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CHLOROQUINE ENHANCEMENT OF ANTICANCER DRUG CYTOTOXICITY IN MULTIPLE DRUG RESISTANT HUMAN LEUKEMIC CELLS*

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Abstract—Vinblastine-sensitive (CCRF-CEM) and -resistant (CEM/VLB$_{100}$) human T-cell lymphoblasts were treated with the lysosomotropic agent chloroquine. As measured by growth inhibition, this drug enhanced the cytotoxicity of vinblastine in the CEM/VLB$_{100}$ cells but was less effective in the CCRF-CEM cells. Chloroquine also enhanced the cytotoxic activity of vincristine, daunorubicin and doxorubicin and, to a lesser extent, teniposide (VM-26) in the CEM/VLB$_{100}$ cells. Histological examination revealed that the vinblastine-resistant cells contained more cytoplasmic vacuoles than their drug-sensitive counterparts. When the CEM/VLB$_{100}$ cells were treated with chloroquine, vinblastine, or a combination of the two, the cells displayed many more cytoplasmic vacuoles than the controls. Coincident with the increased number of vacuoles, these treated cells stained more intensely than controls for the lysosomal enzyme, acid phosphatase, but not for lipid. The vacuolization did not increase as much in the CCRF-CEM cell line when these cells were exposed to the chloroquine + vinblastine combination. Vacuolization was also associated with vincristine, doxorubicin, and daunorubicin treatments, but not with VM-26. We conclude that chloroquine is a modulator of anticancer drug action in the CEM/VLB$_{100}$ cell line.

One of the major problems associated with the treatment of human neoplastic diseases is the development of resistance to many types of chemotherapy. A cell culture model of this "multiple drug resistance" (MDR‡) is that of *Vinca* alkaloid resistance in human leukemic lymphoblasts [1]. Such resistance is associated with a decrease in cellular drug accumulation that has been attributed to either an alteration in cellular drug binding [2, 3] or an enhanced activity of an efflux "pump" of broad specificity [4–7]. Increases in specific cell membrane glycoproteins have also been associated with MDR in such cells [8–10], but the relationship between the altered cell pharmacology and surface glycoprotein changes is unclear.

Recently, several membrane-interacting compounds, such as verapamil and other "calcium channel blockers" have been shown to enhance the cytotoxic effectiveness of *Vinca* alkaloids [11, 12], anthracyclines [13], epipodophyllotoxins [14], and bleomycin [15], primarily, but not exclusively, in MDR cells. Recent results from this laboratory suggest that the effect of verapamil on the cytotoxicity of *Vinca* alkaloids is far greater than its effect on comparable activities of anthracyclines, epipodophyllotoxins, or other agents [16].

We wished to determine if this enhancement of alkaloid cytotoxicity was relatively specific for these "Ca^{2+}-modifying" agents, or if other classes of drugs could produce similar effects. Since verapamil was shown recently to have an effect on lysosomes or lysosomal function [17–19], we asked if the lysosomotropic agent chloroquine (CLQ) could also enhance the cytotoxicity of "natural product" anticancer drugs in our human lymphoblastoid MDR cell line, CEM/VLB$_{100}$. Our initial observations are the subjects of this paper.

MATERIALS AND METHODS

Cells, culture conditions, and cytotoxicity. CCRF-CEM human leukemic lymphoblasts and their VLB-resistant CEM/VLB$_{100}$ derivatives were grown in minimal essential medium (Earle's salts) containing 10% fetal bovine serum as described previously [8]. The CEM/VLB$_{100}$ cells express the MDR phenotype [1]. Drug cytotoxicity was assessed by the inhibition of cell growth in 48 hr, compared to controls. We have shown before that this type of assay is appropriate for relative comparisons [20], and can reflect actual cell-kill [16]. The cells were incubated at 37° in multiwell plates (Falcon No. 3047) at an initial density of ≈2.5 to 3×10^5 cells/ml with or without drugs. At 48 hr, the cell number was determined by a Coulter Counter (model ZBI) using a channelizer to distinguish cells from debris. The IC_{50} value is defined as the concentration of drug required to inhibit by 50% the 48-hr growth of treated cells

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‡ Abbreviations: MDR, multiple drug resistance; VLB, vinblastine sulfate; VCR, vincristine sulfate; DOX, doxorubicin; DNR, daunorubicin; CLQ, chloroquine; and VM-26 (teniposide; 4′-dimethylepipodophyllotoxinthenylidine-β-D-glucoside).

compared to the controls. The fold-decrease in IC_{50} (enhanced cytotoxicity) was determined by dividing the IC_{50} value of the controls by that of the treated cells.

