Biochemical Pharmacology, Vol. 55, pp. 1229–1234, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00615-1

Vincristine Saturation of Cellular Binding Sites and Its Cytotoxic Activity in Human Lymphoblastic Leukemia Cells

MECHANISM OF INOCULUM EFFECT

Hiroyuki Kobayashi, Yuzuru Takemura,* James F. Holland and Takao Ohnuma†*

Department of Medicine, Division of Neoplastic Diseases, Mount Sinai School of Medicine, New York, NY 10029, U.S.A.

ABSTRACT. Vincristine (VCR) is an active agent in the treatment of acute lymphoblastic leukemia (ALL). We evaluated the relationship between the cytotoxic activity of VCR and the degree of VCR saturation of cellular drug binding sites, using the MOLT-3 ALL cell line. When MOLT-3 cells at a density of 1×10^6 or 1×10^8 cells/mL of pH-controlled medium were exposed to VCR for 1 hr, its cytotoxic activity on cells at high density was 10-fold less than on cells at low density (inoculum effect). The number of VCR binding sites measured by Scatchard analysis was 9.25×10^6/cell. At high cell density, the saturation of VCR binding sites was one log order less than that at low density. Irrespective of cell density, curves of cell-kill versus the degree of VCR saturation of the cellular binding sites overlapped each other. Minimal cytotoxic activity was observed at 0.3% VCR saturation, and nearly maximal cytotoxic activity occurred at about 25% saturation, with the IC_{50} at about 4% saturation. These data show that the VCR-induced cell-kill effect is dependent on the degree of saturation of VCR binding sites rather than on the extracellular VCR concentration. The lesser cell-kill on cells at high density can be explained by the lack of drug molecules to sufficiently saturate cellular binding sites. This phenomenon may be responsible, at least in part, for the poor chemotherapeutic outcome of ALL patients with high leukocyte counts at presentation. BIOCHEM PHARMACOL **55**;8:1229–1234, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. vincristine; cytotoxicity; inoculum effect; cellular binding sites; cell density

The cytotoxic activity of certain antineoplastic agents such as VCR‡ and DXR becomes progressively less efficacious when cell density is increased [1–3]. This phenomenon is termed the inoculum effect. The inoculum effect was demonstrated originally in microbial systems in which the size of the inhibitory zone produced by certain antibiotics was influenced by the inoculum size of the microorganisms tested [4, 5]. The exact mechanisms underlying the inoculum effect observed for antitumor agents have not been fully clarified. Our previous studies suggested that the inoculum effect was the result of the unavailability of VCR or DXR molecules to saturate cellular binding sites when cells at high densities are exposed to the drugs [3]. In the present paper, we demonstrate that VCR-induced cell-kill effect is indeed proportional to the degree of VCR saturation of cellular binding sites.

MATERIALS AND METHODS

Cell Line and Cell Culture

The MOLT-3 ALL cells [6] were maintained in RPMI-1640 medium containing 10% (v/v) heat-inactivated FBS and were fed twice a week with fresh medium. Cells in exponential growth at >90% viability were used for all experiments. Cells were examined periodically for Mycoplasma contamination with the MycoTect kit (LifeTechnologies) and were found to be negative.

Drug Exposure and Determination of Inoculum Effect

Inoculum effect was measured as described previously [1–3]. Briefly, VCR sulfate (Sigma Chemical Co.), supplied as powder, was initially reconstituted according to the accompanying manufacturer's instructions and was diluted further in Dulbecco's PBS. The drug dilutions were freshly prepared for each experiment.

Because previous experiments showed that, at a cell density of 10^8 cells/mL, the pH of the RPMI-1640 medium

*Present address: Department of Laboratory Medicine, National Defense Medical College, Tokorozawa, Saitama 359, Japan.

† Corresponding author: Dr. Takao Ohnuma, Department of Medicine, Division of Neoplastic Diseases, Box 1128, Mount Sinai Medical Center, One Gustave L. Levy Place, New York, NY 10029-6574. Tel. (212) 241-6664; FAX (212) 423-9458.

