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Energy-Dependent Reduced Drug Binding as a Mechanism of *Vinca* Alkaloid Resistance in Human Leukemic Lymphoblasts

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SUMMARY

We studied the accumulation of [^{3}H]vinblastine (VLB) by lines of CCRF-CEM cultured human leukemic lymphoblasts that were either sensitive or resistant to the drug. Neither cell line metabolized VLB, nor selectively retained any radioactive impurities. There was an apparent "instantaneous" accumulation of VLB by cells of both lines, resulting in cell to medium ratios >1.0 within 1 sec after drug addition. Experiments between 0 and 60 sec revealed that the presumed undirectional initial rate of VLB accumulation by resistant cells, termed CEM/VLB$_{100}$, was about one-half that of sensitive CEM cells. In experiments carried out over 60 min, the VLB-resistant cells accumulated considerably less [^{3}H]VLB than did the sensitive cells. Drug accumulation by both cell lines was temperature-sensitive, since incubation of cells at 4° resulted in only minimal uptake beyond that observed at zero time. CEM/VLB$_{100}$ cells retained less drug than did CEM cells, apparently because of a larger fraction of readily releasable VLB compared with CEM cells. The accumulation of VLB by either cell line was related in part to cellular levels of ATP. Although depletion of ATP was associated with decreased accumulation of VLB by CEM cells, it was related to enhanced drug accumulation by CEM/VLB$_{100}$ cells. Restoration of ATP levels to near control values by addition of glucose also had opposite effects on the two cell lines, causing further accumulation of VLB by the sensitive line but leading to apparent drug efflux from the resistant line. Potentially competing substrates (VM-26, colchicine, daunorubicin, and doxorubicin) failed to block this glucose-mediated release of VLB from the CEM/VLB$_{100}$ cells. In experiments with energy-depleted CEM/VLB$_{100}$ cells preloaded with VLB and then incubated in drug-free medium, initial drug loss was shown to be independent of cellular metabolism, being roughly the same for both metabolically intact and metabolically depleted cells. Glucose (energy) was required only for subsequent release of what appeared to be a more tightly bound cell-associated fraction of VLB. Results of zero-time binding studies tended to confirm that VLB binding by resistant cells has two components, one requiring and the other not requiring metabolic energy. Differences in the proportions of these two components between the sensitive and resistant cells suggest a mechanism for resistance to VLB and similar compounds.

INTRODUCTION

The development of drug resistance poses a major obstacle to the successful treatment of hematological malignancies. The study of phenotypic changes associated with this phenomenon in leukemic cells can provide clues to its development. Using VLB[1, 2]-resistant sublines of human leukemic lymphoblasts (CEM/VLB) as prototypes for resistance to "natural products" (*Vinca* alkaloids, anthracyclines, and antibiotics), we identified a surface membrane glycoprotein of ≈180,000 M_r that appears to vary in amount with the degree of resistance to VLB (1). Subsequent studies suggested that the carbohydrate moiety of this glycoprotein may be unnecessary for the expression of resistance (2).

In the past, resistance to *Vinca* alkaloids was attributed to decreased membrane permeability, based largely

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[1] The abbreviations used are: VLB, vinblastine sulfate; VCR, vincristine sulfate; VDS, vindesine sulfate; DOX, doxorubicin; DNR, daunorubicin; CLC, colchicine; VM-26, 4′-dimethyl epipodophyllotoxin thenylidene-β-D-glucoside; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

[2] Despite the fact that VCR is used clinically to treat leukemia, we selected cells for resistance to its congener, VLB, for two reasons: (a) when these studies were begun, [^{3}H]VLB was more readily available commercially than was [^{3}H]VCR; and (b) [^{3}H]VLB has greater radiochemical stability than does [^{3}H]VCR, and does not decompose as rapidly. Subsequent experiments with [^{3}H]VCR, not reported here, indicate that the results are comparable to those with [^{3}H]VLB to be reported in this paper.

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on results of cross-resistance studies in which uptake and cytotoxicity of other drugs were the only criteria (3–5). More recent findings indicate that decreased retention of drug may be the basis for such resistance (6–10). A ubiquitous "efflux pump," which apparently can export many structurally different molecules (e.g., anthracyclines, *Vinca* alkaloids, and antibiotics), is thought to be the mechanism of more rapid drug clearance from resistant cells (7, 8, 11–14). However, this concept requires further investigation in view of the apparent lack of molecular specificity of the pump (15). In fact, other mechanisms of resistance toward alkaloids have been suggested. Carlsen *et al.* (16), for example, have proposed that resistance of CHO cells to CLC (and cross-resistance to *Vinca* alkaloids and anthracyclines) is mediated by an active permeability barrier, and Bender *et al.* (17) presented evidence indicating that VCR cytotoxicity (or its lack) correlates best with the amount of drug bound by the cells.

This paper presents experiments designed to examine the accumulation of [^{3}H]VLB by VLB-sensitive and -resistant CCRF-CEM cells. Although they do not define the precise mode of cellular accumulation of drug, our results offer an alternative explanation for resistance to *Vinca* alkaloids: energy-dependent alterations in apparent drug binding leading to less retention of drug by resistant cells. A preliminary account of some of this work has been presented (18).

MATERIALS AND METHODS

Cells. CCRF-CEM cells selected for VLB resistance were grown as described previously (1). CEM/VLB$_{100}$ is a subline selected for its ability to grow in 100 ng of VLB per milliliter (110 nM) and is ≈100–500 times more resistant to VLB than is the sensitive parent line, CEM.

