLB for only limited periods of time in vivo as a consequence of the metabolism and excretion of the drug.

We have developed a sensitive quantitative transplantation procedure that may be used to determine the fraction of transplanted tumor (lymphoma) cells that have proliferative capacity (Bush and Bruce, 1964). We have also developed a method to measure the growth-inhibiting activity in the serum of mice and have used this method to measure the concentration of VLB activity in the serum of mice injected intraperitoneally with VLB (Valeriote and Bruce, 1965). The availability of these two assays together with the results of studies with VLB in vitro has permitted us to develop and test a model for the action of VLB in vivo. The model assumes that VLB in a concentration higher than a critical value leads to the irreversible loss of proliferative capacity by those cells that pass into mitosis. Experimentally, the data for survival of lymphoma cells following various doses of VLB and various durations of exposure to VLB fit the model well. The data to be presented thus support our assumptions that the action of VLB is similar in vitro and in vivo. They suggest a simple method for measuring generation times of cells in vivo and provide a rationale for the use of VLB in the treatment of rapidly proliferating tumors.

MATERIALS AND METHODS

Mice. Five- to 6-week-old AKR/J mice were obtained from the Jackson Memorial Institute, Bar Harbor, Maine. In the experimental groups 5-week-old females weighing between 21 and 24 g were used. They were injected via the tail vein with 10^6 lymphoma cells and 96 hr later were injected intraperitoneally with VLB. For assay of cell suspensions mice of either sex were used. All mice were kept 5 to a cage.

Transplanted Cell Lines. The transplanted cell line used in these experiments arose from a spontaneous lymphoma in an 8-month-old female AKR/J mouse. The properties of the line have been described previously (Bruce and Meeker, 1964). The line has been retransplanted into new AKR hosts weekly. Cells from transplantations 114 to 130 were used as the source of lymphoma cells in the experiments reported here. Growth of the cells through this period was similar to that described previously.

Preparation of Cell Suspensions. Cell suspensions were prepared from 10 femoral marrows of lymphomatous mice by a method previously described (Bruce and Meeker, 1964). A fraction of the suspension was diluted, and 500 to 1000 cells were counted in a hemocytometer. The initial suspension was then diluted to achieve the concentrations required for the subsequent injections. All suspensions were kept in icewater prior to use and were injected within a period of 2 hr following this preparation.

Assay for Lymphoma Colony-Forming Cells. Cell suspensions from animals bearing lymphoma cells were assayed for the number of cells which were capable of producting colonies in the spleens of mice by a method described in detail previously (Bush and Bruce, 1964). A measured fraction of the suspension was injected intra-

venously into a group of 20 mice. The mice were killed 8 days later and the spleens were examined for macroscopic colonies. The mean number of colonies per spleen was determined and the number of lymphoma colony-forming units (CFU) in the initial suspension was calculated.

Vinblastine (VLB). The vinblastine sulfate (E. Lilly and Co., Toronto, Ontario) was obtained as a lyophilized powder and was dissolved in distilled water. All solutions were injected as a single injection intraperitoneally in a volume of 1.0 ml.

MODEL FOR THE ACTION OF VLB

The model for the action of VLB on proliferating cells in vivo rests upon the following 6 assumptions:

(*a*) The number of proliferating cells increases exponentially with time. The assumption is valid in the case of the colony-forming lymphoma cells used in this investigation since these cells have been shown to increase exponentially in number. Following the transplantation of 10^6 cells into AKR hosts the number of colony-forming units increases from about 5 to 10^5 per femur in a period of 7 days (Bruce and Meeker, 1964).

(*b*) All cells are in cell cycle with the same generation time, T_G. Data obtained in studies of generation times of mammalian cells in vitro show that some cells in a population may have generation times shorter or longer than the mean generation time (Hsu, 1960). Since the fraction of such cells is presumably quite small and since the cell line used in these studies has grown in exponential phase with a doubling time, T_D, of 11 hr for many generations, we have chosen to assume that all cells have the same generation time in order to simplify the mathematical treatment. The effect of a distribution of generation times on the model is discussed below. It may be noted that we distinguish between the generation time, T_G, and doubling time, T_D, of cells since death and loss of cells in cycle may lead to a situation where T_D is greater than T_G.

(*c*) There is no important net migration of proliferating cells in vivo after treatment with VLB. While some migration of cells may certainly occur, this assumption is reasonable since the cells to be studied form colonies in vivo and thus demonstrate a propensity to remain localized.