‡ *Abbreviations:* VCR, vincristine; DXR, doxorubicin; ALL, acute lymphoblastic leukemia; FBS, fetal bovine serum; and MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium.

Received 3 February 1997; accepted 25 September 1997.

containing 10% FBS decreased to 6.3 by the end of a 1-hr incubation period and the change in the pH influenced the concentration–response curve of VCR [3], we used pH-controlled medium throughout the present experiments. The pH of the medium could be maintained by adjustment of the pH to 7.4 with 0.35 M of HEPES. A 1-hr exposure to the medium containing HEPES did not affect cell viability or subsequent cell growth, and additions of drug solution at the concentration ranges studied did not alter the pH.

The cells were suspended in the pH-controlled RPMI-1640 medium containing 10% FBS at a cell density of 1.1×10^6 or 1.1×10^8 viable cells/mL. Aliquots of 0.9 mL of cell suspension were placed in tissue-culture tubes (Falcon 3033, Becton-Dickinson) and exposed to 0.1 mL of graded concentrations of VCR for 1 hr at 37° in a shaking incubator. For control, 0.1 mL of Dulbecco's PBS was added to the cell suspension instead of the drug solution.

At the end of the incubation period, the cells were washed twice with medium containing 10% FBS, and the cell density was readjusted to 5×10^4 cells/mL. Each 0.2-mL aliquot of cell suspension was placed in a 24-well flat-bottomed plate (Falcon 3047) in triplicate and then incubated for 3 days at 37° in a humidified atmosphere consisting of 5% CO_2 and 95% air. Following incubation, viable cells were quantified using the MTT (Sigma) assay as described previously [3]. Concentration–response curves were drawn by plotting the absorbance, expressed as a percentage of the untreated control value, against the drug concentrations tested.

Measurement of Intracellular VCR Contents

Cellular contents of VCR were measured by the method described by Totsuka *et al.* [7]. Various concentrations of [^{3}H]VCR (sp. act. 7.2 mCi/mg, Amersham Radiochemicals, final concentration 0.0625 to 1 μg/mL) with or without 500-fold excess unlabeled VCR were added to the cell suspension at a final cell density of 4×10^6 viable cells/mL of the medium containing 10% FBS. After a 4-hr period of incubation at 37° in a shaking incubator, 0.5-mL aliquots of the reaction mixture were removed and were superimposed on the mixture of mineral oil (Sigma) and silicon oil (Fluka), of which the final density was adjusted to 1.031 g/mL, followed by immediate centrifugation at 12,000 *g* for 1 min to separate the cells from the supernatant under the oil layer. After saving the supernatants and removal of the oil, the cell pellets were solubilized in 100 μL of 1% Triton X-100 (v/v). The supernatants saved and the cell pellets solubilized were transferred to counting vials containing 5 mL of the scintillant BCS (Amersham). Radioactivity was determined by a liquid scintillation counter (Packard, model 1900CA). The residual binding in the presence of excess unlabeled VCR was assumed to represent nonspecific binding. In this case, the nonspecific VCR binding sites may include membrane protein/lipid and cellular low affinity acidic proteins distinct from tubulin. The difference between the total binding and the nonspecific binding was taken to be specific VCR binding. For Scatchard analysis to calculate the overall number of VCR binding sites per cell, the VCR concentration in the supernatants after a 4-hr incubation was regarded as free VCR concentration.

Cellular Retention of VCR

Cells at a density of 1×10^6 or 1×10^8 viable cells/mL of pH-controlled medium were incubated with [^{3}H]VCR supplemented with non-radioactive VCR for 1 hr at 37° in a shaking incubator. At the end of the incubation, 0.5 mL of reaction mixture was removed and placed on the oil mixture (final density 1.031 g/mL) in a microcentrifuge tube, followed by immediate centrifugation at 12,000 *g* for 1 min. Cell pellets were harvested for the measurement of radioactivity as described above. The remainder of the cell suspension was washed twice with ice-cold Dulbecco's PBS plus 1% bovine serum albumin and 1 mM of EDTA, resuspended in drug-free culture medium at a cell density of 10^6 cells/mL, and then incubated again at 37°. After 0.5, 1, 2, and 3 hr of incubation in drug-free medium, 0.5 mL of the cell suspension was harvested for the measurement of radioactivity retained in the cells.