Flow cytometry and mitotic index. After exposure to drugs for different times, cells were harvested for flow cytometry. DNA was stained with propidium iodide for DNA histograms and cell cycle analysis. Mitotic cells were stained with acetocarmine. Staining methods, DNA analysis, and enumeration of mitotic cells were done as described earlier [16].

Histochemistry. Cytospin preparations of the control or drug-treated CCRF-CEM and CEM/VLB_{100} cells were stained with an Ames automated Hematek slide stainer (Wright's stain), for lipid with Oil Red O or Sudan Black B and for acid phosphatase activity [21].

Chemicals and supplies. VLB and VCR were obtained from Eli Lilly & Co. (Indianapolis, IN). DOX and DNR were from Adria Laboratories (Wilmington, DE), and CLQ was purchased from the Sigma Chemical Co. (St. Louis, MO). All these compounds were dissolved in 0.9% NaCl solution. VM-26 was obtained from Bristol-Myers (Syracuse, NY), dissolved in 100% dimethyl sulfoxide (DMSO) and subsequently diluted with 0.9% NaCl solution. The final DMSO concentration of 0.3% had no independent effect on cell growth.

RESULTS

Effect of CLQ on anticancer drug cytotoxicity. A concentration of CLQ (5×10^{-5} M) that had little effect on cell growth enhanced the ability of VLB to inhibit the growth of the VLB-resistant CEM/VLB_{100} cells, decreasing the VLB IC_{50} by ≈13-fold (Table 1). However, the same concentration of CLQ had little effect on the cytotoxicity of VLB in the drug-sensitive CCRF-CEM cells (Table 1). A similar type of preferential enhancement of cytotoxicity of anticancer drugs on resistant cells but not sensitive cells has been observed with verapamil [22]. The enhancement of VLB cytotoxicity by CLQ in the MDR CEM/VLB_{100} cells indicates that CLQ has a greater effect on the resistant cells than on the sensitive cells.

Although CLQ by itself at the concentrations used was somewhat cytotoxic to both cell lines (Table 2), the effect of the CLQ + VLB combination on the inhibition of cell growth was greater than additive in the CEM/VLB_{100} cells (Table 2). This apparent synergism was observed only when CLQ caused some growth inhibition. This effect was not seen with equitoxic (i.e. IC_{50}) concentrations of VLB and CLQ in the VLB-sensitive CCRF-CEM cells; rather, growth inhibition by VLB (10^{-9} M) and CLQ (10^{-4} M or 5×10^{-5} M) was additive in these cells (Table 2). Equimolar concentrations of CLQ always had a slightly greater inhibitory effect on the CEM/VLB_{100} cells compared to the CCRF-CEM cells, but this difference was not statistically significant.

Table 2. Enhancement of VLB cytotoxicity in CEM/VLB_{100} cells by CLQ

Drug treatments		Percent of control 48-hr cell growth*	
[VLB]†	[CLQ]†	CCRF-CEM	CEM/VLB_{100}
1×10^{-9}	0	81 ± 12‡	NA§
1×10^{-7}	0	NA	94 ± 6
0	5×10^{-5}	83 ± 11	75 ± 4
0	1×10^{-4}	70 ± 12	53 ± 4
1×10^{-9}	5×10^{-5}	64 ± 12	NA
1×10^{-9}	1×10^{-4}	51 ± 16	NA
1×10^{-7}	5×10^{-5}	NA	37 ± 6
1×10^{-7}	1×10^{-4}	NA	31 ± 8

* Cell growth and its inhibition by drugs (cytotoxicity) were monitored as detailed in Materials and Methods.
† Molar concentration.
‡ Mean ± SD of four to eight experiments.
§ Not assayed.

The CEM/VLB_{100} cell line is cross-resistant to other natural product compounds (or their semisynthetic derivatives) such as VCR, DOX, DNR, and VM-26 [1, 23]. CLQ (5×10^{-5} M) also enhanced the cytotoxicity of DOX, DNR, and VCR by approximately 3-, 5- and 10-fold respectively (Table 3). Of considerable interest was the observation that CLQ (5×10^{-5} M) did not greatly potentiate the cytotoxicity of the epipodophyllotoxin VM-26 (Table 3). Moreover, the synergistic effects of CLQ + VCR mixture were greater than the effects of CLQ with either of the two anthracyclines or with VLB.