Accumulation and retention of [^{3}H]VLB. Cells taken for study at a density of 5–8 × 10^5/ml were removed from culture, washed twice, and resuspended in flasks or glass vials at ≈1 × 10^6/ml in a buffer (pH 7.2) modified slightly from that described by Bleyer *et al.* (19). The buffer contained (millimolar): NaCl, 125; KCl, 4.0; K_2HPO_4, 1.1; $NaHCO_3$, 16.0; $CaCl_2$, 1.9; $MgCl_2$, 1.0; Hepes, 10.0; and phenol red at a final concentration of 10 mg/liter. When necessary, sugars and metabolic inhibitors were added as indicated in the text. Unless otherwise indicated, all experiments were carried out in the presence of glucose.

Standard uptake experiments were begun by adding [^{3}H]VLB to the cell suspension after a 10-min preincubation; three 0.9-ml aliquots were then removed and placed immediately in 1.5-ml microcentrifuge tubes containing 0.5 ml of silicone oil (20). The remaining cell suspension was incubated, usually at 37°, in a shaking water bath. The tubes were centrifuged immediately at 15,000 × *g* for ≈1 min in a microcentrifuge (Brinkmann Instruments, Model 5412). At various times thereafter, other aliquots were removed from the flasks, and the cells were centrifuged as before. Rapid uptake (0–60 sec) experiments were performed directly in microcentrifuge tubes, as detailed in the legend to Fig. 2.

In experiments measuring drug retention, we collected cells either immediately or at 60 min after drug addition, diluted the cell suspensions with 3–5 volumes of ice-cold buffer, and centrifuged them at 4° at 1000 × *g* for 5 min. The supernatant fluid was decanted and the cell pellet was resuspended in the appropriate volume of drug-free medium at the desired temperature and incubated as before. Aliquots were taken at various times thereafter for the determination of cell-associated radioactivity.

Centrifugation of the cell suspension through silicone oil rapidly separated the cells from the incubation medium containing radioactive drug. Both the incubation medium and the silicone oil were then aspirated, and the cell pellet was solubilized overnight at room temperature in 1 ml of 1% SDS. The SDS aliquot, containing solubilized cells, was then transferred quantitatively to a scintillation vial, 10 ml of scintillation cocktail were added, and the samples were counted for radioactivity in a Searle Mark III or Beckman LS7000 liquid scintillation spectrometer. All points represent experiments performed in triplicate, and the standard deviations were usually less than ±15% of the mean values.

Lack of metabolism of [^{3}H]VLB. To determine whether [^{3}H]VLB was metabolized by the cells, we performed three types of experiments: (a) Cells were incubated in [^{3}H]VLB for 60 min, washed, and resuspended in drug-free medium for 60 min as described above. The "efflux" medium was then collected, aliquots were spotted directly on thin-layer sheets of aluminum oxide (with fluorescent indicator), and chromatograms were developed in ethyl acetate/absolute ethanol (3:1) (21). (b) In similar experiments, a modification of the method of Creasy and Chou (22) was followed: The efflux medium was lyophilized, reconstituted in 0.05 M NaCl, put on a Sephadex G-25 column, and eluted with 0.05 M NaCl. Aliquots of the radioactivity were then analyzed by thin-layer chromatography as above. (c) The method of Skovsgaard (11) was employed, with VLB substituted for VCR. After incubation with [^{3}H]VLB for 60 min, the cells were sonicated, and 10 ml of ice-cold absolute ethanol, containing 1 mg of nonradioactive VLB (EtOH/VLB), were added to the sonicates. Controls were cells that were incubated and sonicated before [^{3}H]VLB was added. The ice-cold EtOH/VLB was then added, and these latter preparations were subsequently treated in the same manner as the others. The pH was adjusted to 8.0 with NH_4OH, and the sonicates were centrifuged at 10,000 × *g*. The supernatants were saved, and the pellets were reextracted twice more. The pooled supernatants were subsequently extracted twice with equal volumes of methylene chloride, which was then evaporated to dryness. The residue was dissolved in methanol, and aliquots were chromatographed on sheets of aluminum oxide, as described above. By these methods, 92–94% of the radioactivity was recovered as unchanged VLB. It was concluded from all of these experiments that neither the sensitive CEM cells nor the resistant CEM/VLB$_{100}$ cells metabolized VLB to any significant extent.

Lack of preferential accumulation of ^{3}H-labeled impurities. We were concerned that radioactive impurities in the [^{3}H]VLB (<5% of the total ^{3}H) might be preferentially accumulated or released by the cells (23) and hence affect interpretation of the results. We tested this possibility by determining how much of the cell-associated ^{3}H could be displaced by nonradioactive VLB. We found that ≈97–99% of all the cell-associated ^{3}H could be displaced by an excess of unlabeled VLB, indicating that (a) most of the cell-associated ^{3}H was in fact [^{3}H]VLB, and (b) the nondisplaceable fraction of ^{3}H did not contribute in any significant way to the total drug accumulated.