Determination of Total Protein Content

Total protein content was determined as described by Harlow and Lane [8]. Exponentially growing cells (1×10^7 cells) were collected by centrifugation, resuspended in 200 μL of lysis buffer (2% sodium dodecyl sulfate, 100 mM of dithiothreitol, 60 mM of Tris, pH 6.8) by mixing vigorously, and boiled for 5 min. After shearing DNA by sonication, the sample was centrifuged at 10,000 *g* for 10 min. The concentration of total protein in the supernatant was measured by the pyrogallol red-molybdate method as described by Watanabe *et al.* [9].

RESULTS

Inoculum Effect

Figure 1 shows the sensitivity to VCR of MOLT-3 cells as measured at a cell density of 1×10^6 (low) or 1×10^8 (high) cells/mL in the pH-controlled medium. The cell-kill activity on cells at high density was one log order less than that at low density. These results are confirmatory of our earlier observations [1–3].

Number of VCR Binding Sites in MOLT-3 Cells

Table 1 shows the primary VCR binding data obtained from measurements of intracellular VCR contents, as well as specific binding values. These data were used for Scatchard analysis (Fig. 2). Bound/free (B/F) versus bound (B) values were plotted to determine the number of VCR binding sites in MOLT-3 cells [10]. The linear least square regression equation was $y = -0.025x + 0.384$, and the line

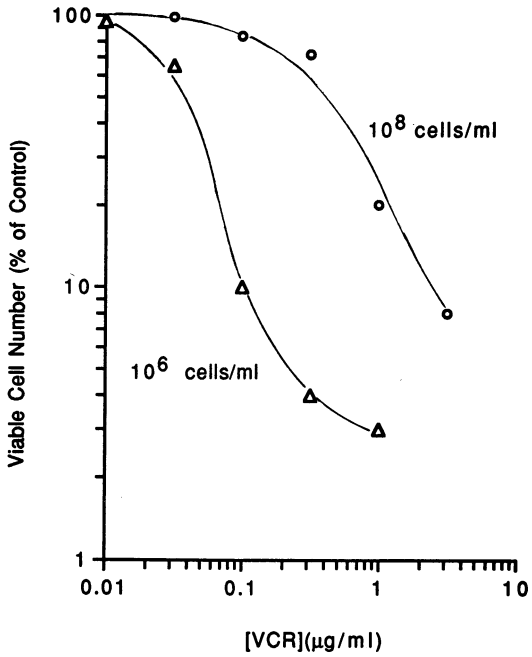

FIG. 1. VCR sensitivity of MOLT-3 cells at a cell density of 1 × 10^6 (△) or 1 × 10^8 (○) cells/mL in pH-controlled RPMI-1640 medium containing 10% FBS. Each data point represents the mean value for 3 independent experiments performed in triplicate. All experimental values were within 15% of the mean.