(*d*) The activity of VLB in the fluid surrounding cells in vivo decreases exponentially with time and, at any time, depends directly upon the injected dose. This assumption is based on data for VLB activity in the serum of mice from 1 to 10 hr following the intraperitoneal injection of from 0.2 to 2.0 mg VLB per mouse (Valeriote and Bruce, 1965). We are therefore assuming that the concentration surrounding the cells is directly proportional to that in the serum.

(*e*) Only cells passing through mitosis are killed by exposure to VLB. We here assume that the action of VLB in vivo is similar to its action on mammalian cells in vitro (Bruchovsky et al., 1965).

(*f*) The dose-response curve for proliferating cells exposed to VLB is discon-

tinuous. Below a critical concentration of VLB, C_k, there is 100% survival of cells while above this concentration there is 0% survival. This assumption is also based on observations made with cells growing in vitro. It has been found that such cells lose proliferative capacity over a relatively short range of concentrations of VLB near 3×10^{-9} g per ml (Valeriote and Bruce, 1965).

In brief, we are assuming that the growth of cells in vivo is uniform, that the cells in the animal are exposed to the levels of growth-inhibiting activity of VLB that are measured in the serum, and that the action of VLB is the same in vivo and in vitro.

When cells are growing exponentially, are in uniform cycle, and are not migrating (assumptions *a*, *b*, and *c*) the number of viable cells passing through mitosis (or any other point in the cell cycle) in a time, *dt*, at time *t*, is

$$dN = A \cdot 2^{t/T_D}\, dt \tag{1}$$

where A is a constant corresponding to the rate of appearance of new cells due to division at time $t = 0$ and T_D is the doubling time for the cells. If cells lose their proliferative capacity only when exposed to VLB during mitosis (assumption *e*) and if the cells are so exposed for a length of time equal to or greater than the generation time, T_G, of the cells, then all proliferating cells will be inactivated. If, however, they are in contact with the drug for a period, τ, less than T_G then a fraction of the cells will survive. The fraction, $F(\tau)$, which survives when cells are in contact with the drug for the period τ is

$$F(\tau) = \int_{t=\tau}^{T_G} A \cdot 2^{t/T_D}\, dt \Big/ \int_{t=0}^{T_G} A \cdot 2^{t/T_D}\, dt = (2^{T_G/T_D} - 2^{\tau/T_D})/(2^{T_G/T_D} - 1) \tag{2}$$

In the situation in which there is no death of cells occurring so that $T_G = T_D$, this equation reduces to:

$$F(\tau) = 2 - 2^{\tau/T_G} \tag{3}$$

Both equations (2) and (3) have a value $F = 1$ when $\tau = 0$ and decrease to $F = 0$ when $\tau = T_G$.

We have assumed (assumption *d*) that the concentration of growth-inhibiting activity in the serum, C, at a time, t, following a single, intraperitoneal injection of VLB of dose, D mg, is given by:

$$C(t) = B \cdot D \cdot 2^{-t/T_{1/2}}$$

where B is a constant relating growth-inhibiting activity and injected dose and $T_{1/2}$ represents the half-life of the growth-inhibiting activity of VLB in the serum. If VLB kills cells passing through mitosis only when the concentration, C, is greater than C_k (assumption *f*), the duration of this inhibition, that is the time, t_k, required for the VLB concentration to reach C_k, will depend on the dose of VLB injected

and will be given by:

$$t_k = 0 \quad \text{for} \quad D < D_k = C_k/B$$

and

$$t_k = T_{1/2} \log_2 (D/D_k) \quad \text{for} \quad D > D_k \tag{4}$$

Equations (3) and (4) may be combined by substituting t_k for τ to give the fraction of cells, $G(D)$, which survive a dose, D, after exposure to VLB for a time T_G:

$$\begin{aligned} G(D) &= 1 \quad \text{for} \quad D < D_k \\ G(D) &= 2 - 2^{(T_{1/2}/T_G)(\log_2 [D/D_k])} \quad \text{for} \quad D_k \leq D \leq D_k \cdot 2^{T_G/T_{1/2}} \\ G(D) &= 0 \quad \text{for} \quad D > D_k \cdot 2^{T_G/T_{1/2}} \end{aligned} \tag{5}$$

The model thus predicts the extent of survival of cells exposed to VLB in vivo both for various doses of VLB, when the cell survival is assayed at time T_G [equations (5)] and for various durations of exposure to VLB, when the concentration is above C_k [equation (3)]. If, in the former case, the fraction surviving is assayed at a time greater than T_G, the survival curve will have the same form and the same end-points as equations (5) although the dose-response curve will have a slightly different shape since some surviving cells may pass through mitosis more than once in this case. In our studies we chose to examine survival to the various doses of VLB after a 24 hr exposure since we had no prior knowledge of the value of T_G. We have also chosen to assume that the generation time and doubling time were equal in our fit of the experimental data. Our results suggests that they are, indeed, nearly equal.

