Further studies immediately adaptable to analysis of the reciprocal relationship between seminiferous epithelium and anterior pituitary include assay of pituitaries removed at weekly intervals postimmunization to time of full development of aspermatogenesis (2 months) and then until repopulation of seminiferous epithelium occurs (6–8 months) (1).

Summary. Adenohypophyses from normal and aspermatogenic (ASP) guinea pigs were assayed for FSH (modified Steelman-Pohley assay) and for LH (OAAD assay) content. FSH content of adenohypophyses from normal guinea pigs was 0.38 times FSH content of adenohypophyses from ASP guinea pigs. Relative to NIH-FSH-S3 there was 40 and 108 μg of FSH per gland, respectively. There was no significant difference in luteinizing hormone content between adenohypophyses from normal and ASP guinea pigs. Relative to Armour's LH preparation, there is approximately 5 μg equivalents of LH per adenohypophysis. The increased pituitary content of FSH in the ASP guinea pig is compatible with the thesis that FSH production is affected, either directly or indirectly, by a feedback mechanism associated with tubular elements.

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Kinetics of the Effect of Vincristine Sulfate on the Reproductive Integrity of Proliferating Cultured Leukemia L1210 Cells* (32718)

LEE J. WILKOFF, ELIZABETH A. DULMADGE, AND GLEN J. DIXON
(Introduced by Frank M. Schabel, Jr.)
Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35205

In order to cure leukemia, successful chemotherapy is dependent upon the eradication of every leukemia cell present in the host by drug dosages nonlethal to the host (1, 2). If host defense mechanisms are involved in leukemia cell eradication (3, 4), the total number of leukemia cells must at least be reduced to the number that can be eradicated by these processes. Recent investigations have indicated that certain antimetabolites of chemotherapeutic interest may be more effective in reducing *in vivo* leukemia cell populations when they are used on a multiple dose schedule than on a daily optimal dose schedule (5, 6). Pertinent to this multiple daily dose scheduling was the observation that when proliferating cultured L1210 leukemia cell populations were exposed to selected drugs which were effective only during a particular phase of the cell cycle, the kinetics of the reduction in the number of viable cells did not occur at a first-order rate (the fraction of cells killed

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per unit of time was not constant) (7).

Considerable interest has been shown in the use of vincristine sulfate (VCR) as a chemotherapeutic agent in the treatment of leukemias and other neoplasms (8). Furthermore, recent studies have shown that VCR may exhibit cell cycle specificity. Cardinali *et al.* demonstrated that VCR arrested cells in metaphase in normal bone marrow and in L1210 leukemia cells in DBA/2 mice (9). George, *et al.* (10), has shown that VCR may block cell division by its effect on spindle tubules resulting in aberrant chromosome movement. If cells are sensitive to VCR only during a particular phase of the cell cycle, this would be reflected in the kinetics of the loss of proliferative capacity of L1210 populations exposed to VCR. Therefore a study was undertaken to determine the kinetics of the lethal effect of this agent. Leukemia L1210 cells proliferating *in vitro* were exposed to selected concentrations of VCR for different periods of time and the number of cells surviving drug treatment was estimated by an animal bioassay method. The rate of cell killing in populations treated with this agent deviated from first-order kinetics. The kinetics could be described by a Gompertz equation of the form $N = N_0 \exp[-\beta/\alpha\ (1-e^{-\alpha t})]$ where N_0 is the number of viable cells present at time t, t is the duration of drug exposure, β is the rate of population reduction, and α is a constant which expresses the rate of change of β (11, 12).

Materials and Methods. Drug concentrations were selected in an attempt to match the theoretical maximum *in vivo* concentration in mice assuming rapid equal distribution in all body fluids following the administration of a single-dose LD_{10} of the drug (13). The total unbound body water in a mouse is approximately 75% of the body weight (14). Thus, in a 20-gm mouse about 15 gm would be water. Assuming complete and instantaneous equilibration following the administration of a single-dose LD_{10} of a drug, the concentration in body water in μg/ml (μg/ml is approximately equivalent to mg/kg) is 1.3 $\times$ LD_{10} in mg/kg of body weight. This theoretical maximum concentration which can be attained in the body fluids of a mouse is about 3.8 μg/ml for VCR. The agent was tested at several concentration levels based on this value.

