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The effect of vinca alkaloids in enhancing the sensitivity of a methotrexate-resistant (L1210/R7A) line, studied by flow cytometric and chromosome number analysis

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Summary. *Two L1210 murine lymphoma cell lines sensitive and resistant to methotrexate (L1210 and L1210/R7A, respectively) and previously shown to exhibit collateral sensitivity to the vinca alkaloids have been studied by flow cytofluorimetric techniques following propidium iodide staining of the DNA. Following treatment with a range of concentrations of vincristine, both cell lines showed a build-up of fluorescence in the 4n position. However, the methotrexate-resistant cell line exhibited this effect at lower doses of vincristine. On an equimolar basis, the vinca alkaloids ranked for intensity of this effect in the order vinblastine > vindesine > vincristine. DNA fluorescent histograms following various times of continuous exposure to vincristine showed an accumulation of material at the 8n position, which was shown by chromosome analysis to be due to polyploidy. It was concluded that the methotrexate-resistant cells (L1210/R7A) experience difficulty in traversing mitosis and this difficulty is enhanced by the vinca alkaloids.*

Introduction

The technique of flow cytofluorimetric analysis has been used to study the effects of various agents on cell-cycle progression [21, 22]. In the case of methotrexate acting on cultured human leukaemia cells (CCRF-CEM), an accumulation in early S phase linked to inhibition of DNA synthesis has been demonstrated [20]. The vinca alkaloids [3] have been shown to inhibit microtubular assembly, leading to metaphase arrest and in turn to polyploidy [1] during chromosome duplication. However, other cytotoxic actions have also been shown to occur during the G1 and S phases of the cell cycle [11, 16, 18]. Using flow cytometry, a block in G2 + M phase has been associated with cell kill [6]. On the other hand, the lethal effects of vincristine and vindesine have been shown [7] to occur with the S phase in logarithmically growing N1L8 cells.

In the present work, we have used flow cytometry to study the effects on the L1210 parental line and a methotrexate-resistant line (L1210/R7A) of three vinca alkaloids previously shown to exhibit collateral sensitivity [14].

Materials and methods

Materials. The vinca alkaloids (vincristine, vinblastine, and vindesine) were supplied by Eli Lilly. The propidium iodide was obtained from Calbiochem.

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Cell culture. The cell lines (L1210 and L1210/R7A) and culture conditions were exactly as described in the previous paper [14]. For the growth-inhibition studies used in this work cells in the exponential phase were treated with vincristine and vinblastine and incubated in suspension at 37° C in a 5% CO_2 atmosphere. Cell counts were assayed at intervals of 24 h on an electronic cell counter (Coulter Electronics).

Flow cytometry. The cell suspensions (10^5 cells/ml in RPMI 1640 medium supplemented with 10% horse serum) were treated with vinca alkaloids at the requisite concentration for the times stated at 37° C in a 5% CO_2 atmosphere. The cells were centrifuged at 160 *g*, 4° C, for 5 min, and the pellet washed in phosphate-buffered saline (PBS), pH 6.9, and fixed in acetone : ethanol (1 : 1) at 4° C for 1 h prior to rehydration in 0.25 *M* sucrose, 5 m*M* magnesium chloride, 20 m*M* sodium chloride pH 6.4 (SMT). After RNA digestion (RNase, 1 mg/ml SMT, pH 6.4, 37° C, 40 min), cellular DNA was stained with propidium iodide (50 µg/ml, 30 min, 4° C). Intracellular fluorescence was measured on a modified flow cytofluorimeter [9] with an excitation wavelength of 488 nm and an emission wavelength of 600 ± 20 n*M*. Histograms for the vincristine dose response were then normalised to the same total number of cells observed, i.e., 10,000.