Flow cytometric findings. To determine whether CLQ was enhancing VLB cytotoxicity or exerting an

Table 1. Effect of chloroquine on the cytotoxicity of vinblastine in CCRF-CEM and CEM/VLB_{100} cells*

Treatment	CCRF-CEM			CEM/VLB_{100}		
	IC_{50}† (M)	Fold-decrease‡	P values	IC_{50} (M)	Fold-decrease	P values
VLB	$3.5 \pm 1.1 \times 10^{-9}$§			$5.5 \pm 1.3 \times 10^{-7}$		
CLQ	$2.3 \pm 0.9 \times 10^{-4}$			$1.1 \pm 2.2 \times 10^{-4}$		
VLB + CLQ (50 μM)	$2.2 \pm 0.9 \times 10^{-9}$	1.6	0.09	$4.1 \pm 2.0 \times 10^{-8}$	13.4	<0.001
VLB + CLQ (100 μM)	$1.5 \pm 1.6 \times 10^{-9}$	2.3	0.06	$2.0 \pm 1.8 \times 10^{-8}$	27.5	<0.001

* Cells were grown for 48 hr in the presence or absence of drugs, as detailed in Materials and Methods.
† Molar concentration of drug that inhibits 48-hr cell growth by 50%, compared to control.
‡ Calculated by dividing the IC_{50} value of the control cells by the IC_{50} value of drug-treated cells.
§ Mean ± SD of four to eight experiments.

Table 3. Effect of chloroquine on the cytotoxicity of vincristine, daunorobicin, doxorubicin and VM-26 in CEM/VLB_{100} cells*

Treatment	IC_{50}† (M)	Fold-decrease‡	P values
CLQ	$1.7 \pm 0.6 \times 10^{-4}$§		
VCR	$1.8 \pm 1.3 \times 10^{-6}$		
VCR + CLQ (1×10^{-5} M)	$9.0 \pm 11.0 \times 10^{-7}$	2.0	>0.10‖
VCR + CLQ (5×10^{-5} M)	$1.9 \pm 1.1 \times 10^{-7}$	9.5	<0.05
VCR + CLQ (1×10^{-4} M)	$1.9 \pm 1.4 \times 10^{-8}$	95	<0.01
DNR	$1.4 \pm 1.1 \times 10^{-6}$		
DNR + CLQ (5×10^{-5} M)	$2.9 \pm 1.5 \times 10^{-7}$	4.8	0.06‖
DNR + CLQ (1×10^{-4} M)	$1.3 \pm 1.0 \times 10^{-7}$	11	<0.05
DOX	$2.1 \pm 0.9 \times 10^{-6}$		
DOX + CLQ (5×10^{-5} M)	$7.4 \pm 4.3 \times 10^{-7}$	2.8	<0.05
DOX + CLQ (1×10^{-4} M)	$2.3 \pm 1.3 \times 10^{-7}$	9.1	<0.01
VM-26	$2.5 \pm 0.8 \times 10^{-6}$		
VM-26 + CLQ (5×10^{-5} M)	$1.5 \pm 1.2 \times 10^{-6}$	1.6	>0.10‖
VM-26 + CLQ (1×10^{-4} M)	$6.6 \pm 6.7 \times 10^{-7}$	3.8	<0.01

* Cells were grown for 48 hr in the presence or absence of drugs; see Materials and Methods for details.

† Molar concentration of drug that inhibits 48-hr cell growth by 50%.

‡ Calculated by dividing the IC_{50} of the control cells by that of the treated cells.

§ Mean ± SD of five to six experiments.

‖ Not statistically different from anticancer drug controls.

independent effect of its own, we measured the cell number and cell distribution of treated CEM/VLB_{100} cells at various times over 24 hr. We attempted to measure these parameters at 48 hr, but there were few or no cells left in the flasks of the CLQ + VLB or the VLB (5×10^{-6} M)-treated cultures. Therefore, the analysis is only shown through 24 hr. As can be seen in Fig. 1C, relatively noncytotoxic concentrations of VLB (1×10^{-7} M) and CLQ (5×10^{-5} M) caused a decrease in cell number between 12 and 16 hr, indicating that this combination is cytotoxic to the CEM/VLB_{100} cells and not simply growth inhibitory. This decrease in cell number paralleled that seen with a cytotoxic concentration of VLB alone (5×10^{-5} M). This decrease was accompanied by an increase in the number of cells in the G_2 + M phase of the cell cycle (Fig. 1A) as well as by increases in the mitotic index (Fig. 1B). Since CLQ itself (5×10^{-5} M) had little or no effect on cell number, cell cycle distribution, or mitotic index, and the effects of CLQ + VLB on these parameters appeared identical to those produced by VLB alone, it is likely that the cytotoxicity of the combination is due to a potentiation of VLB cytotoxicity by CLQ. These results are very similar to our recent findings that verapamil potentiates the cytotoxicity of VLB [16].