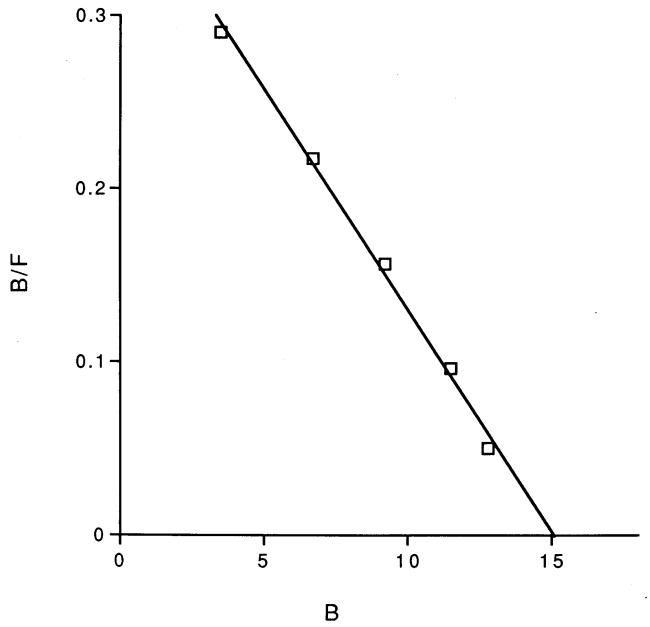

FIG. 2. Bound/free (B/F) vs bound (B, expressed as picomoles per 10^6 cells) plot.

intersected on the x (B) axis at 15.36 (pmol/10^6 cells). Hence, MOLT-3 cells had VCR binding sites of 15.36 × 10^{-12}(/10^6 cells) multiplied by Avogadro's number of 6.0225 × 10^{23} or approximately 9.25 × 10^6 sites/cell.

The total protein content of the MOLT-3 cells was approximately 1.5 mg/10^7 cells, which can be converted to 150 pg/cell. Based on an estimate of about 2% of total protein being tubulin, the tubulin content of MOLT-3 cells can be calculated as 3 pg/cell. Since the molecular weight of dimeric tubulin is approximately 110 kDa, a single MOLT-3 cell was considered to have 1.6 × 10^7 molecules of total tubulin, or 9.9 to 11 × 10^6 molecules of unpolymerized tubulin assuming 30–40% to be in the polymerized state. This number of tubulin molecules per cell matched beautifully with our result obtained by Scatchard analysis (9.25 × 10^6 binding sites/cell).

Cellular Retention of VCR by MOLT-3 Cells

The retention of VCR was measured in MOLT-3 cells that had been preloaded for 1 hr with increasing concentrations of VCR, washed, and transferred to drug-free medium (Fig. 3). The retention of VCR reached a plateau after a 3-hr incubation in drug-free medium. The pattern and values of VCR retention were in agreement with previous findings measured in different leukemic cells [11, 12]. For the same VCR concentrations used in the medium, the plateau levels of VCR retained in cells at high cell density were approximately 1/10 as compared with those at low cell density.

Relationship between VCR-Induced Lethality of MOLT-3 Cells and the Degree of VCR Saturation of Cellular Binding Sites

The plateau levels of VCR intracellular concentrations in the retention study were assumed to represent the fractions of VCR tightly bound to their binding sites (mainly tubulin) [13]. The degree of VCR saturation of the cellular

TABLE 1. Primary VCR binding data used for Scatchard analysis

VCR concentration in the medium (μg/mL)	Total binding (pmol/culture tube)	Non-specific binding (pmol/culture tube)	Specific binding (pmol/culture tube)	VCR in the supernatant after 4-hr incubation (pmol/tube)
0.0625	17.6	3.6	14.0	48.4
0.125	33.6	6.8	26.8	123.6
0.25	46.0	9.2	36.8	236
0.5	57.6	11.6	46.0	480
1.0	64.0	12.8	51.2	1024

MOLT-3 cells were incubated at 37° with the indicated concentrations of VCR for 4 hr. Each culture tube contained 4 × 10^6 cells/mL. Each data point represents the mean of 3 independent experiments performed in duplicate. All experimental values were within 15% of the mean. Specific binding values, expressed as pmol per 10^6 cells, correspond to B values in Fig. 2.

