Acta Paediatrica Hungarica 32 (2), pp 223-231 (1991)

DECREASED SENSITIVITY OF CYTOSTATIC DRUGS IN GLUCOCORTICOID RECEPTOR-FREE ACUTE MYELOID LEUKAEMIA CELLS. CLINICAL AND EXPERIMENTAL OBSERVATIONS

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Received 4 Dec 1989

Preliminary clinical observations have suggested that low cellular glucocorticoid receptor (GR) levels might have been connected with multidrug resistance in children with acute myeloblastic leukaemia (AML). To test this possibility, we have developed glucocorticoid resistant subclones of two recently established human myeloid leukaemic cell lines. The cause of glucocorticoid resistance was GR negativity in these subclones. GR positive parent cell lines or GR negative subclones were incubated for 1 h in the presence of Adriamycin, Cytosine-arabinosid, Etoposide or Vincristine, respectively. After short-term (1 h) incubation in suspension cultures cells were washed and plated in clonogenic agar cultures. Each anticancer drug was more potent against both GR positive parent cell lines than against the GR negative subclones. The results of this study suggest that the absence of GRs is a useful marker of multidrug resistance in childhood AML.

INTRODUCTION

Drug resistance is a major reason for treatment failure in childhood acute myeloblastic leukaemia (AML) /8/. Mechanisms of resistance are not well understood /5/, although the overexpression of a plasma membrane glycoprotein, P-glycoprotein, has been observed in several multidrug resistant cell lines and clinical specimens including AML cells /1, 10/. On the other hand, Adriamycin resistance of the promyelocytic human leukaemia cell line HL-60 has been reported in the absence of detectable P-glycoprotein /11/. Recent clinical observations have suggested that glucocorticoid resistance might also have been connected with pleiotropic drug resistance

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in leukaemic children resulting in treatment failure /12/.

In the present study, glucocorticoid receptors (GR) of primary AML cells were investigated and GR level of the cells was related to remission induction. Moreover, in vitro sensitivity in two recently established human myeloid leukaemia cell lines, BRM and DD, and in their glucocorticoid resistant subclones, BRMd and DDd, was studied using 4 anticancer agents: Adriamycin (ADR), Cytosin-arabinoside (ARA-C), Etoposide (VP-16) and Vincristine (VCR).

MATERIALS AND METHODS

Chemicals. VP-16 and its solvent consisting of 150 mg benzyl-alcohol and 3250 mg polyethyleneglycol-300 in 5 ml destillated water was provided by Bristol Laboratories. RPMI-1640 fetal calf serum (FCS) and phosphate buffered saline pH 7.4 (PBS) were purchased from GIBCO. Lymphoprep was a product of Nycomed. (1,2,4 ^{3}H)-Dexamethason was made by Amersham. Agar was purchased by Difco. All other chemicals were purchased by Sigma. ADR and VCR were dissolved in ethanol-PBS at 1:1 v/v. ARA-C was dissolved in RPMI-1640. Solvents were present in control flasks at equivalent levels.

Patients. 10 children with AML participated in the study. Two patients were examined both at the onset of the disease and in relapse. Diagnosis was established according to FAB criteria /2/. All patients were treated according to the protocols of the Hungarian Leukaemia Study Group for Children.

Cell lines and culture conditions

BRM (CD13+, CD14+, CD33+, VIM2+, HLA-DR-, TdT-, myeloperoxidase-) and DD (CD24+, VIM2+, HLA-DR-, TdT, myeloperoxidase-) are recently established myeloid leukaemia cell lines. DD cells were provided kindly by Dr. G. Szegedi (Department of Internal Medicine III of the Medical University of Debrecen). Two variants, BRMd and DDd, resistant to growth inhibitory action of dexamethasone were isolated by culturing BRM cells for 62 subcultures and DD cells for 54 subcultures (3- to 4-day intervals), respectively, in the presence of increasing concentrations of dexamethasone /6/. Both parent cell lines and glucocorticoid-resistant variants were grown in plastic Petri dishes (Nunc) at 37 ^{o}C in a humidified atmosphere of 5% CO_2 in air in RPMI-1640 supplemented with 2mM L-glutamine, 100 U/ml Penicillin, 100 ug/ml Streptomycin and 10 % FCS (complete culture medium; CCM). Cells in exponential growth phase were used.