Analysis of intracellular DNA distributions. Analysis of the histograms was based on the method of Dean and Jett [4] and Fried [5]. This involves the fitting of a normal curve to the G1 peak, another to the G2 + M peak, and between one and 10 such curves to the S peak, according to the formula:

$$y(x) = \sum_{i=1}^{n} \left[\frac{A_i}{\sigma_i \sqrt{2\pi}} \cdot e^{-\frac{1}{2}} \cdot \left(\frac{x - \mu_i}{\sigma_i} \right)^2 \right],$$

where $i = 1$ corresponds to the G1 peak, $i = n$ corresponds to the G2 peak, A_i is the area under the ith curve, μ_i is the mean of the ith curve, σ_i is the standard deviation of each curve, and $y(x)$ is the number of cells in channel x. A least-squares method was used to solve the equation. All calculations and curve fittings were carried out on a Hewlett-Packard system 45 microcomputer. The coefficient of variation (c.v.) of the data was found by calculation of μ_i from the G1 peak. An estimate of the standard deviation (σ_1) of this peak was obtained by taking the left-hand half width at 0.6065 of the peak height. The c.v. could then be calculated from the relationship: c.v. = $\sigma_{1/\mu 1}$. Alternatively, where the G1 component was small or absent the c.v. was found from the G2 + M fractions, using the

half width at 0.6065 on the right-hand side of the peak maximum. For assessments of material showing higher fluorescence than G2 + M, the total area of the histogram was calculated and the area of fluorescence greater than the G2 peak was expressed as a percentage of the total.

Chromosome analysis. The parental L1210 and L1210/R7A lines were treated for 48 h with 10^{-7} *M* vincristine prior to the preparation of chromosome spreads as described in the previous paper [14].

Results

Growth inhibition

The growth inhibition curves of L1210 and L1210/R7A cells following 24 h exposure to vincristine are shown in Fig. 1 and represent aliquots taken from the flow cytofluorimetry dose-response experiments. The ID_{50} (that dose resulting in 50% growth inhibition following 1-day treatment) of the parent cell line is 1.15×10^{-8} *M* vincristine and corresponds very closely to that of the LD_{50} obtained in the plating experiments (10-day treatment) described in the previous paper. The ID_{50} of the methotrexate-resistant cell line, 4.22×10^{-9} *M* was approximately 54% of the LD_{50} obtained in the previous plating experiment, however, representing a ratio of 2.7, which is significantly greater than that observed in the colony-forming experiments (1.3). The treatment given in the present study, however, was shorter (1 day) than in the plating experiments previously reported (10 days) to allow any cell-cycle perturbations to be seen.

Flow cytofluorimetry

Dose-response studies. The effect of vincristine at different dose levels on the propidium iodide fluorescence distribution is shown in Fig. 2. The histograms in this figure represent the pattern obtained from 10,000 cells in each case, plotted with identical axes parameters to allow a direct visual comparison.

Good definition of G1, S, and G2 + M phases of the cell cycle can be observed in the control (untreated) cells (Fig. 2, A and I). The proportions of cells in each of the phases of the cell cycle after treatment with varying doses of vincristine are shown in Table 1. Due to the problem of separating the late S phase from the G2 + M phase of the cell cycle in grossly perturbed systems to computer-derived values for the proportion of cells in S and G2 + M have been combined. Coefficients of variation of between 2% and 5% were obtained from the data.

In the L1210 cell line only minimal or no cell-cycle perturbations can be observed at doses up to 4×10^{-9} *M*

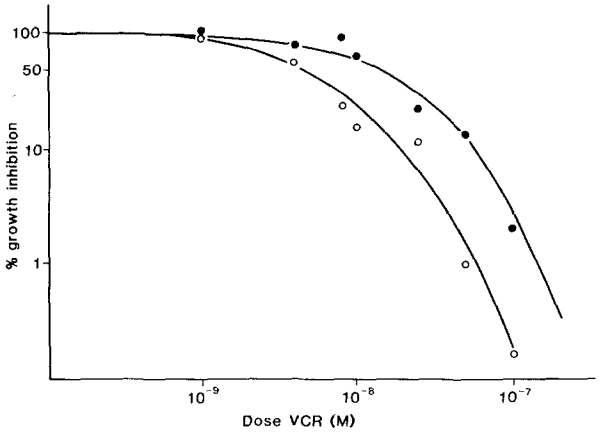

Fig. 1. Growth inhibition curves of L1210 (●———●) and L1210/R7A (○———○) cell lines following 24 h treatment with vincristine