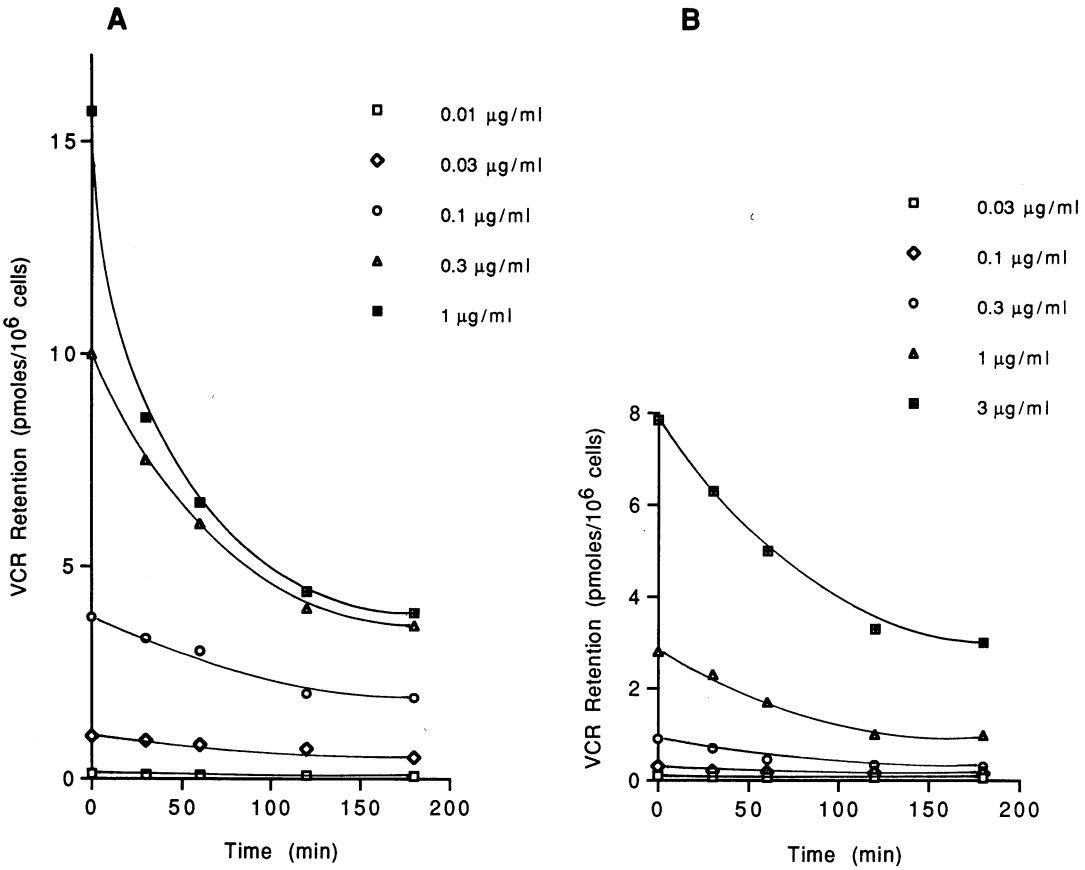

FIG. 3. Retention of VCR by MOLT-3 cells. Cells at a cell density of 1×10^6 (panel A) or 1×10^8 (panel B) cells/mL were preloaded with the indicated concentrations of VCR for 1 hr, washed, and then incubated in drug-free medium at a cell density of 1×10^6 cells/mL. The intracellular VCR was measured at the indicated time points and expressed as picomoles VCR per 10^6 cells. Each data point represents the mean of 3 independent experiments performed in duplicate. All experimental values were within 15% of the mean.

binding sites can be calculated by the plateau level of intracellular VCR molecules divided by 9.25×10^6 (VCR binding sites/cell). In Fig. 4, the degree of VCR saturation of the cellular binding sites and the VCR-induced cell-kill effect (measured by the MTT assay 72 hr after a 1-hr exposure to VCR) were plotted. Irrespective of the cell density during the exposure to VCR, curves of cell-kill versus the degree of VCR saturation of the cellular binding sites overlapped each other. Thus, the minimal cell-kill activity was observed at 0.3% VCR saturation. With increases in drug saturation, the cell-kill activity increased progressively, and near maximal cell kill occurred at about 25% saturation. The VCR concentration producing 50% inhibition of cell growth (IC_{50}) was at the VCR saturation of approximately 4%.