Calculation of cell volume. Cell volumes were determined from measurements made with (a) ^3H_2O, (b) packed cells, or (c) a Coulter counter, Model ZBI.[3] Where appropriate, corrections were made for intercellular water space either with [^{14}C]inulin or by determining dry weight and using an approximate specific volume for protein of 0.75 (24). There was good agreement among the methods. The mean uncorrected cell volumes, in microliters per 10^6 CEM or CEM/VLB$_{100}$ cells, were as follows: by Method a, 1.42 ± 0.03 to 1.64 ± 0.03; by Method b, 0.72 ± 0.18 to 1.69 ± 0.06; and by Method c, 0.75 ± 0.19 to 1.21 ± 0.23. When corrected as described above, the intracellular free water space ranged from 3.7 × 10^{-13} to 5.7 × 10^{-13} liters/cell for CEM and 1.8 × 10^{-13} to 4.2 × 10^{-13} liters/cell for CEM/VLB$_{100}$. Volumes for the two lines varied with the cell density of the culture.

Determination of cellular levels of ATP. ATP was measured in cell extracts according to the method of Stanley and Williams (25), using desiccated firefly tails as the source of the luciferase enzyme, and a

[3] Calculation of cell volume with the Coulter counter was as described in the Coulter instruction manual. Thus, $V = K \cdot I \cdot A \cdot T_L$, where K is a calculated machine constant, I and A are the switch settings for (aperature current)$^{-1}$ and (amplification)$^{-1}$, respectively, and T_L is the setting of the lower threshold for half-count.

Packard Tri-Carb liquid scintillation counter, Model 3003, with the coincidence gate switched off for detection of photons.

Materials. [^{3}H]VLB (specific activity 5–25 Ci/mmole) was purchased from either Moravek Biochemicals (Brea, Calif.) or Amersham Corporation (Arlington Heights, Ill.) and was ≥95% pure when analyzed by thin-layer chromatography as described above; if necessary, the [^{3}H]VLB was repurified by preparative thin-layer chromatography. [*carboxyl*-^{14}C]Inulin (specific activity 1.91 mCi/g) and [^{3}H]$_2$O (specific activity 1.0 mCi/g) were obtained from New England Nuclear Corporation (Boston, Mass.). Silicone oil (Versilube F-50 or Nyosil 50) was obtained from Harwick Chemical Corporation (Memphis, Tenn.) or William F. Nye, Inc. (New Bedford, Mass.), respectively; minimal essential medium (Earle's salts) was from Gibco (Grand Island, N.Y.); scintillation cocktail (RIA-Solve II or Budget-Solve) was from Research Products International (Downer's Grove, Ill.). Desiccated firefly lanterns, ATP, Hepes, D-glucose, 2-deoxy-D-glucose, D-mannose, D-galactose, L-fucose, and CLC were purchased from Sigma Chemical Company (St. Louis, Mo.), and thin-layer sheets of aluminum oxide (with fluorescent indicator) were obtained from Brinkmann Instruments (Westbury, N.Y.). Microcentrifuge tubes and tissue culture flasks were purchased from standard sources, as were other chemicals and reagents. VLB, VCR, and VDS were generous gifts of Eli Lilly and Company (Indianapolis, Ind.) and were supplied through the courtesy of Dr. Gerald L. Thompson. DOX and DNR were obtained from Adria Laboratories (Wilmington, Del.), and VM-26 was provided by Bristol Laboratories (Syracuse, N.Y.) through the courtesy of Dr. William T. Bradner.

RESULTS

Accumulation of [^{3}H]VLB. Figure 1 compares the uptake of [^{3}H]VLB, added at a final concentration of 200 nM, by CEM and CEM/VLB$_{100}$ cells. When cells were incubated at 37°, drug accumulation in the resistant line was clearly decreased relative to the sensitive line. Both lines showed appreciable drug accumulation at "zero time." Incubation of cells at 4° did not abolish this instantaneous accumulation, and resulted in only minimal further uptake during the remainder of the 60-min experiment. These findings indicate that a portion of [^{3}H]VLB accumulation by both cell lines is temperature-sensitive.

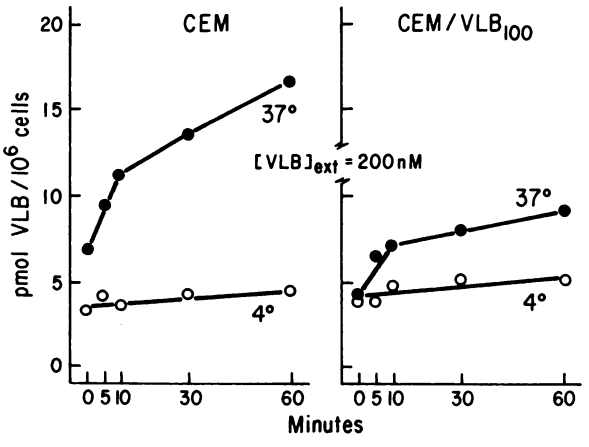

FIG. 1. *Accumulation of [^{3}H]VLB by VLB-sensitive (CEM) and -resistant (CEM/VLB$_{100}$) cells*

Cells were incubated in buffer containing 10 mM glucose and [^{3}H]VLB at a final concentration of 200 nM either at 37° (●) or at 4° (○), and triplicate aliquots were taken for the determination of radioactivity at the times indicated. See Materials and Methods for details. Depicted is a representative experiment showing the mean values. The standard deviations were ≤15% of the means.