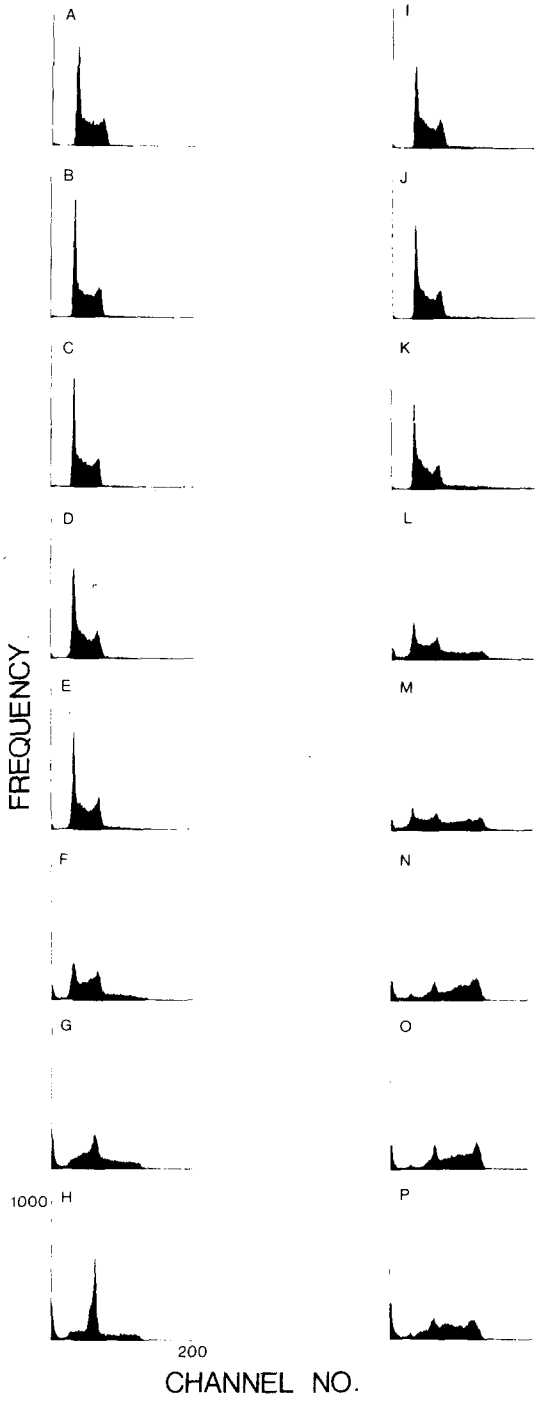

Fig. 2. Flow cytofluorimetric DNA fluorescence histograms following treatment of L1210 (A–H) and L1210/R7A (I–P) cells with vincristine: A + I Control; B + J 10^{-9} *M*; C + K 4×10^{-9} *M*; D + L 8×10^{-9} *M*; E + M 10^{-8} *M*; F + N 2.5×10^{-8} *M*; G + 0 5×10^{-8} *M*; H + P 10^{-7} *M*

Table 1. The percentage distribution in the different stages of the cell cycle observed from analysis of DNA fluorescent histograms following treatment with various concentrations of vincristine

Cell line	Cell phase	Control	Concentration VCR (M)						
			10^{-9}	4×10^{-9}	8×10^{-9}	10^{-8}	2.5×10^{-8}	5×10^{-8}	10^{-7}
L1210	G_1	30 ± 3	34.5 ± 6	30 ± 0	39.5 ± 9	31 ± 1	23.5 ± 1.0	6 ± 3	4.5 ± 1
	S	56.5 ± 4	52.5 ± 5	56.5 ± 4	44 ± 6	50 ± 1	46 ± 3	94 ± 15	95.5 ± 4
	G_2 + M	14.5 ± 0.7	14 ± 1	13.5 ± 4	16.5 ± 4	14 ± 3	30 ± 4		
L1210/R7A	G_1	33 ± 4	34.5 ± 8	34.5 ± 2	26 ± 4	16.5 ± 2	7.5 ± 1	6 ± 0	8 ± 0
	S	45.5 ± 5	49.5 ± 9	49 ± 9	46.5 ± 4	55.5 ± 8	82.5 ± 5	43.5 ± 6	12 ± 2
	G^2 + M	21.5 ± 1	16 ± 1	16 ± 6	27 ± 0	27 ± 6			