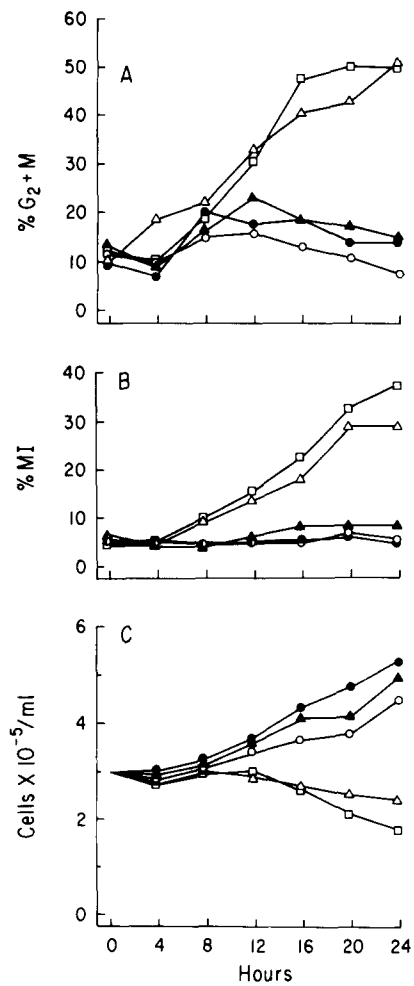

Fig. 1. Time course of CLQ action in CEM/VLB_{100} cells. Large flasks of cells were prepared, and at zero time the following drug additions were made in small volumes to individual flasks: (●) 0.9% NaCl solution; (○) CLQ (5×10^{-5} M); (▲) VLB (1×10^{-7} M); (□) VLB (5×10^{-6} M); and (△) CLQ + VLB (1×10^{-7} M). At the times indicated, aliquots were taken for (A) flow cytometric analysis, (B) mitotic index, and (C) cell number. Shown is a representative experiment.