The high zero-time values for [^{3}H]VLB associated with both cell lines ranged between 3 and 6 pmoles/10^6 cells. In these experiments, 5–10 sec had usually elapsed from the time when [^{3}H]VLB was added to the cell suspension (zero time) until samples were removed and centrifuged. Since plasma membrane transport of nucleosides and their antitumor analogues is extremely rapid (26, 27), we wondered whether the high initial levels of cell-associated [^{3}H]VLB might be due to either rapid transport of the alkaloid or processes other than transport, such as binding. These possibilities were tested by performing rapid uptake assays directly in the microcentrifuge tubes, which allowed us not only to take time points every 2 or 5 sec, as shown in Fig. 2, but also to measure zero-time values for cell-associated [^{3}H]VLB within 0.5–1.5 sec after addition of drug to the cell suspension. It is clear from Fig. 2 that the initial rate of uptake of [^{3}H]VLB by the resistant cells, presumably a reflection of undirectional influx (26, 27), is about one-half that of the sensitive cells, as determined from the slopes of the regression lines. Most important, the zero-time values for cell-associated VLB in sensitive and resistant cells were 3.5–3.8 pmoles/10^6 cells, for apparent cellular drug concentrations of 6.1–9.0 μM, respectively. That these high concentrations of cell-associated VLB at zero time apparently represent binding is demonstrated in Fig. 3. Increasing concentrations of nonradioactive VLB caused a progressive decrease in the percentage of [^{3}H]VLB accumulated by both cell lines.

Retention of [^{3}H]VLB. The decreased accumulation of [^{3}H]VLB by resistant cells could be a consequence of its decreased permeability and/or decreased retention. Shown in Fig. 4 are typical results of experiments de-

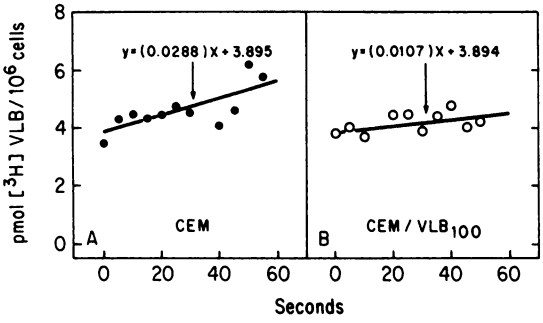

FIG. 2. *Initial accumulation of [^{3}H]VLB by CEM and CEM/VLB$_{100}$ cells*

These experiments were conducted directly in the microcentrifuge tubes in the 37° warm room; additionally, cell suspensions and drug solutions were kept in a 37° water bath until needed. After 200 μl of silicone oil were added to the tubes, which were then warmed to 37°, 200 μl of the 37° cell suspension (in glucose-containing buffer) were carefully layered over the oil. Assays were begun by the addition of 200 μl of buffer containing glucose and [^{3}H]VLB, the final concentrations being 10 mM and 200 nM, respectively. In all cases, additions to the tubes were in reverse order to the time of incubation; within 0.5 sec after the addition of drug to the last tube, the microcentrifuge was started and the cells were immediately separated from the medium by pelleting them through the silicone oil; this last tube was the "zero-time" point. Shown are the results of three experiments in which single aliquots were taken at 5-sec intervals. Each point represents the mean of three determinations. Regression lines, fit by the method of least squares, are defined by the equations in each box.

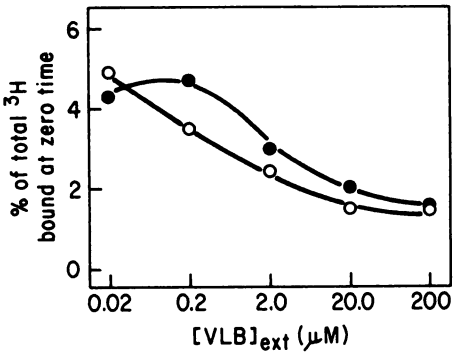

FIG. 3. *Reduction of instantaneous [^{3}H]VLB binding by nonradioactive VLB*

Cells were preincubated in glucose-containing buffer for 10 min at 37°. Different concentrations of nonradioactive VLB and a constant amount of [^{3}H]VLB were added simultaneously to the cell suspensions, which were then diluted immediately with ice-cold buffer and processed as described under Materials and Methods. Shown is a representative experiment, each point being the mean of three separate determinations. Cell lines: ●, CEM; ○, CEM/VLB$_{100}$.

signed to quantitate the amount of drug retained by each cell line. Whereas the VLB-resistant line lost about two-thirds of its accumulated drug during the washing procedure, the sensitive line lost little or none under these conditions. Reincubation of cells in drug-free buffer at 37° led to drug loss from both cell lines until only presumably tightly bound drug remained (19, 28). This nonreleasable, bound fraction accounted for ≈4–10% of the drug accumulated by resistant cells, contrasted with ≈40–50% by sensitive cells.