vincristine, but at doses greater than this a build-up of cells in the 4n position is apparent. However, in the methotrexate-resistant cell line increases in the proportion of cells between the 2n and 4n positions (presumably S phase) are apparent for doses up to 10^{-8} M. In this case, at doses above 4×10^{-9} M vincristine a cell-cycle arrest at the 4n position becomes evident. Treatment of both cell lines with concentrations of vincristine resulting in such an arrest show fluorescence corresponding to DNA contents greater than expected from normal diploid cells (i.e., 4n). This effect is more apparent with the L1210/R7A cells, in which the higher fluorescence tends to reach a peak at 2.5×10^{-8} M vincristine and then decrease (Fig. 3).

Comparative vinca studies

The comparative effect of the vincristine with vinblastine and vindesine at 10^{-8} M for 22 h under the same culture conditions as above is shown in Fig. 4. It can be seen that the cell-cycle arrest in the G2 + M position is more pronounced in the L1210/R7A than in the L1210 cell line. At equivalent molar doses the effect was more pronounced with vinblastine than with vincristine; vindesine was intermediate between the two. The order of effect compares with the cytotoxic differences observed in the earlier study of the three vinca alkaloids.

Time-course studies

The time course of the effect of vincristine (10^{-7} M continuous exposure for up to 120 h) on the propidium iodide fluorescence in each of the two cell lines is shown in Figs. 5 and 6. It can be seen that the differential effect between the two cell lines is apparent at time points up to the 10 h sample. A gradual accumulation of cells with 4n levels of fluorescence can be observed in both the cell lines.

The rate of accumulation of 4n cells is greater in the L1210/R7A line. At 24 h and later continuous treatment with vincristine results in grossly perturbed systems (Fig. 6). However, the appearance of fluorescence at the 8n position becomes apparent in both cell lines, which from chromosome analysis is shown to be due to polyploidy. The median chromosome count in the L1210 was 38.2 ± 1.4 for the untreated sample and 71.2 ± 12.5 for the 48 h vincristine-treated sample. The corresponding values for the L1210/R7A line were 37.2 ± 1.5 and 80.5 ± 19.7, respectively. The L1210 line showed 12% diploid cells, the rest being tetraploid whereas in the L1210/R7A line there are no diploid, 92% tetraploid, and 8% greater than tetraploid cells. The data from flow cytometric analysis shows a larger proportion of cells with DNA contents greater than 4n in the L1210/R7A line

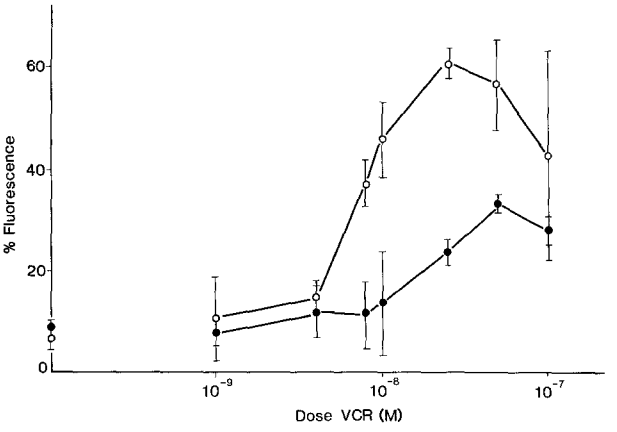

Fig. 3. Effect of vincristine concentration on percentage of total fluorescence appearing above the G2 peak in L1210 (●——●) and L1210/R7A (○——○) cell lines

Table 2. The proportion of cells exhibiting fluorescence greater than 4 n following exposure to the vinca alkaloids at 10^{-7} M for 48 h

Cell line		Percentage of cells greater than 4 n[a]	
		(Incl. < 2 n)	(Excl. < 2 n)
L1210	Control	5.38	6.08
	Vincristine	7.14	8.51
	Vindesine	8.85	10.65
	Vinblastine	17.6	27.4
L1210/R7A	Control	3.03	3.1
	Vincristine	22.3	37.3
	Vindesine	15.36	26.2
	Vinblastine	27.6	53.9

[a] In column headed 'Incl. > 2 n' a proportion attributed to debris is included; 'Excl. > 2 n' excludes this proportion

(Fig. 3, Table 2). Even though only a very small number of cells was available following 120 h continuous exposure a degree of fluorescence is observable at the 2n position, possibly reflecting a small subpopulation of surviving cells (Fig. 6, D).