Dexamethasone binding assay

Glucocorticoid binding of whole cells was carried out with (1,2,4-^{3}H)-Dexamethasone as described previously /7/. Briefly, various mixtures of (^{3}H)-Dexamethasone and cold dexamethasone were added to 0.1 ml cell suspensions at 37 ^{o}C for 30 min.

After terminating the reactions by rapid cooling and washing in ice cold PBS, cells were resuspended in 5 ml of scintillation fluid. Radioactivity was measured in a Nuclear ISOCAP 300 radiospectrofluorimeter. All assays were outlined in triplicates. Specific dexamethasone binding was defined as the difference in radioligand binding in the absence and presence of unlabeled hormone. Binding sites per cell (GR level) were calculated according to Scatchard /13/ or to the abbreviated single point assay /4/ if the number of available cells was limited. Dissociation constants (K_D) were determined from the Scatchard graphs.

In vitro drug treatment. BRM, DD, BRMd and DDd cells at a density of $5x10^5$ cells/ml in CCM were exposed to various concentrations of anticancer agents for 1 h at 37 ^oC in 5 % CO_2.

Colony-forming assay. After short-term (1 h) incubation, cells were washed twice in RPMI-1640, counted and subsequently plated at $5x10^2$ cells/ml in 0.3 % agar medium containing RPMI-1640 supplemented with L-glutamine, antibiotics and 20 % FCS. Cultures were incubated for 7 days at 37 ^oC in a humidified atmosphere of 5 % CO_2 in air. Colonies containing more than 15 cells were counted using an inverted microscope (Leitz). Cytotoxicity was determined by comparing colony counts from drug-treated cells with counts from cells incubated only with solvent (controls) in per cent of controls.

Statistics. Agar cultures were performed in triplicates. Mean $\pm$ S.D. of two to three repeated experiments were presented. Group data were compared by Student's t-test after checking the normal distribution of the data by Geary's test and comparing S.D.-s by the t-test.

RESULTS

GRs and treatment results in AML patients. GRs were detected in each primary AML cases (Table I). Patients had GR levels ranging between 114 to 10763 (mean$\pm$S.D.:4623$\pm$3566), while values of control subjects scattered between a remarkably narrower range: 1027 to 9311 (mean$\pm$S.D.:2523$\pm$1646), as determined previously /7/. K_D values of patients were in the nanomolar range as in control persons. Two patients were investigated in relapse (cases N^o 2/b and 5/b in Table I). However, both relapsed patients exhibited very low GR levels. Less than 1000 GRs/cell were observed in three cases (cases N^o 2/b, 5/b and 10 in Table I). These cases did not respond to antileukaemic treatment protocol, while remission induction was successful in 8 out of 9 cases exhibiting more than 1000 GRs per cell.

TABLE I

Glucocorticoid receptors (GR) in acute myeloid leukaemic children

	GR/cell	K_D (nM)		Remission induction	Outcome of the disease
1.	10763	5.7	M5b	successful	2nd CCHR
2/a.	6772	-	M2	successful	relapsed
2/b.	250	-		unsuccessful	died
3.	6474	-	M4	successful	died
4.	6330	-	M6	unsuccessful	died
5/a.	5833	-	M1	successful	relapsed
5/b.	0	-		unsuccessful	died
6.	3416	-	M1	successful	died
7.	2261	-	M1	successful	died
8.	1176	10.3	M1	successful	died
9.	1000	-	M1	successful	died
10.	114	-	M1	unsuccessful	died

CCHR denotes complete clinical and haematologic remission

In vitro anticancer drug sensitivity of GR positive lines and GR negative subclones

BRM and DD cells were characterized by a high number of GRs, 15476 and 7270 GR/cell respectively. K_D values were 5.4 nM in case of BRM cells and 13.4 nM in case of DD cells. BRMd and DDd cells which were resistant to growth inhibitory action of dexamethasone, did not have specific dexamethasone binding sites, i.e. GRs.

The effect of the anticancer drugs on colony formation of glucocorticoid-sensitive and -resistant cell lines were summarized in Fig. 1 and 2. Each drug caused a concentration dependent inhibition of colony formation of the investigated cell lines. ADR and VP-16 were proven to be the most effective agents used in pharmacologic concentrations /3, 14/. VCR was moderately inhibitory. ARA-C was the least effective in these cell lines. Identical concentration of each four drugs resulted in significantly greater decrease in colony formation of BRM and DD cells than of BRMd and DDd cells.