Effect of glucose and azide on the accumulation and retention of [^{3}H]VLB. Others have demonstrated that the cellular accumulation of alkaloids and anthracyclines

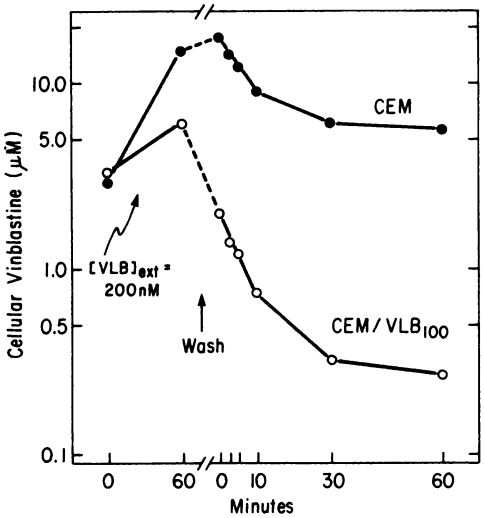

FIG. 4. *Release of [^{3}H]VLB by CEM and CEM/VLB$_{100}$ cells*

Cells were incubated at 37° for 60 min in buffer containing 10 mM glucose and [^{3}H]VLB at a final concentration of 200 nM, after which they were washed at 4° and resuspended in drug-free glucose-containing buffer. See Materials and Methods for details of the procedure. Aliquots of the cell suspension were taken for the determination of cell-associated ^{3}H at the times indicated. Shown is a representative experiment. Each point represents the mean of triplicate determinations; the standard deviations were ≤15% of the means.

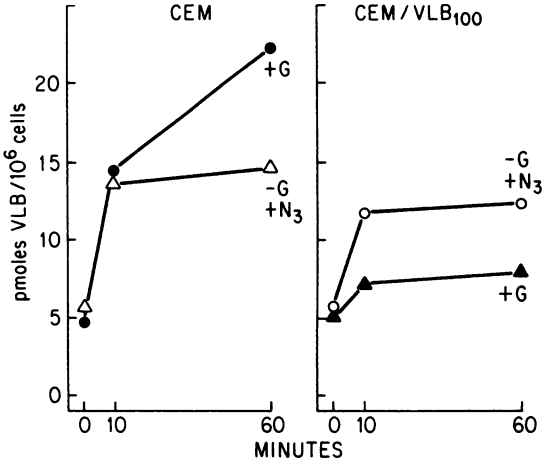

FIG. 5. *Effects of glucose depletion on the accumulation of [^{3}H]VLB by CEM and CEM/VLB$_{100}$ cells*

Cells were incubated in buffer containing [^{3}H]VLB at a final concentration of 200 nM in the presence of 10 mM glucose (●, ▲; *+G*) or in glucose-free buffer containing 10 mM NaN_3 (○, △; *−G +N$_3$*). Aliquots were taken at the times indicated, and cells were processed for the determination of ^{3}H as described under Materials and Methods. Shown is a typical experiment. Each point represents the mean of triplicate determinations; the standard deviations were ≤15% of the mean values.

is altered in the presence of metabolic inhibitors (11, 12, 29). In general, metabolic inhibitors such as azide, cyanide, and iodoacetate permit a greater accumulation of drug by resistant cells, an effect that has been attributed either to inhibition of an ATP/energy-requiring "efflux pump" (7, 8, 11–14) or to an active, energy-requiring "permeability barrier" (16). In our resistant CEM/VLB$_{100}$ cells, sodium azide likewise increased the steady-state level of cell-associated VLB (Fig. 5, right). However, addition of azide to the drug-sensitive CEM cells inhibited accumulation of [^{3}H]VLB, and the steady-state level of drug was qualitatively and quantitatively similar to that of the azide-treated resistant cells (Fig. 5, left). Addition of glucose to the medium after cells were allowed to accumulate VLB in the presence of azide caused the resistant cells to lose drug until a new steady-state plateau was reached (Fig. 6A). By contrast, glucose caused the further accumulation of [^{3}H]VLB by CEM cells to a higher steady-state level (Fig. 6B).

The effects of glucose shown in Fig. 6 could be mimicked by mannose, another metabolizable sugar, but neither 2-deoxyglucose nor galactose nor fucose affected steady-state levels of cell-associated [^{3}H]VLB (data not shown). Such results are in agreement with observations of See *et al.* (29) and, more recently, Seeber *et al.* (30), and suggest that cell metabolism is involved in the accumulation of [^{3}H]VLB by both cell lines. Indeed, when measured under the same conditions that were imposed in the preceding experiment, cellular levels of ATP decreased until glucose was added (Fig. 7). The addition of sugar caused an immediate increase of cellular ATP to ≈79–85% of initial values. That ATP levels did not return to normal may reflect the continued presence of azide in the system.

Evidence for two VLB binding compartments. The relationship of drug binding to metabolic state of the cell is shown in Fig. 8. In the presence of glucose, only ≈10%

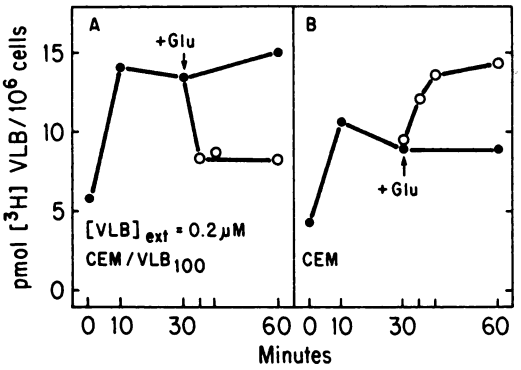

FIG. 6. *Effect of glucose on the levels of [^{3}H]VLB in azide-treated VLB-resistant (A) and -sensitive (B) cells*

Cells were incubated at 37° in glucose-free buffer containing 10 mM NaN_3 and [^{3}H]VLB at a final concentration of 200 nM. After 30 min, glucose was added to some flasks to a final concentration of 10 mM. Aliquots were taken at the time points indicated and processed for the determination of ^{3}H as described under Materials and Methods. Shown are two representative experiments. Each point represents the mean of triplicate aliquots; the standard deviations were usually ≤15% of the mean values.