DISCUSSION

To clarify the mechanisms of inoculum effect, we investigated the relationship between the degree of drug saturation of the cellular binding sites and drug-induced cytotoxic activity at two different cell densities. At the high cell density, the cytotoxic activity of VCR was 10-fold less than that at low cell density, and this reduced biological activity was correlated with a one log order decrease in the degree of VCR saturation of the cellular binding sites. To achieve VCR saturation necessary for equivalent cytotoxic activity, 10-fold higher drug concentrations were required for cells at high cell density compared with those at low density. Irrespective of cell density, however, the cytotoxic activity of VCR correlated with the degree of drug saturation of the cellular binding sites. These observations indicate that VCR-induced cytotoxicity is dependent on the degree of saturation of VCR binding sites rather than the extracellular VCR concentrations.

The number of VCR binding sites per one MOLT-3 cell was shown to be 9.25×10^6, which can be converted to 9.25×10^{14} sites for 10^8 cells/mL of cell suspension. The numbers of VCR binding sites for MOLT-3 cells were essentially identical to those of vindesine receptors reported in MOLT-4 cells [7]. A 1 μg/mL (or 1.0845×10^{-6} M) concentration of VCR corresponds to 6.53×10^{14} VCR molecules/mL. In the retention study, MOLT-3 cells at a density of 10^8 cells/mL exposed to 1 μg/mL of VCR retained 1 pmol VCR/10^6 cells or 6.022×10^{13} VCR molecules/10^8 cells. Therefore, approximately 10% of VCR in the medium bound to the cellular binding sites. This resulted in 6% saturation of the cellular binding sites,

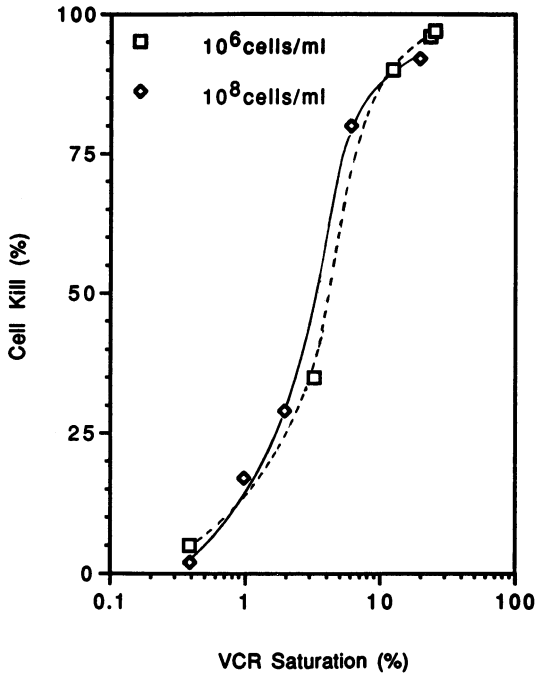

FIG. 4. Relationship between VCR-induced cytotoxicity of MOLT-3 cells and the degree of VCR saturation of cellular binding sites.

producing 80% cell-kill. One microgram of VCR molecules/mL appeared to be insufficient to saturate critical levels of the cellular binding sites when the high density of cells was used. Similar calculations for the cell density of 10^6 cells/mL resulted in 25% saturation and close to 100% cell kill. Therefore, our data clearly show that the lesser cytotoxic activity of VCR against cells at high cell density can best be explained by the lack of enough drug molecules to saturate cellular binding sites. In this context, the lack of enough drug molecules to saturate critical levels of leukemic cells' drug-binding (or target) sites proves to be the cause of the inoculum effect.

Singer and Himes [14] reported that the presence of about 1 tubulin–Vinca alkaloid complex molecule per 100 tubulin molecules or 1% binding was sufficient to produce 50% inhibition of proliferation in murine B16 melanoma cells. In our study, the VCR saturation at IC_{50} was about 4%. This difference in IC_{50} values between those reported by Singer and Himes and our results may be related to the different cell lines used and the different manner of VCR exposure (43 vs 1 hr).