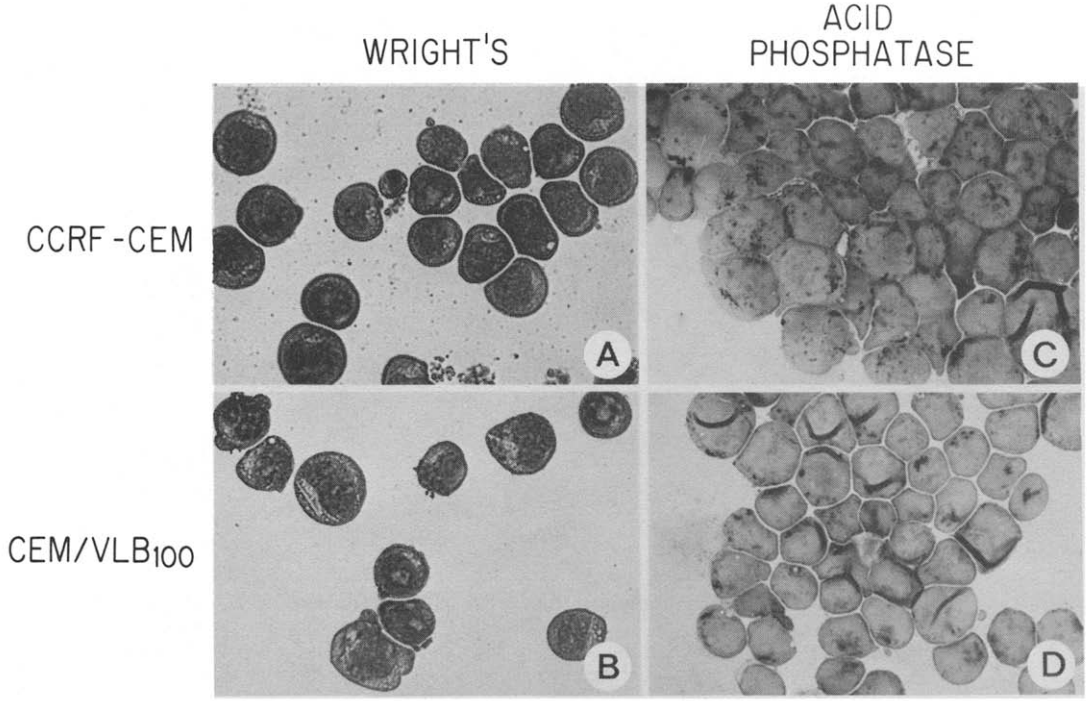

Fig. 2. Cytospin preparations of CCRF-CEM (A and C) and CEM/VLB$_{100}$ (B and D) cells. Shown are photomicrographs of: Wright's (A and B)- and acid phosphatase (C and D)-stained cells. See Materials and Methods for further details.

Cell morphology. Microscopic examination of cytospin preparations of both the CEM/VLB$_{100}$ and CCRF-CEM cells revealed that the drug-resistant line appeared to contain more vacuoles than the drug-sensitive line (Fig. 2, A and B). Further, the perivacuolar areas appeared to stain for the lysosomal enzyme, acid phosphatase (Fig. 2, C and D). When the CEM/VLB$_{100}$ cells were treated with 5×10^{-5} M CLQ (Fig. 3C), they were seen to contain many more vacuoles than the untreated CEM/VLB$_{100}$ controls (Fig. 3A). It is of interest that these vacuoles often increased in number when the cells were treated with noncytotoxic concentrations of VLB (1×10^{-7} M; Fig. 3B; Ref. 16). The effects of the CLQ + VLB combination were greater than those of either drug alone (Fig. 3D).

The intensity of the staining for the lysosomal enzyme, acid phosphatase, which appeared to be associated with many of the vacuoles, was enhanced greatly by the treatments with CLQ (Fig. 3G) and VLB (1×10^{-7} M) (Fig. 3F). However, those cells treated with the highly cytotoxic combination of VLB + CLQ had, in fact, less intense staining for acid phosphatase (Fig. 3H), most likely as a consequence of cell damage (cytotoxicity) and leakage of the enzyme from these vacuoles [24]. The control vacuoles did not stain for lipids with either Oil Red O or Sudan Black B (data not shown) as has been reported for two drug-resistant murine cell lines [25, 26], suggesting that in the CEM/VLB$_{100}$ cells they are not lipid droplets.

Cytospin preparations of the drug-*sensitive* CCRF-CEM cells revealed that CLQ (5×10^{-5} M; Fig. 4C) as well as VLB (1×10^{-9} M; Fig. 4B) both caused vacuoles similar to those seen in the CEM/VLB$_{100}$ cells treated with either 5×10^{-5} M CLQ or 1×10^{-7} M VLB (Fig. 3, B and C). However, it is of interest that, in the drug-sensitive CCRF-CEM cells, the CLQ + VLB combination (Fig. 4D) did not cause those gross morphological changes seen in CEM/VLB$_{100}$ cells after treatment with either drug alone. This suggests that the vacuoles are in some way related to the synergistic effect of the CLQ + VLB in the CEM/VLB$_{100}$ cell line but not in the CCRF-CEM cell line.

We have found that treatment of CEM/VLB$_{100}$ cells with VCR (Fig. 5B) also caused vacuole formation, but treatment with DOX (not shown), DNR or VM-26 did not. However, simultaneous CLQ treatment of CEM/VLB$_{100}$ cells with VCR, DNR, or DOX (not shown) was grossly cytotoxic and caused these morphological changes (Fig. 5), but the combination of CLQ + VM-26 did not cause any gross morphological changes that differed from that of either drug alone. If these morphological changes are related to the cytotoxic effectiveness of the combination, then they confirm the results in Table 3.

DISCUSSION

We have shown that the lysosomotropic agent CLQ can enhance the cytotoxicity of VLB in a human MDR cell line, but not in the drug-sensitive counterpart. CLQ (5×10^{-5} M) also enhanced the cytotoxicity of VCR, DNR and DOX, but not VM-26, in these drug-resistant cells. The enhanced cytotoxicity of the combination of CLQ + VLB appears to be related to a potentiation of the action of VLB,