Discussion

Possible modes of action of the vinca alkaloids other than those interactions associated with metaphase arrest have been suggested; for example, several groups have reported cytotoxic

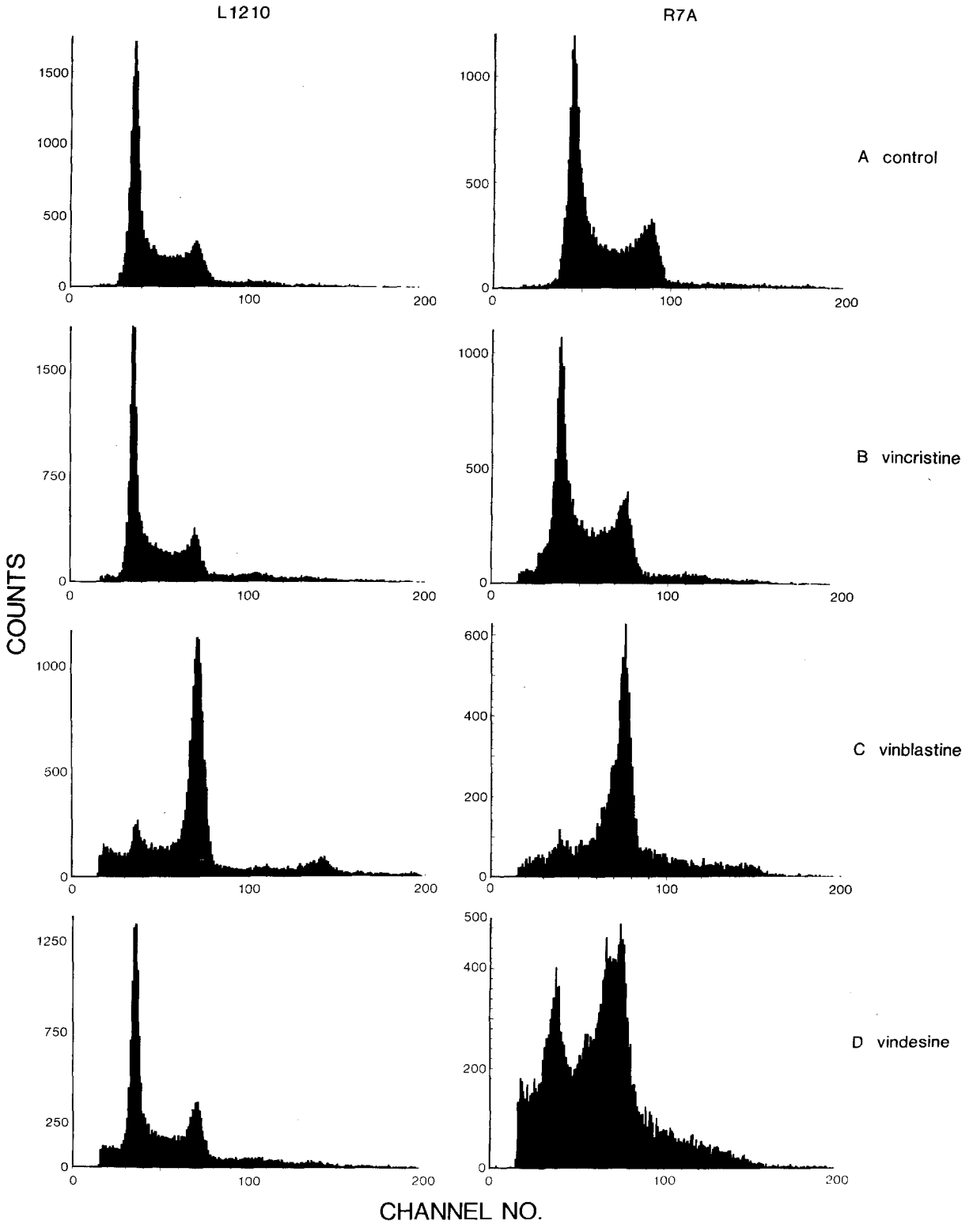

Fig. 4A–D. DNA fluorescence histograms following treatment of cells with 10^{-8} *M* vinca alkaloid