Vinca alkaloids were shown to mediate cytotoxicity in mammalian cells by binding to tubulin, the protein subunit of microtubules, and to prevent the polymerization of tubulin to form microtubules with arrest of cells in mitosis [15]. Vinca alkaloids are widely used for the treatment of malignant diseases, particularly ALL [16]. Admittedly, *in vitro* data cannot be extrapolated directly to leukemic patients; however, our observations may have the following clinical implications. At the time ALL is first diagnosed or at subsequent clinical recrudescence of the disease, the body contains approximately 10^{12} leukemic cells/m^2 of body surface area [17]. Therefore, the number of VCR binding sites in the total leukemic cells/m^2 equals $9.25 \times 10^{18}/m^2$, assuming fresh ALL cells have approximately the same number of binding sites as MOLT-3 cells. A clinical dose of 1.4 mg of VCR/m^2 corresponds to approximately 1×10^{18} molecules. Therefore, even if all the VCR molecules entered leukemic cells and bound to the binding sites, only 10% of cellular binding sites could be saturated. Moreover, VCR has been shown to bind to many host organ tissues, making the whole body a potentially infinite sink for the drug [18]. If one adds host cell mass and various pharmacokinetic barriers until the drug reaches target sites within individual cells, far fewer leukemic cellular binding sites are expected to be saturated by the standard doses of VCR.

Clinically achievable VCR concentration × time product was reported to be 0.025 to 0.103 μg · hr/mL [19, 20], while we found that *in vitro* VCR concentration × time for IC_{90} of MOLT-3 cells was 2 or 0.2 μg · hr/mL at a density of 10^8 or 10^6 cells/mL, respectively. Because 10^6 leukemic cells/mL, the low cell density we studied, corresponds to only 10^3 leukemic cells/μL in peripheral blood, even from the viewpoint of concentration × time, the VCR molecules available clinically appear to be insufficient. It is possible that this phenomenon is responsible, at least in part, for poor chemotherapeutic outcome of ALL patients with high leukocyte count at presentation [21, 22].

In the treatment of patients with ALL, VCR is usually administered in a combination with other antileukemic agents. VCR is known to interact with other agents with synergistic or antagonistic effects, depending on the drugs combined and the sequences used [23]. In addition, in the clinic VCR is given weekly. The effects of insufficient concentrations of VCR in combination with other drugs and of repetitive VCR exposure on cytotoxic activity are yet to be determined. Our data may serve as a basis to study this concept in a more realistic *in vivo* model.

This work was supported, in part, by the T. J. Martell Memorial Foundation for Leukemia, Cancer and AIDS Research, New York, NY; by the United Leukemia Fund, Lake Success, NY; by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan; and by the Chemotherapy Foundation, New York, NY. We thank Mr. Sean Savitz for technical assistance.

References

1. Ohnuma T, Arkin H and Holland JF, Effects of cell density on drug-induced cell kill kinetics *in vitro* (inoculum effect). *Br J Cancer* **54:** 415–421, 1986.
2. Takemura Y, Kobayashi H. Miyachi H, Hayashi K, Sekiguchi S and Ohnuma T, The influence of tumor cell density on cellular accumulation of doxorubicin or cisplatin *in vitro*. *Cancer Chemother Pharmacol* **27:** 417–422, 1991.
3. Kobayashi H, Takemura Y and Ohnuma T, Relationship between tumor cell density and drug concentration and the cytotoxic effects of doxorubicin or vincristine: Mechanism of