DISCUSSION

Low GR level is a proven unfavourable prognostic factor in childhood acute lymphoid leukaemia (ALL) /9/. In our previous study we have chosen the arbitrary value of 1000 GR/cell in childhood ALL /7/. All children having less than 1000 GR/cell exhibited a significantly worse outcome of the disease than patients with GR levels between 1000 to 10000 /7/. The observations made by AML children suggest that less than 1000 GR/cell may be a useful marker of poor disease outcome in childhood AML as well. Because of the limited number of patients we did not perform statistical evaluation. In order to study the possible connection between low cellular GR level and multidrug resistance in AML, as suggested by the clinical observations, an in vitro model was developed using glucocorticoid sensitive, GR positive human myeloid leukaemia cell lines and their glucocorticoid resistant, GR negative

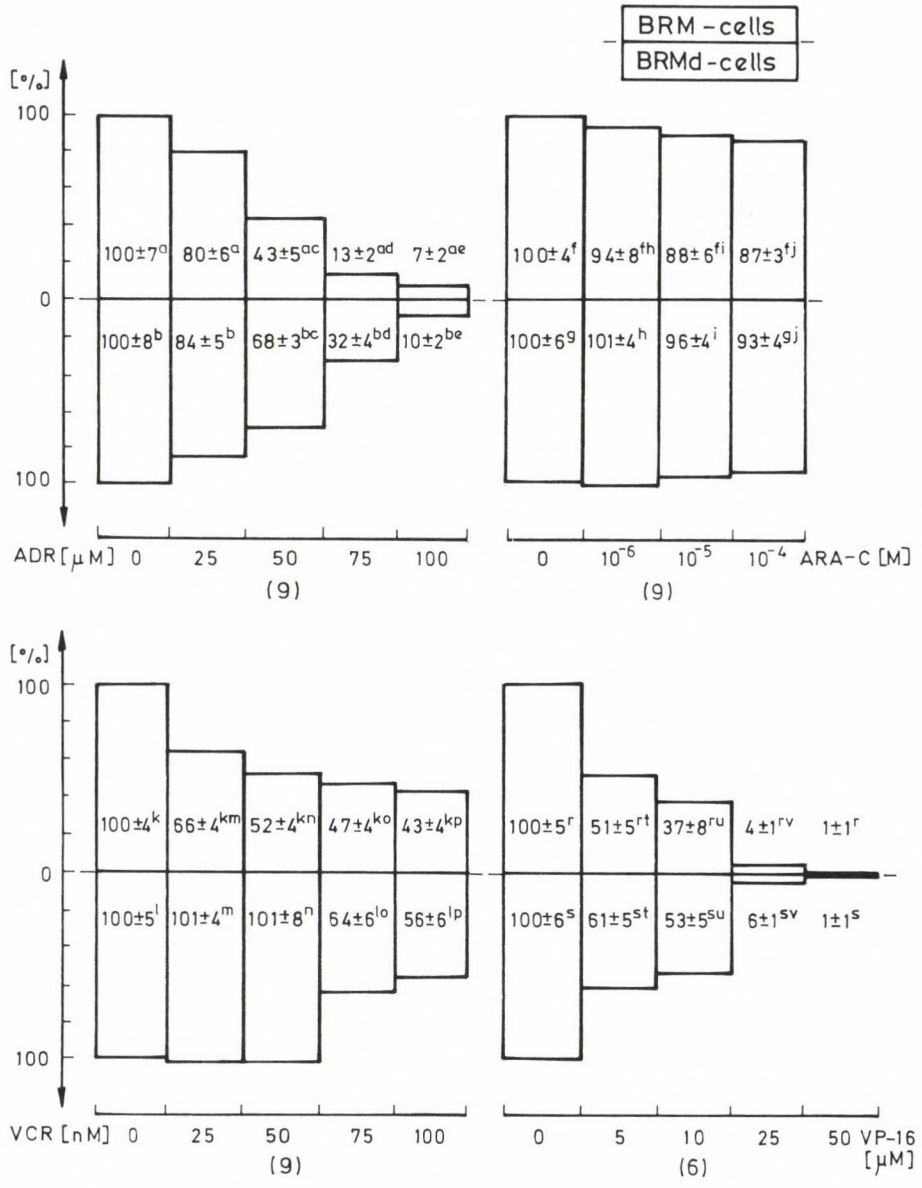

Fig. 1. Effect of antileukaemic drugs on colony formation of BRM and BRMd cells.
Mean ± S.D. values of relative plating efficiencies are given in per cent.
$^{a-v}$indicate statistically significant differences between the corresponding groups

