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Susceptibility of Multidrug-resistant Human Leukemia Cell Lines to Human Interleukin 2-activated Killer Cells

Astrid Kimmig, Volker Gekeler, Manfred Neumann, Gerd Frese, Rupert Handgretinger, Gabriella Kardos, Heyke Diddens, and Dietrich Niethammer[1]

Department of Hematology and Oncology, Children's University Hospital [A. K., R. H., G. K., D. N.], and Physiological Chemistry [V. G., G. F., M. N.], University of Tuebingen, Tuebingen, and Medical Laser Center, Luebeck [H. D.], West Germany

ABSTRACT

Considering the possibility to overcome drug resistance by other treatment strategies than chemotherapy we investigated the susceptibility of three independently selected multidrug-resistant sublines of the T-lymphoblastoid leukemic cell line CCRF-CEM to lymphokine-activated killer (LAK) cells. We found that two of the multidrug-resistant sublines were significantly less susceptible targets to LAK cells. A third one, however, was as susceptible as the parental CCRF-CEM cell line. Moreover, a multidrug-resistant subline that reverted to an almost drug-sensitive phenotype was observed to be also revertant for resistance against LAK cells. We found an inverse relationship between the expression of the *mdr*1 gene (P-glycoprotein) and the susceptibility to LAK cells. Verapamil, a calcium channel blocker, while increasing the drug sensitivity of a multidrug-resistant subline, did not induce a reversal of the suppression of LAK susceptibility. The possibility of enhanced resistance to LAK cells of multidrug-resistant cells should be taken into account when one is looking for therapy strategies to overcome multidrug resistance.

INTRODUCTION

Drug resistance, often acquired during therapy and followed by failure of chemotherapy, is a serious problem in the treatment of malignancies. One way to overcome this problem could be an immunotherapeutic approach. Immunocompetent cells are able to kill malignant cells (see for review Ref. 1). In several murine tumor models adoptive immunotherapy could be proven to be effective in tumor therapy (2) and currently clinical studies are being carried out. The results are still limited so far, but some of them should encourage further research (3-5).

A decreased number and activity of natural killer cells in leukemia patients during active disease (6-9) were reported, but this deficiency could be corrected by Il-2[2] *in vitro* (8, 10, 11). The experimental data on LAK cells suggest that adoptive immunotherapy may be effective and useful for treatment of leukemia (11, 12). Most of the patients who are treated by immunotherapy have previously received a variety of chemotherapeutic agents, and have developed resistance to the conventional therapy. A few reports about the development of MDR (see for review Refs. 13 and 14), together with the amplification of the *mdr*1 gene and the expression of the P-glycoprotein in adult patients (15, 16) and children (17) suffering from leukemia have been published recently. It seems to be important to know more about the relationship between MDR and the susceptibility to immunocompetent cells. There are already a few investigations about this problem with different results. Two investigations showed MDR cells to be less susceptible targets to NK cells (18, 19); one of them reported an inverse relationship between the P-glycoprotein expression and the susceptibility to NK-like cells (18). In contrast, three studies using the same drug-resistant cell line did not find any difference in LAk cell or activated-monocyte-mediated lysis of drug-resistant cells (20-22). In order to get more information about the susceptibility of MDR leukemic cells to LAK cell-mediated lysis, we compared three MDR sublines selected with different chemotherapeutic agents and a revertant MDR cell line with the parental drug-sensitive cell line.

MATERIALS AND METHODS

Leukemic Cell Lines. We used the human T-lymphoblastoid cell line CCRF-CEM (ATCC CCL 119, purchased from the American Type Culture Collection, Rockville, MD). The cells were exposed initially to a drug dose that represented approximately the ID_{50} in order to select a drug-resistant subline. Then the drug dose was increased stepwise in regard to the cell proliferation. No further mutagenic agent was added. The following multidrug-resistant sublines were used: CCRF ACTD400, selected by actinomycin D, CCRF ADR5000, selected by Adriamycin, and CCRF VCR1000, selected by vincristine. The numbers indicate the amount of drug in ng/ml continuously present in the culture medium for each subline. If a subline was transiently cultured without the selecting agent, this fact was expressed by adding a "−" sign. A drug treatment of sublines after a period of drug-free culturing was illustrated by a "−+" sign. In addition a subline designated CCRF ACTD (REV) was used which has lost the MDR phenotype almost completely after culturing without the selecting agent for 17 months (23, 24).

Plasma Membrane Preparation, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, and Western Blotting. At least 5×10^7 cells were used for the preparation of a plasma membrane-enriched microsomal fraction from disrupted cells by differential centrifugation according to the method of Gerlach *et al.* (25). The protein content of the samples was determined by the method of Bradford (26) with bovine serum albumin as a standard. For Western blotting, 35 μg protein/lane were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a modification of Fairbanks' technique, as described (25, 27); in modification of this method we used 7% acrylamide gels.

Electrophoresis was carried out at constant current at 4 mA/cm^2 for 16 h. Proteins were transferred onto Immobilon -P (Millipore, Bedford, MA) transfer membranes by electroblotting at constant current at 0.8 mA/cm^2 for 1.5 h using the NovaBlot (Pharmacia LKB, Freiburg, West Germany) electrophoretic transfer unit as described by the suppliers.