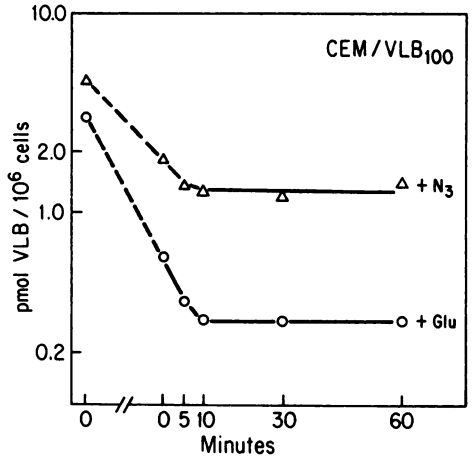

FIG. 8. *Effects of metabolic status on the "instantaneous" binding of [^{3}H]VLB by CEM/VLB$_{100}$ cells*

Cells were preincubated at 37° for 10 min in the presence of 10 mM glucose (○) or 10 mM NaN_3 (△). [^{3}H]VLB was added to a final concentration of 200 nM, after which the cells were immediately collected and washed as described under Materials and Methods. The cells were then resuspended in drug-free medium at 37°, and aliquots were taken for the determination of ^{3}H at the times indicated. Shown is a represenative experiment. Each point represents the mean of triplicate aliquots, the standard deviations being ≤15% of the mean values. The initial (prewash) amounts of cell-associated drug were 3.04 ± 0.07 and 4.46 ± 0.42 pmoles of VLB/10^6 cells for glucose- and NaN_3-treated cells, respectively.

of the [^{3}H]VLB "instantaneously" associated with resistant cells is bound and not readily released when the cells are resuspended in drug-free medium. By contrast, in azide-treated, glucose-free cells, 3 times more drug (31%) is tightly bound at zero time. Thus, VLB binding by CEM/VLB$_{100}$ cells can be considered to have two compartments—one dependent on and the other independent of the metabolic status of the cell.

Further evidence for this two-compartment concept is presented in Fig. 9. Metabolically depleted CEM/VLB$_{100}$ cells were preincubated with [^{3}H]VLB for 60 min, washed, and resuspended in drug-free buffer at 37° with or without glucose (but containing azide) or with glucose added after 30 min. (That no drug was lost from these cells during the washing procedure is most likely a reflection of their energy-depleted state, in contrast to the cells in the experiment shown in Fig. 4.) Regardless of their metabolic state, the cells lost drug rapidly when resuspended in drug-free medium. After 10 min, drug loss by cells lacking glucose began to reach a plateau at a higher steady-state level than did cells containing glucose. Addition of sugar at 30 min led to a further loss of [^{3}H]VLB, to the same level seen with the original glucose-supplemented cells. Thus, cellular metabolism has little or no effect on the *initial* loss of [^{3}H]VLB, but does seem

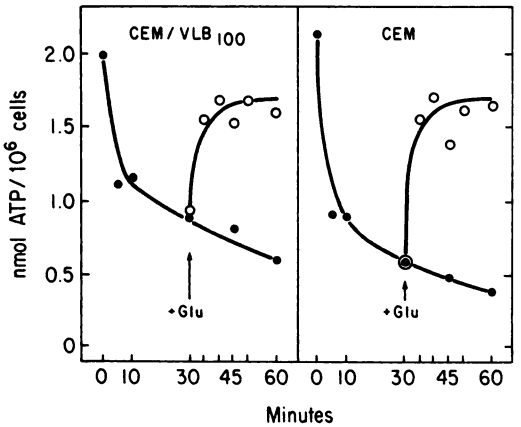

FIG. 7. *Effects of azide and glucose on the ATP levels of VLB-resistant and -sensitive cells*

CEM/VLB$_{100}$ (left) and CEM (right) cells were incubated at 37° in glucose-free buffer containing 10 mM NaN_3. After 30 min, glucose (*+Glu*) was added to some flasks to a final concentration of 10 mM. Aliquots were taken at the time points indicated and processed for the determination of ATP as described under Materials and Methods. Shown are two representative experiments. Each point represents the average of duplicate determinations.

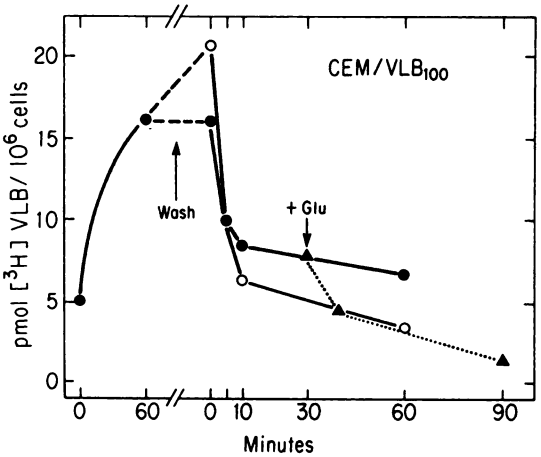

FIG. 9. *Release of [^{3}H]VLB from CEM/VLB$_{100}$ cells*

See text for experimental details and Materials and Methods for details of the processing of samples. ○, Cells incubated or resuspended in buffer containing sodium azide (10 mM) but no glucose; ●, cells resuspended in buffer containing glucose (10 mM); ▲, cells resuspended in buffer containing sodium azide and no glucose, but to which glucose was added at the time indicated to a final concentration of 10 mM.

to be able to cause the release of another (bound) fraction of the drug. Subsequent experiments indicated that the release of [^{3}H]VLB was not due to cell death, inasmuch as 77–91% of the cells retained their capacity to exclude trypan blue under these conditions. This experiment also suggests that [^{3}H]VLB is lost from resistant cells down its concentration gradient by a passive, energy-independent process.