The drug was weighed into a flask. Immediately before adding the drug to the test culture, it was dissolved in a minimum amount of water and diluted with L1210 growth medium (15) to the desired concentration.

Stock cultures of L1210 cells were grown *in vitro* in L1210 growth medium supplemented with 5% fetal bovine serum and maintained in logarithmic growth phase by periodic dilutions (15). Cultures used for drug studies were initiated with approximately 5×10^4 cells per ml and allowed to grow to a population density of 2×10^5 to 8×10^5 cells/ml.

Several 190-ml samples from the stock L1210 culture were placed into 500-ml spinner flasks (Bellco Glass Company, Vineland, New Jersey). An additional 200-ml sample was placed into a spinner flask which served as the control culture. At zero time (immediately prior to the addition of the drug) a 10-ml sample was removed from the control culture and appropriately diluted for titration in BFD_1 (C57/Bl ♀ $\times$ DBA/2 ♂) mice (15). A 10-ml sample from each drug solution was then added to the appropriate spinner flask and 10 ml of medium was added back to the control culture. At specific time intervals after the addition of the drug, an 8-ml sample was removed from each flask and bioassayed in mice. Five BDF_1 mice of approximately the same age (6–8 weeks old) and weighing 18–21 gm each were used for bioassay at each drug concentration-time interval. One ml of cell suspension was inoculated intraperitoneally into each mouse. All operations were conducted at 37° C. Glassware, medium, and drug solutions were equilibrated at 37°C before use. Samples in transit from the cell culture laboratory to the animal laboratory for bioassay were held in a 37°C water bath, and inoculated into animals within 4 minutes after removal from the culture flasks.

Unpublished results based on a KB cell culture assay method (Dixon *et al.*) indicate that VCR is stable in the cell culture system for at least 24 hours.

The basis for estimations (from life span and survival data) of the approximate num-

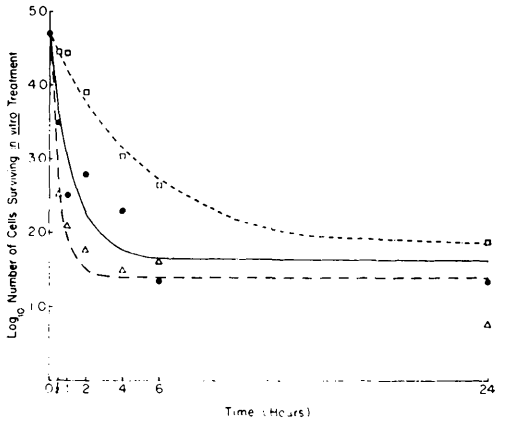

FIG. 1. Effect of vincristine sulfate on the kinetic behavior of proliferating cultured L1210 leukemia cells. The number of cells surviving *in vitro* drug treatment was determined by bioassay of 1 ml samples and estimated from the average life span of the bioassay animals. □ 1 μg/ml ● 5 μg/ml; and △ 25 μg/ml. The logarithms of the numbers of cells estimated on the basis of life span data were approximately normally distributed. For the groups of five mice used and at the drug concentrations employed, the standard deviation of the logarithm of the mean cell number averaged about 0.285. The values of the constants β and α of the Gompertz function calculated from the data for each drug concentration were:

Conc. (μg/ml)	β	α	Level of confidence of α (%)
1.0	0.021	0.003	99.9
5.0	0.092	0.013	95.0
25.0	0.229	0.030	97.5

ber of leukemia cells surviving a given drug dose have been presented by Skipper, *et al.* (1). It is assumed that drug treatment of a cell population usually does not induce or select cells with generation times greatly different from those of the untreated population, and that the surviving cells begin proliferating at the normal rate immediately upon inoculation into mice. Experimental evidence for this assumption has been presented by Skipper *et al.* (1,16), Wilcox *et al.* (17), and Schabel (18).