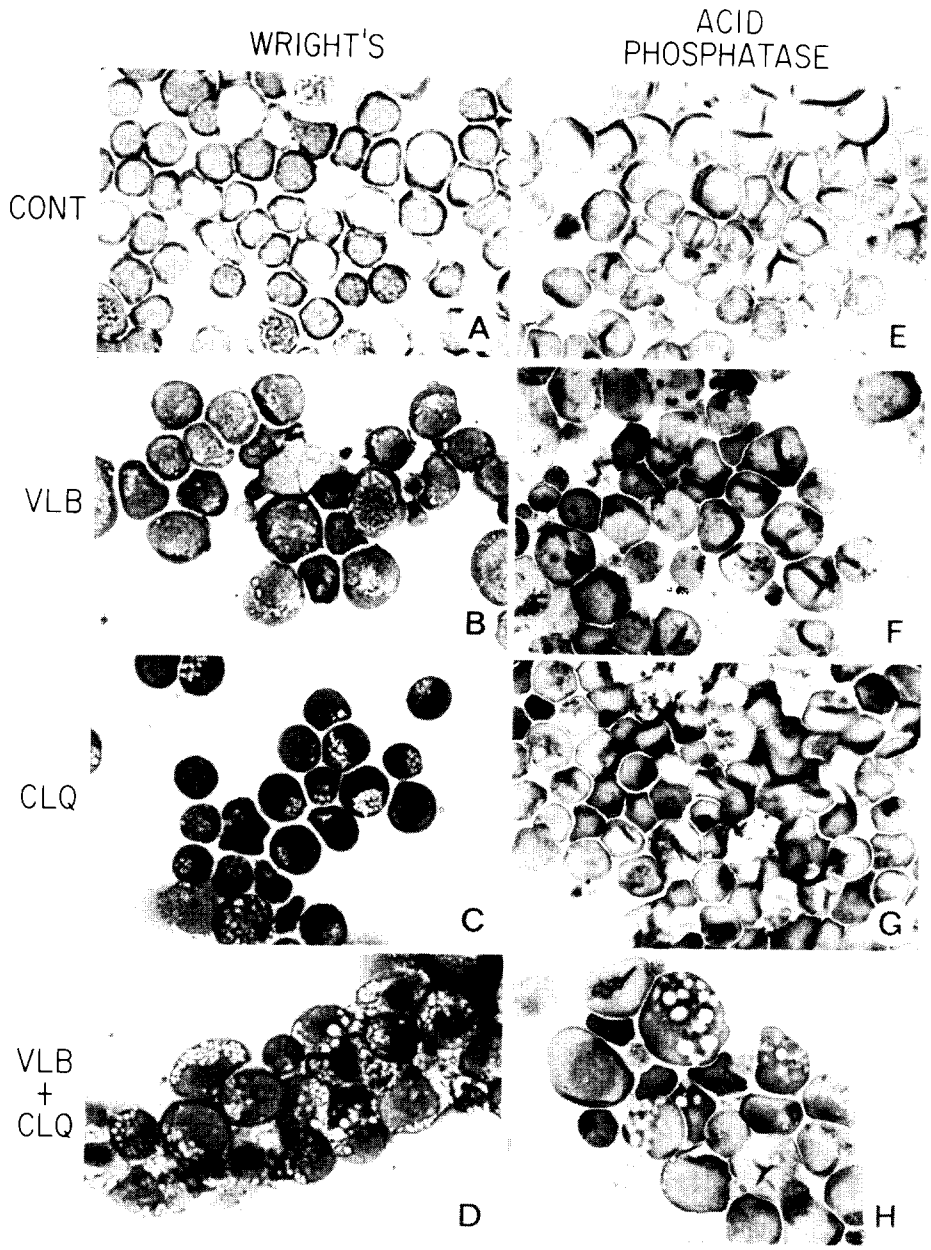

Fig. 3. Morphology and acid phosphatase staining of CEM/VLB$_{100}$ cells after treatment with CLQ and VLB. Key: (A and E) control; (B and F) VLB, 1×10^{-7} M; (C and G) CLQ, 5×10^{-5} M; and (D and H) VLB (1×10^{-7} M) + CLQ (5×10^{-5} M). (A–D) Wright's stain; (E–H) acid phosphatase. See Materials and Methods for details.