effects in the G1 and S phases of the cell cycle [16, 18]. It has also been suggested that the action of vinblastine and vincristine could be attributed to binding to tubulin in phases of the cell cycle other than metaphase, or to the existence of cytotoxic actions on other cellular processes [11]. Electron-microscope studies have indicated that vindesine has the same cellular action as vinblastine and vincristine in arresting P388 cells in mitosis, with blockage of production of the microtubules and precipitation of tubulin in the form of paracrystal [13]. The apparent order of three vinca alkaloids according to the number of paracrystals formed from the tubulin pool of P388 cells is vinblastine > vindesine > vincristine. However, it has been concluded that the small differences in the interaction of the three vinca alkaloids with

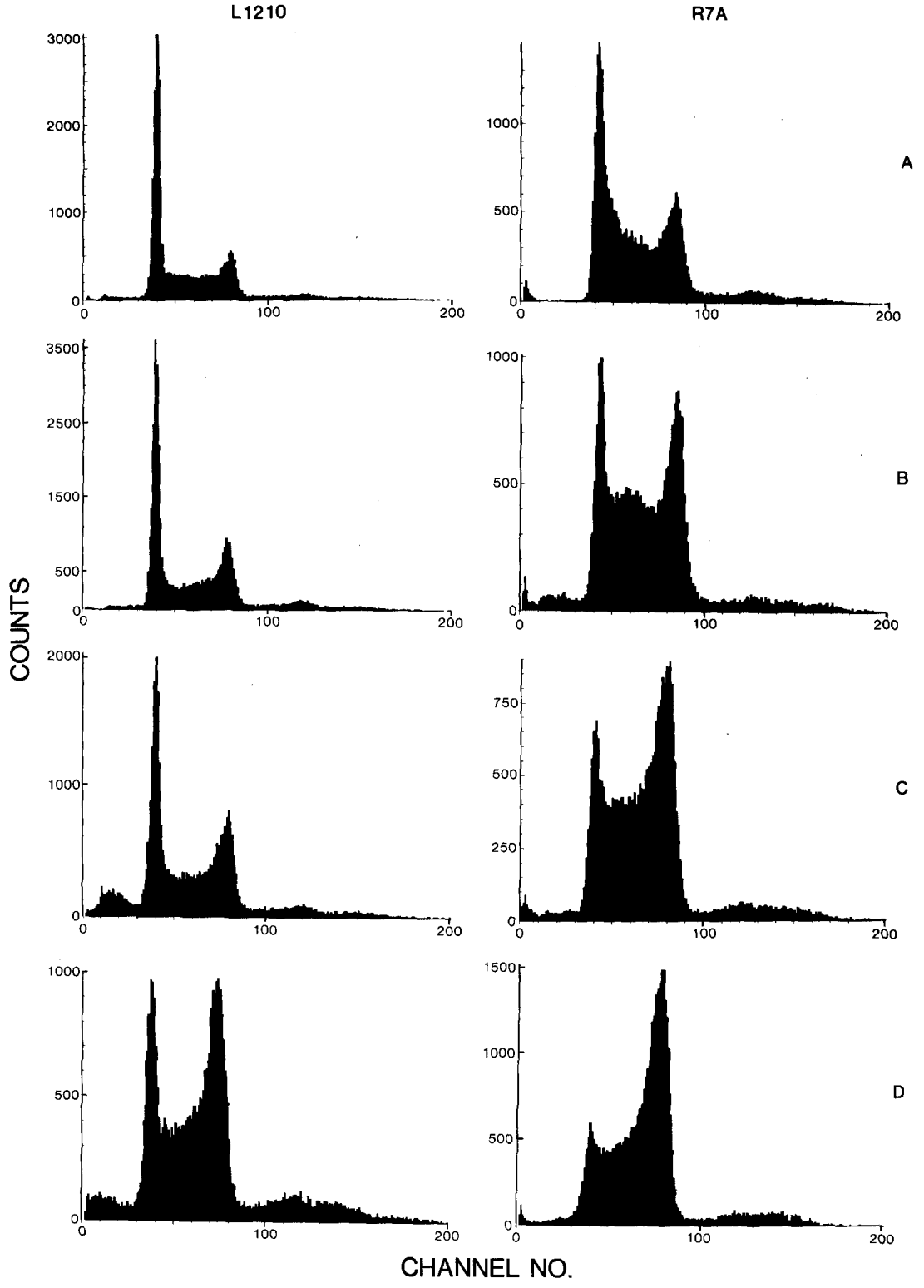

Fig. 5A–D. DNA fluorescence histograms following treatment of cells with 10^{-7} *M* vincristine for 1 h (**A**), 5 h (**B**), 7.5 h (**C**), 10 h (**D**)

the microtubules in vivo cannot explain their different chemotherapeutic effectiveness or toxicity [13].

The DNA distribution histograms obtained from the cytofluorimeter show dose-dependent perturbations in the cell cycle. In the methotrexate-resistant cell lines (Fig. 2) an increase in the proportion of cells in the S phase of the cell cycle can be seen at doses of vincristine up to 10^{-8} *M*. This may possibly demonstrate a cytotoxic action other than metaphase arrest. At higher doses, an increase in the proportion of cells containing 4n DNA is observed. This is in agreement with the data of other workers [1], who suggest that their findings were consistent with the kill of S-phase cells. However, in the original (sensitive) cell line no corresponding increase in the proportion of cells in the S phase is seen at doses up to 10^{-8} *M*.