Cell survival was determined as a function of the duration of drug exposure. If the parameter describing the rate of cell killing (β) is not constant but decreasing exponentially with time, then a plot of the $\log_{10}$'s of the number of cells which survived the *in vitro* drug treatment (at a specific drug concentration) against time can best be described by a Gompertz equation of the form $N = N_0\exp[-\beta/\alpha(1-e^{-\alpha t})]$ where α is the parameter describing the rate of change of β (12). If the value of α does not differ significantly from zero, then the Gompertz equation does not describe the data any better than a simple exponential decay equation. In other words as the value of α approaches zero, the Gompertz equation degenerates to $N = N_0e^{-\beta t}$, a form of the first-order rate equation (11,12). Thus the constant α can be used as an index which measures the deviation from first-order kinetics. If we assume that the variance about the Gompertz regression curve is constant and that the time measurements are free of error, the parameters α and β and the confidence limits of α can be calculated according to Deming (19). In the case where the value for the constant α is significantly different from zero (a level of confidence of 95% or greater is considered to be significant) the Gompertz equation will describe the kinetic data. If the constant α does not differ significantly from zero, then a plot of the $\log_{10} N$ versus time t of the experimental data should result in points scattered about a straight line. This is indicative of a first-order rate where the Gompertz equation has degenerated to $N = N_0e^{-\beta t}$ (11,12). A Control Data LGP-30 computer was programmed to evaluate the data.

Results. Proliferating L1210 populations were treated *in vitro* with different concentrations of VCR. When the number of cells surviving drug treatment was determined (by bioassay) as a function of the duration of drug exposure, the kinetics of population reduction was not first order (Fig. 1). The constant α used as an index of the deviation of the data from first-order kinetics was at least significant at the 95% level of confidence. Since there is evidence that VCR acts at the cellular level on cells entering or in mitosis (9,10) the kinetics of population reduction was determined for proliferating cultured L1210 cells exposed to colchicine

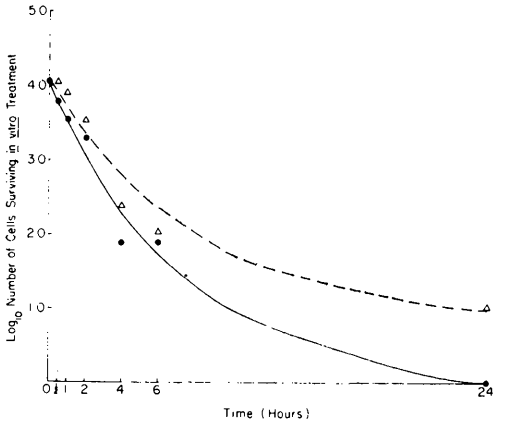

FIG. 2. Effect of colchicine on the kinetic behavior of proliferating cultured L1210 leukemia cells. The number of cells surviving *in vitro* drug treatment was determined by bioassay of 1 ml samples and estimated from the average life span of the bioassay animals. △ 0.0625 μg/ml; ● 0.125 μg/ml. The logarithms of the numbers of cells estimated on the basis of life span data was approximately normally distributed. For the groups of five mice used and at the drug concentrations employed, the standard deviation of the logarithm of the mean cell number averaged about 0.346. The values of the constants β and α of the Gompertz function calculated from the data for each drug concentration were:

Conc. (μg/ml)	β	α	Level of confidence of α (%)
0.0625	0.015	0.002	99.5
0.1250	0.022	0.002	99.9

(Fig. 2). The constant α was significant at the 99.5% level of confidence indicating that the kinetics of population reduction with this mitotic inhibitor was also not first order.