inoculum effects. *Cancer Chemother Pharmacol* **31:** 6–10, 1992.
4. Basker MJ, Edmondson RAE and Sutherland R, Comparative antibacterial activity of azlocillin, mezlocillin, carbenicillin and ticarcillin and relative stability to beta-lactamases of *Pseudomonas aeruginosa* and *Klebsiella aerogenes*. *Infection* **7:** 67–73, 1979.
5. Bodey GP and Pan T, Mezlocillin: *In vitro* studies of a new broad-spectrum penicillin. *Antimicrob Agents Chemother* **11:** 74–79, 1977.
6. Minowada J, Ohnuma T and Moore GE, Rosette-forming human lymphoid cell lines: 1. Establishment and evidence for origin of thymus-derived lymphocytes. *J Natl Cancer Inst* **49:** 891–895, 1972.
7. Totsuka K, Oshimi K and Mizoguchi H, Vindesine receptors in cells of a human leukaemia cell line. *Br J Cancer* **46:** 392–396, 1982.
8. Harlow E and Lane D, Preparing total cell lysates for immunoblotting. *Antibodies. A Laboratory Manual*, pp. 481–482. Cold Spring Harbor Laboratory Press, Plainview, NY, 1988.
9. Watanabe N, Kamei S, Ohkubo A, Yamanaka M, Ohsawa S, Makino K and Tokuda K, Urinary protein as measured with a pyrogallol red-molybdate complex manually and in a Hitachi 726 automated analyzer. *Clin Chem* **32:** 1551–1554, 1986.
10. Rosenthal HE, Graphical method for the determination and presentation of binding parameters in a complex system. *Anal Biochem* **20:** 525–532, 1967.
11. Ferguson PJ and Cass CE, Differential cellular retention of vincristine and vinblastine by cultured human promyelocytic leukemia HL-60/Cl cells: The bases of differential toxicity. *Cancer Res* **45:** 5480–5488, 1985.
12. Pierré A, Pérez V, Léonce S, Boutin JA, Saint-Dizier D, Hautefaye P, Lavielle G and Atassi G, Relationship between the cellular accumulation and the cytotoxicity of S12363, a new vinca alkaloid derivative. *Cancer Chemother Pharmacol* **29:** 367–374, 1992.
13. Bai RL, Pettit GR and Hamel E, Binding of dolastatin 10 to tubulin at a distinct site for peptide antimitotic agents near the exchangeable nucleotide and vinca alkaloid sites. *J Biol Chem* **265:** 17141–17149, 1990.
14. Singer WD and Himes RH, Cellular uptake and tubulin binding properties of four Vinca alkaloids. *Biochem Pharmacol* **43:** 545–551, 1992.
15. Luduena RF, Banerjee A and Khan IA, Tubulin structure and biochemistry. *Curr Opin Cell Biol* **4:** 53–57, 1992.
16. van Tellingen O, Sips JHM, Beijnen JH, Bult A and Nooijen WJ, Pharmacology, bio-analysis and pharmacokinetics of the vinca alkaloids and semi-synthetic derivatives (review). *Anticancer Res* **12:** 1699–1716, 1992.
17. Freireich EJ, Gehan EA, Sulman D, Boggs DR and Frei E III, The effect of chemotherapy on acute leukemia in the human. *J Chronic Dis* **14:** 593–608, 1961.
18. Wierzba K, Sugiyama Y, Okudaira K, Iga T and Hanano M, Tubulin as a major determinant of tissue distribution of VCR. *J Pharm Sci* **76:** 872–875, 1987.
19. Alberts DS and Chen H-SG, Tabular summary of pharmacokinetic parameters relevant to *in vitro* drug assay. In: *Cloning of Human Tumor Stem Cells* (Ed. Salmon S), pp. 351–359. Alan R. Liss, New York, 1980.
20. Owellen RJ, Root M and Hains FO, Pharmacokinetics of vindesine and vincristine in humans. *Cancer Res* **37:** 2603–2607, 1977.
21. Hammond D, Sather H, Nesbit M, Miller D, Coccia P, Bleyer A, Lukens J and Siegel S, Analysis of prognostic factors in acute lymphoblastic leukemia. *Med Pediatr Oncol* **14:** 124–134, 1986.
22. Sather HN, Statistical evaluation of prognostic factors in ALL and treatment results. *Med Pediatr Oncol* **14:** 158–165, 1986.
23. Kano Y, Ohnuma T, Okano T and Holland JF, Effects of vincristine in combination with methotrexate and other antitumor agents in human acute lymphoblastic leukemia cells in culture. *Cancer Res* **48:** 351–356, 1988.