EXPERIMENTAL RESULTS

Effect of Dose of VLB upon Survival of Colony-Forming Ability. The model which we have developed for the action of VLB in vivo predicts 100% survival of cells when the animals are injected with doses less than or equal to the dose D_k, which gives the critical concentration, C_k. Above this dose, it predicts that survival will decrease to zero after a time T_G when the cells are exposed to a dose $2^{T_G/T_{1/2}}$ times the critical dose. The data of previous studies permit us to estimate these parameters and thus the shape of the dose-response curve. Mouse L-cells grown in tissue culture and exposed to concentrations of greater than 3×10^{-9} mg VLB per ml die in mitosis; this concentration is reached in the serum of mice injected with 1.0×10^{-3} mg VLB (Valeriote and Bruce, 1965). The doubling time of the cells which we are studying is 11.2 hr; the half-life of VLB is 3.5 hr. The ratio $T_G/T_{1/2}$ is thus expected to be approximately 3.1. We would therefore expect that mice exposed to doses of less than 1.0×10^{-3} mg VLB would contain the same number of proliferating cells as the controls while animals receiving $2^{3.1}$ or approximately 8 times this dose would contain no proliferating cells.

To test these predictions, groups of 5 mice each were injected with 10^6 lymphoma cells. Ninety-six hours later the groups were given single, intraperitoneal injections of VLB of from 10^{-3} to 1 mg/mouse. Twenty-four hours following the injection they were killed and pooled cell suspensions from femoral marrow were assayed for their content of colony-forming cells. Five separate experiments were carried out, the results of which appear in Fig. 1. The data are well fitted by equations (5) where $D_k = 5.5 \times 10^{-3}$ mg/mouse and $T_G/T_{1/2} = 3.0$, with $T_D = T_G$. A single experiment in which the dose-response curve for lymphoma cells in the spleen was measured yielded the same results. The range of doses over which the survival of lymphoma cells decreases from the control value to zero is in excellent agreement with that predicted. However, the D_k for these cells was found to be 5.5 times greater than that expected from the in vitro data with mouse L-cells. This difference will be considered in the Discussion section.

Effect of Duration of Exposure to VLB upon Survival of Colony-Forming Ability. The model proposed for the action of VLB in vivo also predicts that the survival of cells following an exposure to VLB should be independent of dose for doses greater than $D_k \cdot 2^{T_G/T_{1/2}}$; and that when the injected dose is larger than this dose, the surviving fraction reaches zero when the duration of exposure to VLB is equal to or greater than the generation time. Data from the previous section would therefore predict that the survival will decrease to zero at 11 hr when more than 5×10^{-2} mg VLB is injected, and that the shape of the disappearance curve would be independent of dose.

To test these predictions, groups of 5 mice each were injected with 10^6 lymphoma

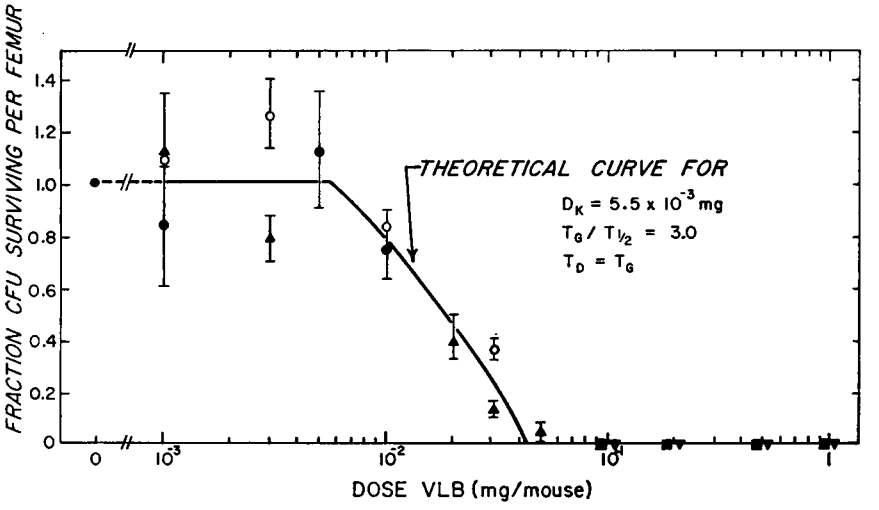

FIGURE 1 Effect of dose of VLB upon survival of lymphoma colony-forming units (CFU) measured 24 hr following administration of VLB. The results shown are from 5 separate experiments. Confidence intervals represent one standard error of the mean.