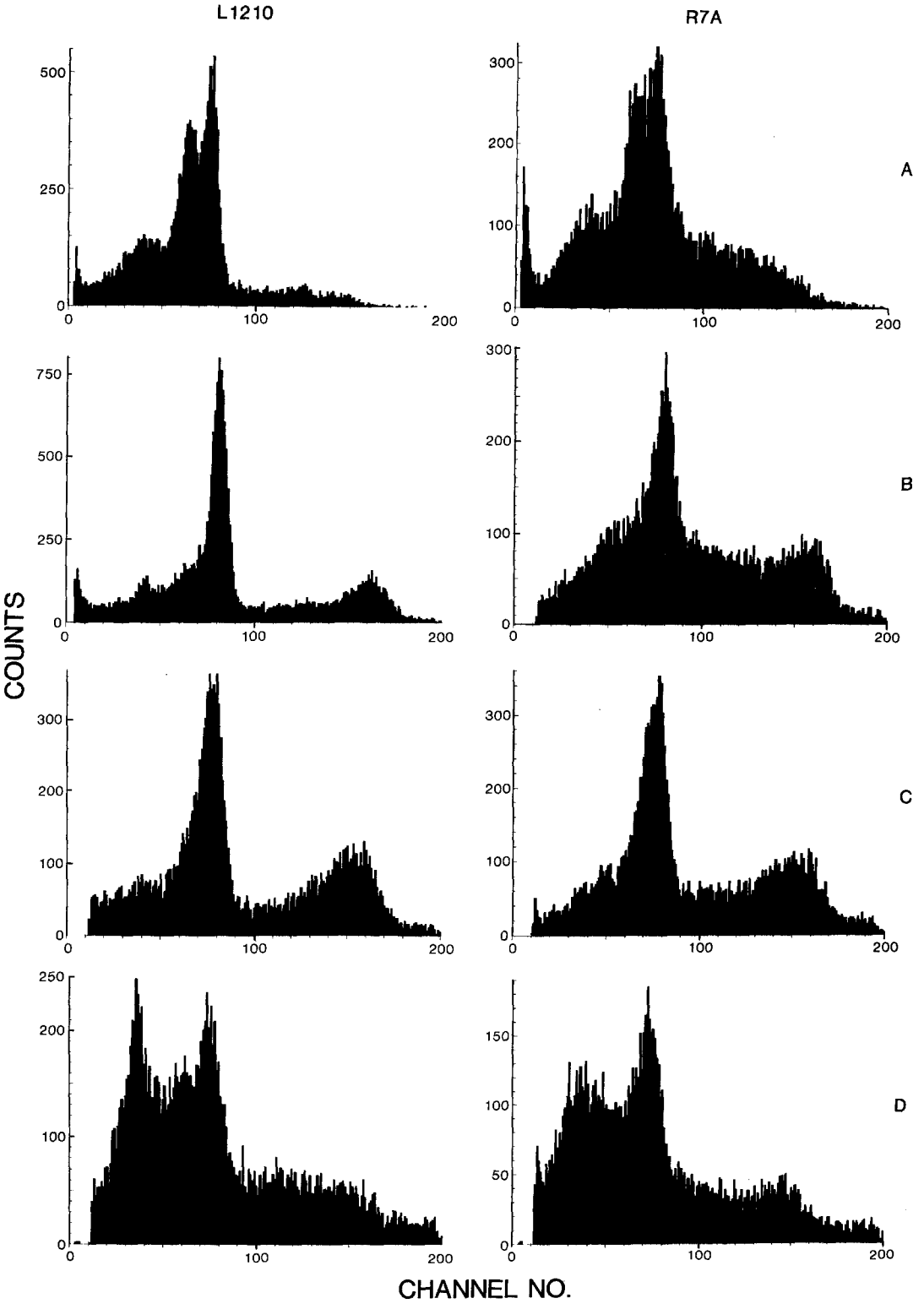

Fig. 6A–D. DNA fluorescence histograms following treatment of cells with 10^{-7} *M* vincristine for 24 h (**A**), 48 h (**B**), 72 h (**C**), 120 h (**D**)

Above this level a build-up at 4n is observed. At the ID_{50} levels for L1210 and L1210/R7A (1.15×10^{-8} *M* and 4.22×10^{-9} *M*) there is no significant difference in the flow cytometry histogram (cf. Figs. 2, E and K, respectively). The time course study with vincristine suggests that initial effects during the first cell cycle may not necessarily be lethal, as there is evidence of a surviving population of cells at time points after 24 h (Fig. 6).

In this study, cells are seen to accumulate with DNA contents greater than 4n to a greater level in methotrexate-resistant (L1210/R7A) line and this accumulation can be attributed to polyploidy. At equimolar concentrations this

effect is produced by the vinca alkaloids in the order vinblastine > vindesine > vincristine. This effect becomes more apparent with longer vincristine incubation times (Fig. 6).

Previous comparative studies in both clinical [12] and experimental [13, 17, 19] systems have shown differences in effectiveness between the vinca alkaloids. Furthermore, vinblastine again appears to be the most effective on a molar basis in the flow cytometric and chromosome studies. In a previous paper [14] the apparent collateral sensitivity of the methotrexate cells to the vinca alkaloids was partially explained by an increased intracellular accumulation of vincristine. However, no such increase was found for vinblastine. The initial build-up of cells in the S phase at low concentrations of vincristine, which