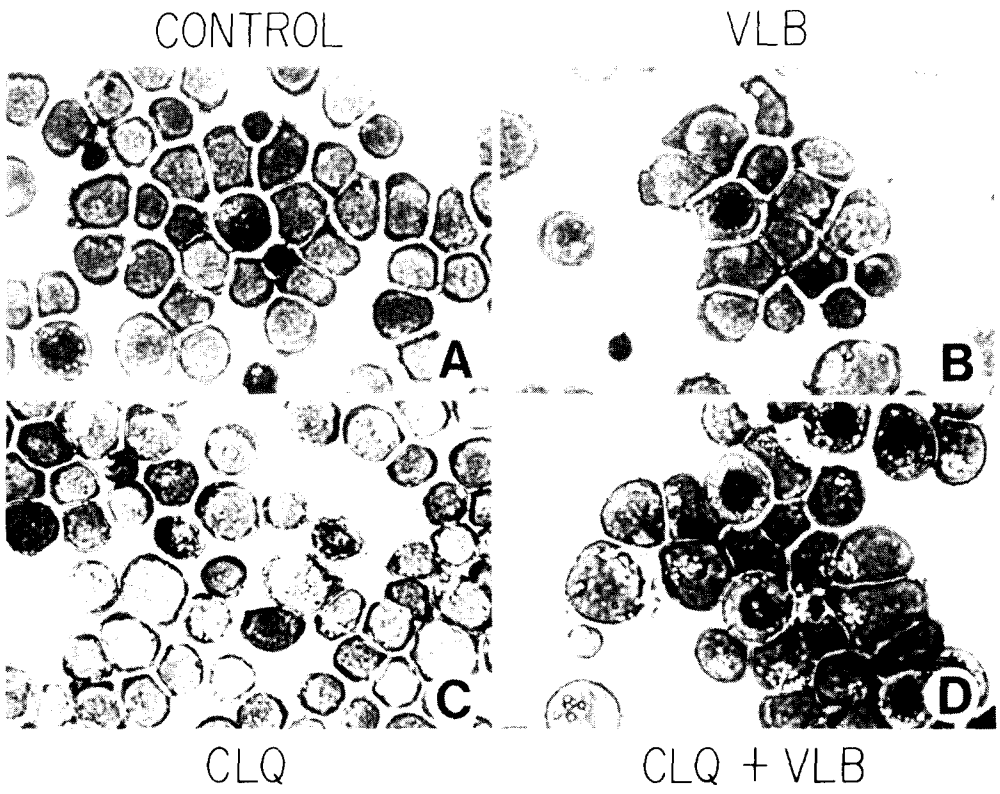

Fig. 4. Cytospin preparations of CCRF-CEM cells 48 hr after drug treatments. Key: (A) control; (B) VLB, 1×10^{-9} M; (C) CLQ, 5×10^{-5} M; and (D) VLB (1×10^{-9} M) + CLQ (5×10^{-5} M). See Materials and Methods for additional details.

as judged by the effects on the cell cycle and mitotic index, and its effects were accompanied by morphological changes.

The apparent potentiation by CLQ of *Vinca* alkaloid cytotoxicity, and, to a considerably lesser extent, anthracycline cytotoxicity is very much like the effects of verapamil, recently noted by us [16] and by Tsuruo *et al.* [27]. It is of interest that, while the CEM/VLB_{100} cells [23] are cross-resistant to *Vinca* alkaloids, anthracyclines and epipodophyllotoxins, the level of reistance is different for the different classes of compounds. The synergistic effect of CLQ also appears to follow the resistance pattern, with VCR + CLQ having the greatest effect and VM-26 + CLQ having the least effect (Tables 1 and 3). The more pronounced effect of CLQ on MDR cells than on drug-sensitive cells is also similar to what has been observed with the "Ca^{2+} channel blockers" [28].

The mechanism by which CLQ, or even verapamil, exerts its action to potentiate alkaloid cytotoxicity is unclear. The morphological changes may provide some guidance for further investigation. Histopathologically, vacuolization is a nonspecific visualization of cell death. It could be that the enlarged vacuoles represent dying cells in the G_2 + M stage of the cycle. However, it is known that VLB treatment is associated with vacuole formation and the inhibition of lysosome degradation in hepatocytes [29]. Vacuole formation has been associated with altered lysosomal function in mammalian cells [30], and there is evidence that CLQ, which also produces vacuoles, is concentrated in lysosomes, consequently altering their function [31, 32]. In this regard, it is of considerable interest that verapamil has lysosomal effects [17–19].

It can therefore be asked if the vacuoles seen in our CLQ-treated (and verapamil-treated [16]) cells are, in fact, lysosomes. We have already noted that these vacuoles do not stain for lipid, but they are associated with the lysosomal enzyme, acid phosphatase. Moreover, our treatments, especially with CLQ, enhanced their number, size and acid phosphatase staining. All these data suggest, but do not constitute proof, that these vacuoles are likely to be lysosomal in nature. Only their purification and study of their various properties will allow for a more complete understanding of any potential role of lysosomes in MDR and in the action of these agents.