cells and 96 hr later were injected with either 0.1 or 0.5 mg VLB. The mice were killed from 0 to 10 hr later and pooled femoral marrow suspensions were assayed for their content of colony-forming cells. The results of three experiments are given in Fig. 2. No difference in survival was found between the two doses used, both curves approaching zero survival at 10 hr. The data are well fitted by equation (3) where T_G = 10 hr and $T_D = T_G$. The generation time of the lymphoma cells in vivo thus appears to be 10 hr, in close agreement with the doubling time.

DISCUSSION

We have proposed a model for the action of VLB in vivo based primarily on previous data obtained in vitro (Bruchovsky et al., 1965, and Valeriote and Bruce, 1965). This model predicts the dependence of survival of colony-forming lymphoma cells as a function of both the dose of VLB, and the duration of exposure of these cells to VLB. Our results are in excellent agreement with the model, thus supporting the assumption that the action of VLB in vivo is the same as its action in vitro.

Two slight discrepancies between the predictions of the model and the experimental results deserve attention. First, it can be seen from Fig. 2 that the survival of lymphoma colony-forming cells does not decrease to zero at the time T_G is predicted by equator (3) but that 5% of the cells still retain proliferative capacity after this duration of exposure. The difference may be understood most readily as

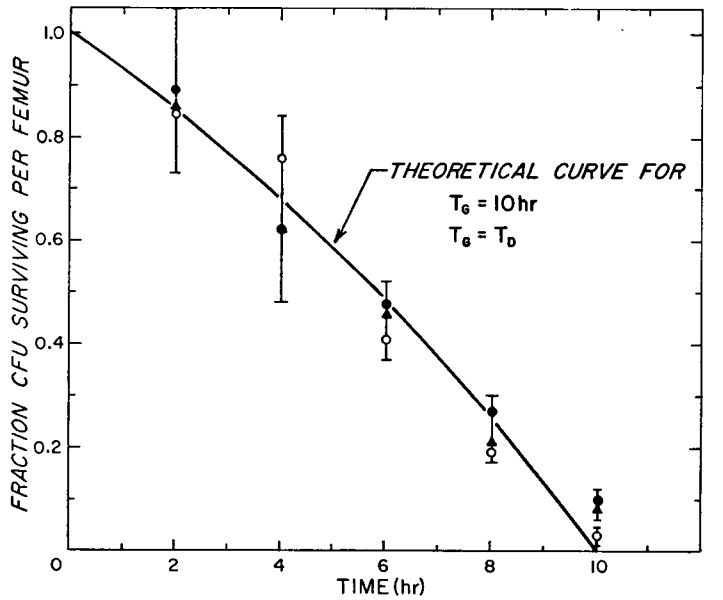

FIGURE 2 Effect of duration of exposure of VLB upon survival of lymphoma colony-forming units (CFU). Solid and open symbols represent an administered dose of 0.5 mg/mouse for the former and 0.1 mg/mouse for the latter. Confidence intervals represent one standard error of the mean.

an inadequacy in assumption *b*, that all cells have the same generation time, T_G. Since some cells have generation times longer than T_G, a fraction of these, when exposed to VLB for a time T_G, will not have passed through mitosis. These cells would presumably survive. Second, lymphoma cells in vivo appear to be somewhat less sensitive to the action of VLB than does a line of mouse L-cells in culture. There was found to be a 5.5-fold difference in sensitivity which may be a consequence of a difference between the intercellular and serum concentrations of VLB (an inadequacy in assumption *d*) or may represent a real but small difference in the sensitivity of the two cell lines studied.

The results of these investigations are of practical interest in two respects. Firstly, the methods used have provided a technique for determining the generation time of lymphoma cells growing in vivo. The survival of these cells is simply determined at intervals for a period of time following the injection of a large dose of VLB and the time at which the survival approaches zero corresponds to the generation time. The method thus provides a means for determining the generation time of cells growing in vivo where conventional autoradiographic techniques cannot be used, but where quantitative assays for cell viability are available. Secondly, the model suggests a rationale for the chemotherapy of rapidly proliferating tumors with agents similar to VLB. It would indicate that the maximum inactivation of the cells of such tumors can be accomplished only when the concentration of the drug exceeds the critical value for the tumor cells for a period of time in excess of the generation time of the cells. To achieve these conditions, estimates of D_k, $T_{1/2}$, and T_G are necessary.

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