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SIMULTANEOUS RESISTANCE TO VINCRISTINE AND ADRIAMYCIN APPEARS AT HIGHER FREQUENCIES THAN TO VINCRISTINE AND ETOPOSIDE IN CHINESE HAMSTER OVARY CELLS

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SUMMARY: Efficacy of combination chemotherapy depends on the probability of a cell being resistant to at least two drugs simultaneously. In this study, we determined the frequencies of double drug resistance in the CHO AA8 cell line under combined exposure to vincristine plus adriamycin, vincristine plus etoposide, and adriamycin plus etoposide. These frequencies were compared to the expected frequencies which are the product of the independent frequencies observed for each drug alone. The results show a high frequency (up to 700-fold) of double resistance to adriamycin plus vincristine, a low increase (up to 30-fold) in frequency of resistance to vincristine plus etoposide, and an intermediate increase (200 to 300-fold) in frequency of cells resistant to adriamycin plus etoposide. The differences observed between combinations seem to be related, at least in part, to the mechanism(s) of resistance generally involved in the resistance to each drug: P-glycoprotein overexpression and/or DNA topoisomerase II alteration.

Cancer chemotherapy regimens generally involve more than one drug. Hence, drug resistance in tumors must develop simultaneously to at least two chemotherapeutic agents. A few reports have shown that, when DNA amplification is involved, observed frequencies of doubly resistant colonies are up to 260 times higher than that predicted from the two independent resistance frequencies (1,2). In both cases, increased enzymes expression via gene amplification was involved in the resistance to each drug: amplified DHFR and CAD genes in resistance to methotrexate and N-phosphonacetyl-L-aspartate (1), or DHFR and MDR genes in resistance to methotrexate and adriamycin (2), respectively. The main conclusion was that the emergence of doubly resistant cells is not the result of independent events (2) and a new phenotype characterized by an increased rate of DNA amplification (amplificator phenotype) has been proposed (1).

In this study, we addressed the question of whether such an increased frequency of doubly resistant cells occurs when the same basic process - i.e. DNA amplification - does not generally account for the resistance of at least one of the two drugs. For this purpose, we chose one drug of a series to which cells

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resist by a mechanism of resistance that does not involve a priori gene amplification, as resistance to this drug is generally related to qualitative alteration or decrased amount/activity of an enzyme. The other drug must select resistant cells in which other mechanism(s) of resistance, possibly involving gene amplification, occur.

We first singled out etoposide (ETO) among epipodophyllotoxin derivatives which are strong poisons of DNA topoisomerase II that have been shown to select resistant cells in which decreased amount (3-6) or qualitative alterations (7,8) of this enzyme were observed. Secondly, we chose vincristine (VCR), a Vinca alkaloid which binds tubulin. Resistant cells to this drug have been shown to exhibit altered molecular properties of its target (9,10) and, more frequently, an overexpression and amplification of MDR gene which codes for the drug efflux pump P-glycoprotein (PGP)(review in ref.11). In addition, we took in consideration adriamycin (ADR), an intercalative drug which poisons topoisomerase II and selects resistant cells in which both DNA topoisomerase II modifications (12,13) and amplification of MDR gene (11) have been found. Association of this drug with either ETO or VCR would give information about the involvement of common mechanisms of resistance in the appearance of doubly resistant cells.

MATERIALS AND METHODS

Cell line and culture. The parental cell line AA8 is a secondary clone isolated from SC1 Chinese hamster ovary cells (2). These cells were grown as described previously in detail (14). Plating efficiency of AA8 cells was 50 to 80%.

Drugs. Vincristine sulfate 1mg/ml aqueous solution (Oncovin^R, Lilly France S.A., Saint Cloud, France) was stored at 4°C. Adriamycin (Adriblastine^R, Laboratoire Roger Bellon, Neuilly-sur-Seine, France) was dissolved in water to 1mg/ml; aliquots stored at -20°C were thawed just before use. Etoposide, kindly provided by Dr C. Dubray, Laboratoires Sandoz, Rueil-Malmaison, France, was dissolved at a concentration of 10^{-2}M in dimethyl sulfoxide and aliquots stored at -20°C.