Effects of other drugs on glucose-mediated VLB release. If the release of [^{3}H]VLB from CEM/VLB$_{100}$ cells under conditions described in Figs. 6 and 9 occurred through the stimulation of an active efflux pump, it should be possible to inhibit the release of the alkaloid with drugs for which an active efflux pump has been postulated as a common basis of resistance and cross-resistance (11, 13, 14, 30, 31), and which should compete with VLB for access to the pump. Conversely, if these compounds compete with [^{3}H]VLB for binding to some other cellular components, they should not impair the glucose-mediated release of [^{3}H]VLB and may even cause its further release. To distinguish between these possibilities, the experiment shown in Fig. 10 was performed. Cells were preincubated for 30 min in buffer containing azide and 20 μM unlabeled drug, as indicated at the *top* of each panel, after which time 0.2 μM [^{3}H]VLB was added (t = 0 in Fig. 10). When steady-state levels of cell-associated [^{3}H]VLB were achieved, glucose was added (*arrows*) and other aliquots were taken at the times indicated. Also shown in Fig. 10 is the net 30-min change (*60′ − 30′*) in cell-associated [^{3}H]VLB caused by glucose. The anthracyclines (DOX, DNR), VM-26, and CLC inhibited neither the accumulation nor the glucose-mediated release of [^{3}H]VLB; indeed, the latter two compounds appeared to enhance the glucose effect. By contrast, all three *Vinca* alkaloids (VCR, VDS, VLB) greatly reduced the accumulation of [^{3}H]VLB and also prevented the glucose effect.

DISCUSSION

Chemotherapeutic strategies designed to overcome resistance to such natural product drugs as *Vinca* alkaloids and anthracyclines will require an understanding of the mechanism(s) by which cells express the drug insensitivity. Accordingly, it is important to determine whether active outward transport or some other phenomenon is primarily responsible for resistance to these agents. We have shown here that alkaloid resistance in leukemic cells may be due primarily to energy-dependent reduced drug binding.

VLB was bound extensively by both sensitive and resistant cell lines. Because of this binding, we suggest that the mechanism of cellular VLB accumulation cannot be determined with certainty, since initial rates of drug uptake, which can be calculated from data such as those depicted in Fig. 2, may not represent actual transport of drug across the membrane. Indeed, although the decreased drug uptake shown in Fig. 2 may indicate that [^{3}H]VLB permeability is impaired in resistant versus sensitive cells, in general agreement with findings for other agents (5, 10, 11), the decreased initial drug uptake may also represent energy-altered drug binding, as discussed below.

Much of the cell-associated VLB appeared to be bound (Fig. 3), and a component of this bound drug was sensitive to the metabolic state of the cell, even at zero time (Fig. 8). More VLB was apparently loosely bound in the resistant cells than in the sensitive cells (Fig. 4). Thus, whereas ≈50% of the VLB accumulated by the sensitive CEM cells was weakly bound and readily releasable, this

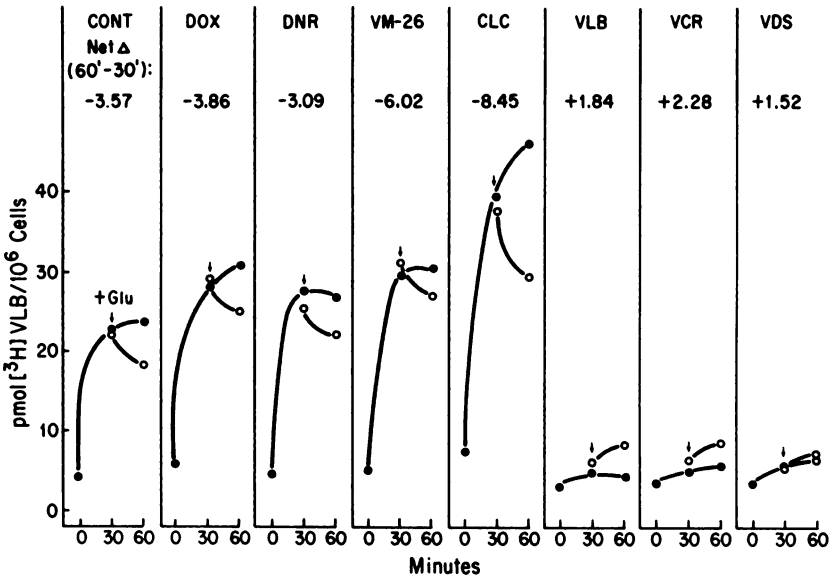

FIG. 10. *Effects of anticancer drugs on the accumulation and glucose-induced release of [^{3}H]VLB from CEM/VLB$_{100}$ cells*

The experimental protocol was as in Fig. 6, except that cells were preincubated for 30 min in glucose-free buffer containing 10 mM NaN_3 and the various drugs shown in each panel, each at a final concentration of 20 μM. At the end of the preincubation period (*t* = 0), [^{3}H]VLB was added at a final concentration of 0.2 μM. After another 30 min, glucose was added to the cell suspension (*arrows*), and aliquots were taken immediately and 30 min later. Net Δ (*60′ − 30′*) values represent the 30-min changes in the amount of cell-associated [^{3}H]VLB in the treated flasks at 60 min relative to the 30-min controls. Each point represents the mean of triplicate aliquots, the standard deviations being ≤15% of the mean values.

fraction was nearly twice as great (≈90%) in the resistant CEM/VLB$_{100}$ cells. That the alkaloid-resistant cells accumulate a larger fraction of loosely bound drug than do the sensitive cells suggests a mechanism by which resistance to VLB may be expressed.