In summary, our data indicate that CLQ enhanced the cytotoxicity of *Vinca* alkaloids and, to a lesser extent, anthracyclines in the MDR cell line CEM/VLB_{100}. CLQ, which has well-known effects in lysosomes, was a modulator of anticancer drug action in our cells. The increased number of acid phosphatase-staining vacuoles in the resistant cells, as well as their alteration after drug treatment, suggests a role for endosomal vesicles in both alkaloid action and resistance, and is a subject of current investigation in our laboratory.

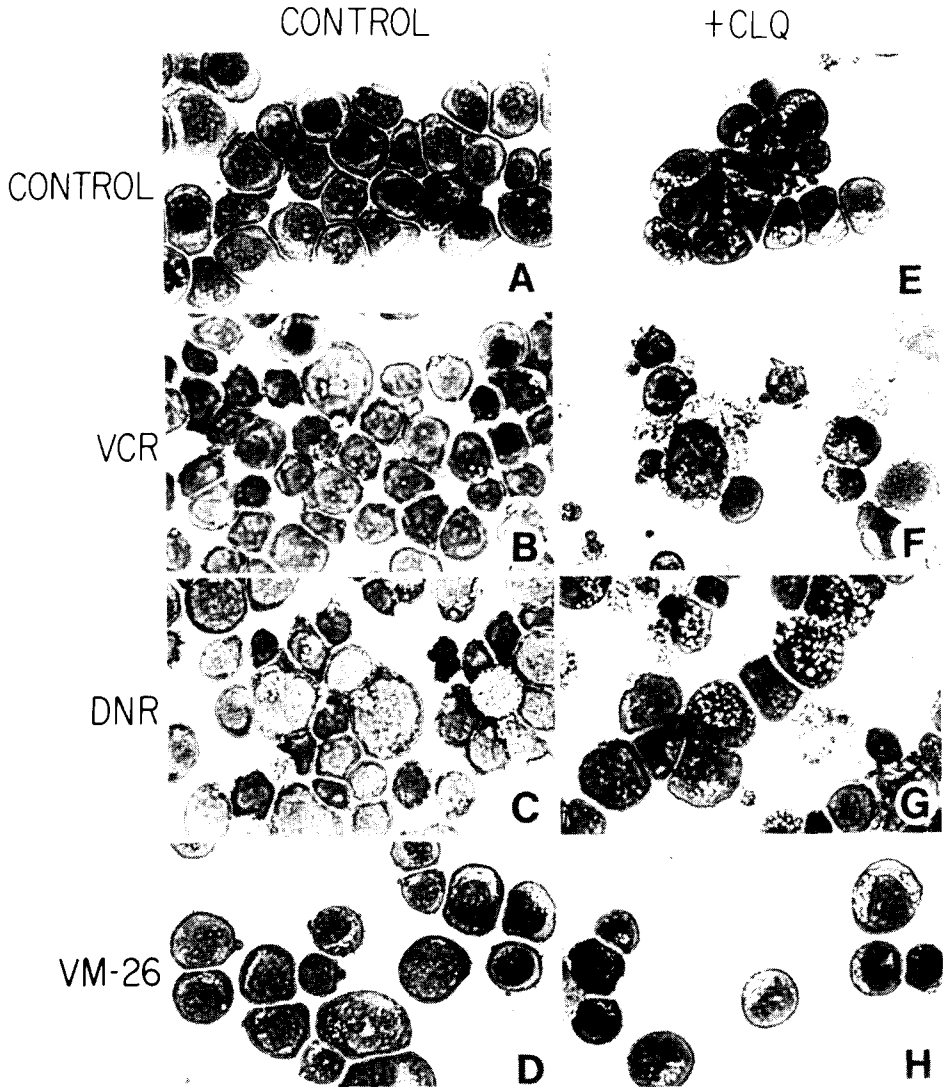

Fig. 5. Cytospin preparations of CEM/VLB$_{100}$ cells 48 hr after drug treatments. Key: (A) control; (B) VCR, 1 μM; (C) DNR, 1 μM; (D) VM-26, 1 μM; (E) CLQ, 50 μM; (F) VCR (1 μM) + CLQ (50 μM); (G) DNR (1 μM) + CLQ (50 μM); and (H) VM-26 (1 μM) + CLQ (50 μM). See Materials and Methods for details.

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