Determination of cell survival frequency. Dose dependent survival was determined by a colony formation assay under continuous exposure to the drugs during 8 days, as described previously in detail (14). In preliminary experiments, no inocculum effect was observed with none of the drugs used in this study when up to 1.8×10^4 AA8 cells/cm^2 were seeded. In the same experiment, cell survival frequency was determined at increasing concentrations of drug A only or in combination with two different concentrations of drug B, as described previously (2). For each couple of drugs, inverting A and B led to two symetrical experiments which were carried out three times. In these experiments, 60mm petri dishes were used and frequencies lower than 1×10^{-6} could not be determined because up to 1.2×10^6 cells were seeded for one determination. Additional experiments, in which up to 50×10^6 AA8 cells were seeded per determination (using 140mm petri dishes seeded at 2.5×10^6 cells) were carried out; 4 to 12 colonies were scored per determination. Colonies were stained with 0.2% aqueous crystal violet solution, washed and counted under a stereomicroscope. Special care was devoted to the morphology of the cells: colonies constituted of a majority of rounded or giant cells were not scored as survivors. Otherwise, only colonies containing 50 cells or more were scored.

Calculations on cell survival frequencies. Each experiment allowed to determine the observed frequencies of surviving cells for drug A only at increasing concentrations (upper curves of the figures), drug B only at two different concentrations (intercept of lower curves with ordinates) and associations of drugs A plus B (other points of lower curves). Expected frequencies for associations of drugs A plus B were calculated from the frequencies observed for drugs A or B only. The ratio:

$$\frac{\text{observed frequency (for drug A plus drug B)}}{\text{calculated frequency (frequency for drug A only x frequency for drug B only)}}$$

was then calculated.

RESULTS AND DISCUSSION

Each panel of Figure 1 shows the frequencies of surviving cells to one drug only (upper curve) or in combination with a second drug used at two different fixed concentrations (lower curves). Comparison of cell survival frequency of doubly treated cells to the expected frequency (calculated from the frequencies observed when cells were treated by each drug only) was made by calculating a ratio "observed frequency/calculated frequency" displayed in Table 1. The results of the various combinations may be compared as comparable range of frequencies were investigated in cells treated with one drug.

As shown in Figure 1A and B, double resistance to ADR plus VCR appeared at very high frequency with ratios reaching 500 to 700-fold (Table 1A and B, first two rows of each). Comparable increase has been observed when methotrexate was combined with N-phosphonacetyl-L-aspartate (1,15) or ADR (2)(see "Introduction"). In AA8 cell line, we have found that MDR gene amplification accounts, in most cases, for VCR and ADR resistance, as observed in several VCR and ADR resistant sublines isolated independently at frequencies of 10^{-4} to 10^{-6} (Souès S., Laval F., and Charcosset J.Y., manuscript submitted). Appearing at these frequencies, cells resistant to ADR plus VCR might exhibit an amplificator phenotype (1) resulting in co-amplification of MDR genes coding for active PGPs (16) which pump these drugs outside the cell (11). However, overexpression of only one PGP in transfectants can confere resistance to both ADR and Vinca alcaloids (17,18). The very high frequency of cells resistant to ADR plus VCR may alternatively result from overexpression of such a PGP.

In contrast to the results obtained when fixed concentrations of ADR were added to increasing concentrations of VCR (Figure 1B), adding fixed concentrations of ETO resulted in low increase of cell survival frequency of doubly treated cells (up to 28-fold, Table 1B, last two rows and Figure 1B'). Comparable results were observed when fixed concentrations of VCR were combined to increasing concentrations of ETO (Figure 1A' and Table 1C, last