The data in Figs. 1 and 2 indicated that 24 hours was the most effective drug exposure time for L1210 populations treated *in vitro* with the above agents. To determine if there was a minimum effective concentration below which VCR had no effect on the proliferative capacity of L1210 populations, a series of L1210 cultures were exposed to different concentrations of the agent (0.000001–10.0 μg/ml) for 24 hours (Fig. 3). At concentrations <0.001 μg/ml, VCR evidently had no effect on the viability of L1210 populations. When the concentration was increased to 0.01 μg/ml a reduction of about one $\log_{10}$ in the number of viable cells occurred. Thus the minimum effective concentration of VCR appeared to be <0.01 but >0.001 μg/ml.

If cell populations were exposed to effective concentrations of VCR for 24 hours, it might be possible to reduce the concentration of VCR and still obtain a maximum degree of cell killing. Proliferating L1210 populations were exposed to 10.0, 1.0, 0.1, and 0.032 μg/ml of VCR for periods of 12 and 24 hours and then assayed for viability. The results (Table I) indicate that a period of drug exposure equivalent to approximately two cell doubling times (24 hours) is ap-

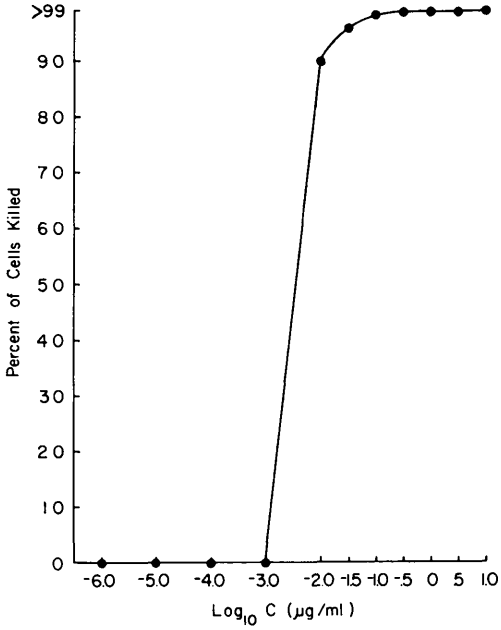

FIG. 3. The effect of vincristine sulfate on the proliferative capacity of cultured L1210 populations at different drug concentrations when the duration of drug exposure was 24 hours. The number of cells surviving *in vitro* drug treatment was determined by bioassay of 1-ml samples and estimated from the average life span of the bioassay animals. Percentage of L1210 cells killed = $(1.0 - N/N_0) \times 100$; N = number of cells surviving drug treatment at 24 hours; N_0 = number of viable cells at time zero. The minimum effective concentration of vincristine sulfate necessary to kill viable cells in the population was <0.01 μg/ml but >0.001 μg/ml.

TABLE I. The Extent of L1210 Population Reduction for Periods of Cell Exposure to Vincristine Sulfate Which Are Equivalent to 1 and 2 Population Doubling Times.[a]

(μg/ml)[b]	Time of exposure (hours)	Approx. no.[c] of surviving L1210 cells	Approx. $\log_{10}$ reduction in cell population
0	0	8000	
0	24	11,000	
10.0	12	<1	>3.9
	24	<1	>3.9
1.0	12	130	1.8
	24	1	3.9
0.1	12	240	1.5
	24	1.3	3.8
0.032	12	1500	0.7
	24	1.8	3.8

[a] The doubling time of L1210 populations *in vivo* and *in vitro* is about 12 hours (1, 11).

[b] The theoretical maximum concentration of vincristine sulfate which can be attained in the body fluids of a mouse is about 3.8 μg/ml (based on the single dose LD_{10} in mg/kg of body weight).