is not observed with the sensitive cells, may suggest that the resistant cell line, although experiencing some difficulty in the organisation of the chromatin and subsequent orientation on the mitotic spindle, nevertheless succeeds in completing the process prior to division in the absence of vinca alkaloids. The longer doubling times of the methotrexate-resistant cell line is reflected in a relatively longer time spent in G2 + M. The sensitive cell line has a 11% G2 + M, i.e., 1.68 h of the 15.7 ± 0.6 h doubling time, whereas the methotrexate-resistant cell line has 15.4% G2 + M, i.e., 2.94 h of the 19.1 ± 0.3 h doubling time. In contrast, the relative time spent in G1 and S are similar. It is also interesting to note that other workers have reported longer doubling times for other methotrexate-resistant cell lines attributed to gene amplification [2, 8]. The present cell line (L1210 R7A) has been shown to have elevated levels of DHFR [15] together with HSRs, and for these reasons gene amplification is strongly suspected. It is possible, therefore, that in this case the suspected gene amplification may be responsible for the difficulty experienced by the L1210/R7A cells in traversing mitosis. Furthermore, a low level of vinca alkaloids may be capable of enhancing this difficulty and thereby interfering with the process more effectively in the resistant than in the sensitive line.

In conclusion, the cell-cycle perturbations occurring in methotrexate-sensitive and -resistant lines following vinca alkaloid treatment parallel the sensitivity difference reported in the earlier paper for this group of agents.

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