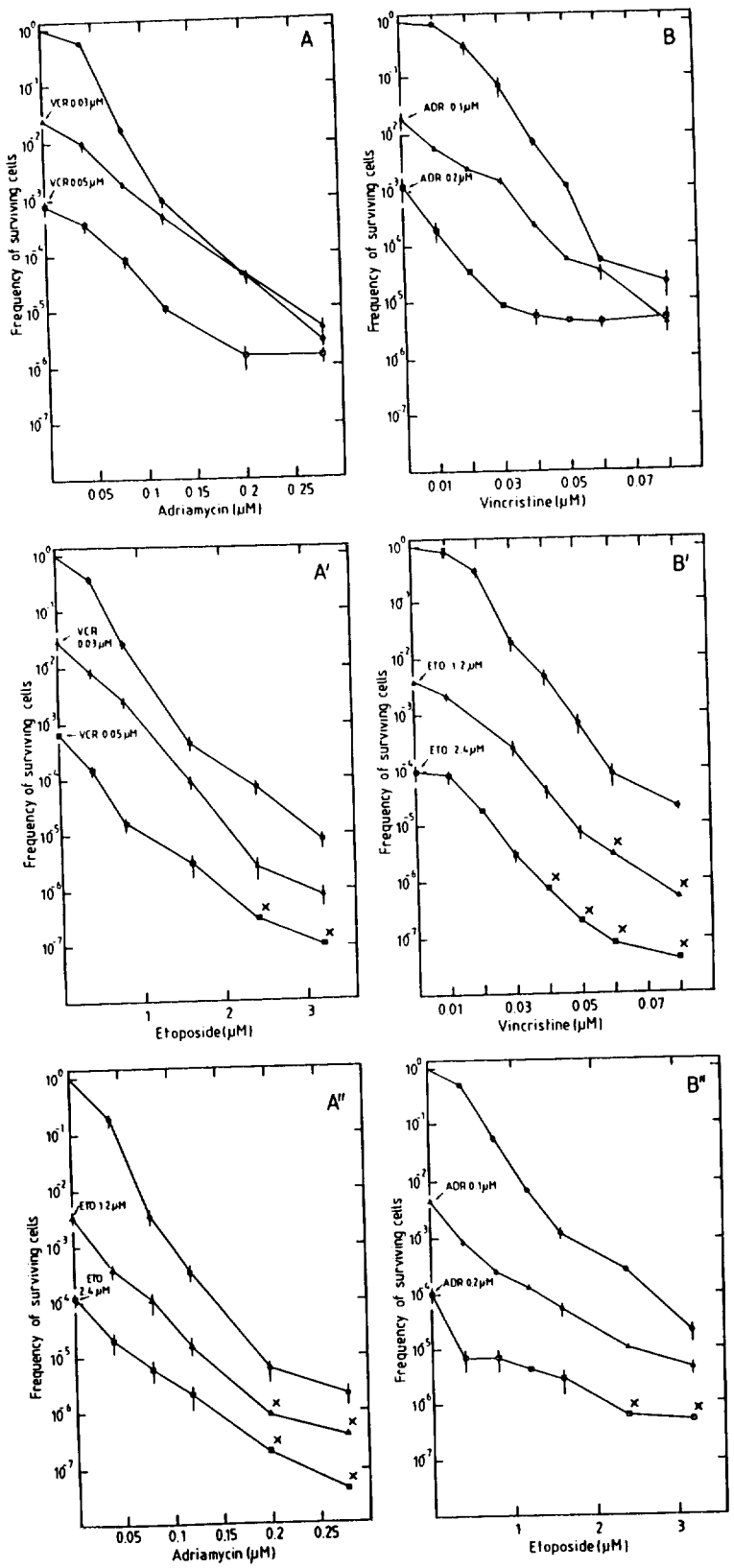
A
B
A'
B'
A''
B''
Frequency of surviving cells
VCR 0.03μM
VCR 0.05μM
ADR 0.1μM
ADR 0.2μM
ETO 1.2μM
ETO 2.4μM
Adriamycin (μM)
Vincristine (μM)
Etoposide (μM)

Table 1 - Ratios "observed frequency/calculated frequency of surviving cells" to the association of two drugs. A, B and C: panels in which ADR, VCR or ETO respectively were used at increasing concentrations in association with either VCR or ETO, ADR or ETO and ADR or VCR at two fixed concentrations. Ratio = observed frequency (drugs A plus B)/calculated frequency (drug A only x drug B only). See "Materials and Methods" for details.