[c] Determined by bioassay of 1 ml samples and estimated from the average life span of the bioassay animals. The logarithms of the numbers of cells estimated on the basis of life span data were approximately normally distributed. For the groups of five mice used and at the drug concentrations employed, the standard deviation of the logarithm of the mean cell number averaged about 0.307.

parently the most effective drug exposure time. For instance, 0.032 μg/ml of VCR produced a reduction in cell population of about 4 $\log_{10}$ in 24 hours, a reduction which is of the same order of magnitude as that produced by 10 μg/ml of VCR in 24 hours.

Discussion. The two-parameter Gompertz equation $N = N_0\exp[-\beta/\alpha(1-e^{-\alpha t})]$ is useful in describing the kinetics of population reduction when the rate of cell killing is not first order. The parameters α and β both reflect the metabolic heterogeneity of an asynchronous cell population. The rate of population reduction β is a reflection of the fraction of the population in or passing through the vulnerable part of the cell replicative cycle with time. Since the rate of population reduction β is not constant, but decreasing exponentially with time, the parameter α may be considered to represent the rate at which the fraction of the cell population in the sensitive portion of the cell cycle becomes smaller. Thus, α can be used as an index which measures the degree of deviation from first-order kinetics.

The kinetics of population reduction when proliferating L1210 cells were treated with VCR was not first order (Fig. 1). Recent investigations indicate that VCR interferes with the normal mitotic processes (10) and, therefore, only that fraction of the population which is entering or in mitosis would be sensitive to the agent. The experiments with colchicine (Fig. 2) confirm the kinetics of population reduction by an agent that affects only that fraction of a cell population that is in the mitotic phase of the cell cycle, since colchicine has been reported to be an inhibitor of spindle formation (20). Recent studies have shown that when proliferating L1210 populations are treated *in vitro* with amethopterin, 5-fluorouracil, and 1-β-D-arabinofuranosylcytosine·HCl the rate of cell killing is not first order (7). These three agents have in common the property of inhibiting mitosis by interfering with some aspect of DNA metabolism (20). Thus nonfirst-order kinetics as regards the rate of cell killing indicate that these agents possess cell cycle specificity; that is, only cells which are in a particular phase of the cell cycle (such as carrying out specific biochemical activities associated with mitosis or preparation for mitosis) are sensitive to the agents. If the population is asynchronous, then a constant fraction of cells will not be killed. Schabel *et al.* (13), have reported that nondividing L1210 leukemia cells *in vitro* were relatively resistant to high concentrations of amethopterin, 5-fluorouracil, and VCR.

Concentrations of VCR less than 0.001 evidently had no effect on the viability of L1210 populations (Fig. 3). Since a one $\log_{10}$ increase in concentration (from 0.001 to 0.01 μg/ml) produces a reduction in population of about 90%, if the employed concentration is greater than the minimum effective concentration, extensive cell killing will result if the duration of drug exposure is about two doubling times. The data in Table I indicate that as a cell proceeds through its replicative

cycle, it will enter that part of the replication cycle in which it will become sensitive to VCR. The L1210 cell has an average doubling time of about 12 hours (1,2,15). Thus with a drug exposure time of 24 hours, essentially all the cells in the population have been able to enter the drug-sensitive phase of the replicative cycle (presumably a particular phase associated with mitosis). Consequently, 0.032 μg/ml of VCR will produce the same degree of population reduction as will 10 μg/ml of VCR. Since the theoretical maximum concentration which can be attained in the body fluids of a mouse is about 3.8 μg/ml, physiologically realistic drug levels of VCR can produce maximum degrees of cell killing *in vitro*.

Schabel (18) reported that when DBA/2 mice implanted with different numbers of leukemia P-1534 cells were treated with VCR (1.3 mg/kg ip single dose) a constant fraction of cells were killed (based on increase in life span at each implant size). In an *in vivo* system a leukemia cell population would be exposed to cytotoxic levels of VCR for a relatively short period of time following a single dose, perhaps 30–40 min. Since this *in vivo* drug exposure time is about 1/24 to 1/18 of the doubling time of the L1210 population, only that fraction of cells in the sensitive part of the replicative cycle would be affected. The fraction of cells sensitive to VCR during this 30–40 min would be killed. Although different numbers of cells were implanted, the sensitive fraction of the cell population at any given time is approximately constant, and the rate of cell killing *in vivo* would be first order in cell number.