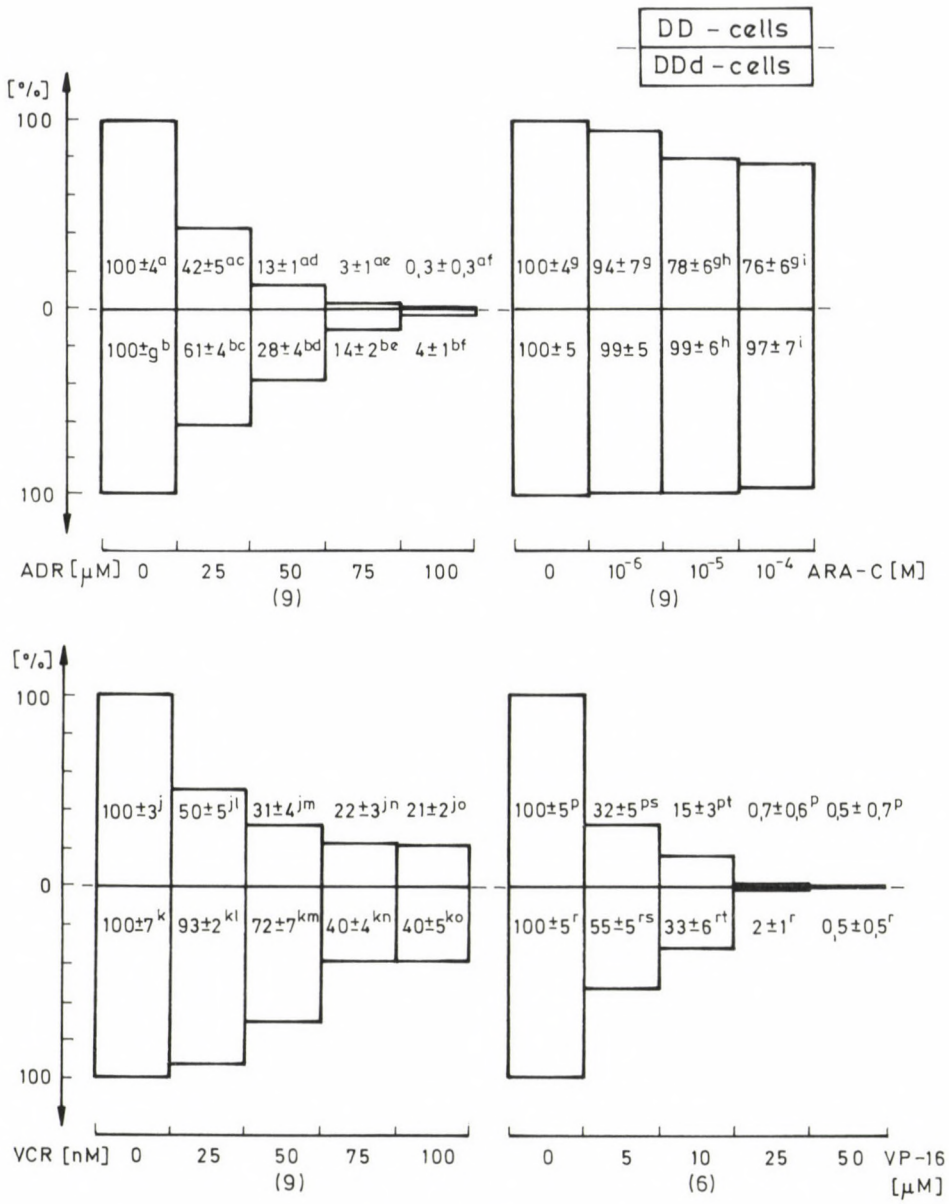

Fig. 2. Effect of antileukaemic drugs on colony formation of DD and DDd cells.
Mean ± S.D. values of relative plating efficiencies are given in per cent.
$^{a-t}$indicate statistically significant differences between the corresponding groups

subclones. In vitro clonogenic assay was performed after short-term incubation with ADR, ARA-C, VP-16 and VCR. Each investigated anticancer drug was significantly less toxic against the GR negative subclones than against the GR positive parent cell lines.

Our results suggest that glucocorticoid resistance caused by the absence of or decrease in the cellular GR content of AML cells may be a useful indicator of pleiotropic drug resistance.

ACKNOWLEDGMENT

C.K. was supported partially by the University of Ulm, F.R.G.

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