A		ADR (μM)				
		0.04	0.08	0.12	0.20	0.28
VCR (μM)	0.03	0.7	5	20	40	70
	0.05	0.8	6.2	16	47	700
ETO (μM)	1.2	0.6	9	13	41	53
	2.4	0.9	15	50	292	212

B		VCR (μM)						
		0.01	0.02	0.03	0.04	0.05	0.06	0.08
ADR (μM)	0.1	0.3	0.2	0.9	1.7	2.3	3.6	10
	0.2	0.2	0.05	0.1	0.7	3.2	180	490
ETO (μM)	1.2	0.7	ND(1)	3.6	2.1	2.8	1	7.1
	2.4	1	1.2	1.7	1.8	3.4	11	28

C		ETO (μM)					
		0.4	0.8	1.2	1.6	2.4	3.2
ADR (μM)	0.1	0.4	1	4	9	9	46
	0.2	0.2	1.4	6.5	24	26	262
VCR (μM)	0.03	0.8	3.5	ND	7	1.3	3.6
	0.05	0.6	1	ND	10	7.2	21

(1) ND, not determined.

two rows). In the work of Souès et al. cited above, we also found that DNA topoisomerase II alteration is generally involved in ETO resistant cells as only one subline selected by ETO displayed very low level of MDR mRNA. On the other hand, VCR does not stabilize the cleavable complex with DNA topoisomerase II and ETO does not bind tubulin (19). An overexpressed PGP that can recognize both VCR and ETO might be involved in the increased frequency of doubly resistant cells to VCR plus ETO which otherwise are presumably double mutants (MDR plus DNA topoisomerase II) when selected at a frequency below 10^{-5} where a subpopulation of resistant cells is observed (20).

Figure 1 . **Top: association ADR plus VCR. Panel A**: ADR only at increasing concentrations (●) or in association with VCR 0.03μM (▲) or 0.05μM (■). **Panel B**: VCR only at increasing concentrations (●) or in association with ADR 0.1μM (▲) or 0.2μM (■). **Middle: association ETO plus VCR. Panel A'**: ETO only at increasing concentrations (●) or in association with VCR 0.03μM (▲) or 0.05μM (■). **Panel B'**: VCR only at increasing concentration (●) or in association with ETO 1.2μM (▲) or 2.4μM (■). **Bottom: association ADR plus ETO. Panel A"**: ADR only at increasing concentrations (●) or in association with ETO 1.2μM (▲) or 2.4μM (■). **Panel B"**: ETO only at increasing concentrations (●) or in association with ADR 0.1μM (▲) or 0.2μM (■). Surviving cells were revealed as colonies under continuous exposure to the drug(s) as described in "Materials and Methods". Each panel shows the results of three experiments in which determinations were made in triplicates excepted the points labelled with a cross which are the result of single determinations (see "Materials and Methods"). Bars, standard error of mean when greater than symbol size.

An increased frequency (200 to 300-fold) of cells surviving to ADR plus ETO was observed (Figure 1A" and B"; Table 1A, last two rows and Table 1C, first two rows). Such an increase may be due to common mechanisms of resistance to these two drugs: DNA topoisomerase II modification (3-6, 7,8,12,13) and possibly PGP overexpression (see above).

Beside the great increased frequency of double resistance to ADR plus VCR observed at the higher concentrations of VCR used, decreased frequencies were found at the lowest concentrations (Figure 1B and Table 1B, first two rows). In this case, 0.2μM ADR induces exponential cell killing when added to concentrations of VCR (0,1 and 0,2μM) to which the cells are not very sensitive. Such a decrease followed by an increase in frequency might be the consequence of different mechanisms which allow the cells to resist to drugs. Because the cells are continuously exposed to drugs, it is unlikely that cell survival is only related to the division probability as previously proposed after a single dose of radiation (21). Even more, while mechanisms of resistance of genetic origin are likely to occur at the highest frequencies in resistant cells (20), we may hypothesize that mechanisms of induction or of epigenetic origin (22) are involved at the lowest frequencies at which cells survive. Moreover, variation in frequencies of cells surviving simultaneously to two drugs, as a function of drugs concentrations, might be involved in conflicting results (synergy versus lack of synergy) found when ETO and cis-diammine-dichloroplatinum(II) were combined in different situations (23,24).

In conclusion, this study shows that the frequency of double drug resistance varies with the drugs involved in the combinations. Taking in consideration the mechanism(s) which generally account(s) for resistance to each drug, it is suggested that, in this study, increased frequency is a consequence of common mechanism(s) of resistance to the two drugs. Whether such hypothesis is valid has to be tested by selecting doubly resistant sublines in one-step selections. Studies are in progress to determine the mechanism(s) of resistance in doubly resistant cells to each drugs combination as such information may be important for the design of chemotherapeutic treatments in the clinic.

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