In the case of the cultured L1210 populations exposed to VCR, the duration of drug exposure is 24 hours (equivalent to about two population doubling times). The kinetics of population reduction over this continuous period of drug exposure depends upon two parameters which reflect the metabolic heterogeneity of an asynchronous cell population. These parameters as described above are: (i) the rate of population reduction (β) which is a reflection of the fraction of the population in or passing through the vulnerable part of the replicative cycle with time, and (ii) the rate at which the fraction of the cell population in the sensitive part of the replicative cycle becomes smaller. Furthermore, this continuous exposure of the population to drug over the 24-hour period, enables cells in the population to progress through the replicative cycle and to reach the VCR sensitive part of the cycle. Thus the rate of cell killing is not first order (a constant fraction of cells is not killed per unit of time).

The kinetics of the lethal action of VCR on proliferating cultured L1210 populations indicate that this agent has cell cycle specificity. This drug may be more effective in reducing *in vivo* leukemia cell populations if the same total dose would be administered at fractional doses in shorter time intervals over a 24-hour period provided that an equal increase in cytotoxicity to normal cells does not result. The total dose administered should be spaced to take into account the mean generation time of the particular cell population being treated. In this way, the probability of effectively treating cells when they are in the drug-sensitive phase of the cell cycle is increased.

Summary. When proliferating cultured L1210 cell populations were exposed to effective concentrations of VCR or colchicine, the kinetics of the reduction in the number of viable cells did not occur at a first-order rate (the fraction of cells killed per unit of time was not constant). The minimum effective concentration of VCR required to produce an effect on the reproductive integrity of L1210 populations was <0.01 but >0.001 μg/ml. If the duration of drug exposure was 24 hours, extensive cell killing could be produced by VCR concentrations which were relatively low when compared to the theoretical maximum concentration attainable in the body fluids of a mouse. The kinetics of the lethal action of VCR on proliferating cultured L1210 populations indicate that this agent has cell cycle specificity.

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Enrichment of Antibody Plaque-Forming Cells of Spleen by Sedimentation at Unit Gravity (32719)

MICHAEL G. MAGE, WARREN H. EVANS, AND ELBERT A. PETERSON
(Introduced by Dean Burk)
Laboratory of Biochemistry, National Cancer Institute, Bethesda, Maryland 20014

Many biological problems, particularly in the area of cell differentiation and maturation, involve the study of complex mixtures of cells, such as spleen or bone marrow. In immunology, such problems as the identification of the cell types involved in various phases of the induction of immune reactions and studies at the molecular level of antibody biosynthesis would be aided by the development of methods for the fractionation of heterogeneous cell populations containing immune cells. One approach to this problem is to exploit physical differences between cells, such as differences in size and density. Szenberg and Shortman (1) have recently studied the distribution of cells active in graft versus host reactions after equilibrium density centrifugation of fowl blood lymphocytes. The present study reports the distribution of hemolytic antibody plaque-forming cells (PFC) and nonplaque formers after sedimentation of spleen cell suspensions from mice immunized with sheep erythrocytes, at unit gravity in a sucrose gradient.

Material and Methods. A method previously developed for bone marrow by Peterson and Evans (2) was employed. It utilizes a cylindrical sedimentation chamber with a conical top and bottom. A chamber of 16-inch diameter (Fig. 1) was used in the present work.

Cell counts were carried out on a Sanborn Electronic Cell Counter, using 4% acetic acid as a diluent for counting nucleated cells. Smears for differential counts were stained with Giemsa, and the criteria of Dunn and of Wintrobe (3) were used to identify the various cell types.

Cells producing high hemolytic efficiency antibodies [H-PFC, considered to be of the