We showed the depletion of ATP permitted increased drug accumulation by the resistant cells but caused decreased accumulation by the sensitive cells (Fig. 5). Addition of glucose to these depleted cells caused a rapid increase in ATP levels (Fig. 7) that was associated with drug loss from the resistant line but with further drug uptake by the sensitive line (Fig. 6). Although the results with resistant cells are similar to those shown by others and ascribed either to inhibition of an ATP-requiring outward drug transport (7, 8, 11–14) or to an active permeability barrier (16), the results with sensitive cells differ from what others have shown (11–14). This enhanced accumulation of drug by the glucose-treated sensitive cells appears to be related to the extracellular concentration of VLB. Decreasing the [VLB]$_{ext}$ resulted in a progressively diminished glucose effect so that, when the cells were incubated in the IC$_{50}$ concentration for CEM cells (≈7 nM[4]), glucose addition neither enhanced nor decreased the amount of [^{3}H]VLB associated with these sensitive cells.[5]

Although our observations regarding cell metabolism and VLB accumulation are not incompatible with the concepts of either an efflux pump or an active permeability barrier, they do support the idea that energy-dependent altered drug binding is important in alkaloid resistance. This was suggested by (a) the effects of glucose on the sensitive cells, discussed above; (b) the demonstration that there appeared to be two cellular VLB-binding compartments, one dependent on and the other independent of cell metabolism (Figs. 8 and 9); and (c) the fact that drug loss from resistant cells appeared to be in part a passive process not associated with inhibition or activation of an efflux pump (Fig. 9).

Support for differential binding and passive loss of drug by resistant cells comes from findings of Minor and Roscoe (6), who studied the release of [^{3}H]CLC from monolayer cultures of mammalian cell lines having different degrees of sensitivity to this drug. They showed that two resistant lines, when incubated in drug-free medium, lost drug three times faster than did a relatively more sensitive line. They attributed the difference to an extra pool of bound drug associated with resistant cells, possibly on the cell surface within the glycocalyx (the glycoprotein and polysaccharide cover that surrounds many cells), which fills and empties rapidly.

In our attempt to distinguish between an active efflux pump and energy-altered drug binding using different drugs for which cross-resistance has been demonstrated, we showed (Fig. 10) that neither anthracyclines, nor VM-26, nor CLC inhibited the glucose-mediated release of [^{3}H]VLB. These results can be interpreted to mean that none of these compounds interferes with any postulated efflux pump. However, all three *Vinca* alkaloids prevented the glucose effect. Although these results could indicate that the *Vinca* alkaloids inhibit an active efflux pump, we believe that this is unlikely because (a) all of the *Vinca* compounds decreased the steady-state accumulation of [^{3}H]VLB by 6- to 11-fold as compared with controls and the other drug-treated cells; this was apparently due to competition for [^{3}H]VLB binding sites by the 100-fold molar excess of nonradioactive VLB, VCR, or VDS (recall Fig. 2). (b) The same effect was not seen with compounds for which cross-resistance has been demonstrated or that are presumably exported by the same efflux pump, *viz.* anthracyclines (11, 13, 14) and epipodophyllotoxins (30). Thus, we conclude that the effect of glucose is to inactivate or impair a VLB-binding process in the resistant cells, rather than to stimulate an active efflux pump.

We have demonstrated that the metabolic state of the cell profoundly influences the extent of accumulation and retention of [^{3}H]VLB. Our data suggest that the accumulation of [^{3}H]VLB by sensitive and resistant cells is governed by an energy-altered, drug-binding mechanism, possibly related to the state of phosphorylation of certain key proteins. Thus, according to our model, certain sugars, rather than activating a drug pump or permeability barrier, may alter the accumulation of alkaloids through an energy-sensitive binding mechanism. There appear to be precedents for such a concept. For example, Costlow and Hample (32, 33) have shown that the specific binding of prolactin by rat mammary carcinoma cells is enhanced in the presence of such inhibitors of cellular metabolism as azide, cyanide, arsenate, and 2,4-dinitrophenol. This observation may indicate that energy is required to keep receptor proteins in an "off" state, perhaps by being phosphorylated; removal of the source of energy may alter the structure of the receptor in some manner. Additionally, Fry *et al.* (34) have presented data showing that 2,4-dinitrophenol and other inhibitors of cellular metabolism increase the nonexchangeable ("bound") fraction of methotrexate in Ehrlich ascites cells, and have suggested that such inhibitors interact directly with the methotrexate carrier in the membrane. Such results may be relevant to the effects of cellular metabolic status on the accumulation of *Vinca* alkaloids and other drugs, as described above. Investigations focusing on the identification of potential energy-sensitive cellular binding molecules and the further characterization of this energy-altered binding phenomenon are currently in progress in our laboratory.

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[5] W. T. Beck and M. C. Cirtain, unpublished results.

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