The Western blots were probed for P-glycoprotein by using the polyclonal antibody mdr (Oncogene Science, Manhasset, NY) or the monoclonal antibody C 219 (Centocor, Malvern, PA) as recommended by the supplier. The filters were washed three times for 5 min at room temperature with a solution of 1% dry milk powder in phosphate-buffered saline and then incubated for 1 h with the same solution containing 0.3 μ Ci/ml ^{125}I-protein A (specific activity, >30 mCi/mg; Amersham, Braunschweig, West Germany). Thereafter the filters were washed three times for 5 min with phosphate-buffered saline at room temperature, dried, and placed on Amersham MP X-ray film.

Estimation of Drug Sensitivity. The "relative resistance" was defined

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[1] To whom requests for reprints should be addressed, at Children's University Hospital, Ruemelinstrasse 21-23, D-7400 Tuebingen 1, West Germany.

[2] The abbreviations used are: IL-2, interleukin 2; MDR, multidrug-resistant or multiple drug resistance; ACTD, actinomycin D; ADR, Adriamycin; VCR, vincristine; NK, natural killer; LAK, lymphokine-activated killer; ID_{50}, 50% inhibition dose.

as the ratio of ID_{50} values of the resistant subline and the parental line. ID_{50} values were determined by the evaluation of cell growth during 72 h as described elsewhere (28). In addition we determined the interference of actinomycin D with RNA biosynthesis and of Adriamycin with DNA biosynthesis by determining the incorporation rates of [^{3}H]uridine or [^{3}H]thymidine, respectively, as described (23).

Effector Cells. Forty-three blood samples were taken from 25 healthy volunteer donors. Peripheral blood mononuclear cells were separated by a standard Ficoll-Hypaque technique (Lymphoprep, Nycomed, Oslo, Norway). The cells were washed twice and resuspended in RPMI 1640 [supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and antibiotics, Seromed, Berlin, West Germany] and adjusted at 1–2 × 10^6 cells/ml; 1000 IU/ml recombinant IL-2 (Bioferon, Laupheim, West Germany) was added and the cells were incubated for 3–4 days at 37°C and 5% CO_2 atmosphere. These cells are referred to as LAK cells. Two of the 25 donors failed to generate LAK activity.

Cytotoxicity Assay. To determine LAK cell activity we used a standard ^{51}Cr release assay by using 5 × 10^3 ^{51}Cr-labeled target cells and effector:target ratios ranging from 40:1 to 2.5:1. The test was done in triplicate and SEM of triplicates was <5%. Spontaneous release (SR, determined by adding medium alone) ranged from 8 to 19%; there was no difference between the various sublines. Total release (TR) was determined by adding 0.1 *n* HCl to the tumor cells. Specific lysis was determined according to the following formula

$$\frac{ER - SR}{TR - SR} \times 100\%$$

where ER is experimental release.

For cold target competition unlabeled target cells were added to the labeled target cells in ratios ranging from 200:1 to 1.5:1. SR was not altered by addition of the unlabeled target cells.

Five to 8 days before starting the cytotoxicity assay, tumor cells were washed and allowed to grow in drug-free medium in order to exclude any influence of the drug itself (29, 30). One day prior to the assay tumor cells were adjusted to 1 × 10^6 cells/ml to allow comparable and optimal growth conditions for the different assays.

In some experiments verapamil (5 μM) was added either 3 days before and during the cytotoxicity assay or only during the 4-h ^{51}Cr release.

Immunophenotyping. Immunophenotyping was done in a standard manner by using monoclonal antibodies purchased from Becton Dickinson, Mountain View, CA. All antibodies were directly fluorescence marked and analysis was done using a FACS scanner (Becton Dickinson).

Statistical Analysis. We used the two tailed Student's *t* test for statistical analysis. Therefore we compared the specific lysis of the differently selected sublines obtained by the same donor in one experiment; 4 to 26 donors were tested in one statistical analysis; data were regarded as significant for $P < 0.05$.

RESULTS

Characterization of Differently Selected Multidrug-resistant Leukemia Cell Lines. The data for cross-resistances to a variety of drugs are summarized in Table 1 [as previously published (17)]. A detailed characterization of the MDR cell lines used in this work will be published elsewhere.[3,4] Each of the resistant sublines shows amplification of the *mdr*1 gene[3] and a distinct expression of the P-glycoprotein (Table 6). The pattern of resistances, however, is quite different for various sublines.[3] The P-glycoprotein expression, *mdr*1 gene amplification, and the degree of drug resistance to one specific drug do not correlate in a simple manner. The level of drug resistance was not substantially altered by culturing the cells in drug-free medium for up to 8 days; only a slight increase of drug sensitivities was noted (data not shown).

Table 1 *Multiple drug resistance of CCRF-CEM sublines*

Relative resistance was determined as the ratio of ID_{50}subline/ID_{50}CCRF-CEM. ID_{50} values were derived from the evaluation of cell growth after 72 h.

Subline	Relative resistance (-fold)			
	Actinomycin D	Adriamycin	Vincristine	VM26
CCRF ACTD400	571	71	2831	83
CCRF VCR1000	102	90	1760	26
CCFR ADR5000	1107	846	1692	249
CCRF ACTD (REV)	12	42	107	ND[a]

[a] ND, not done.

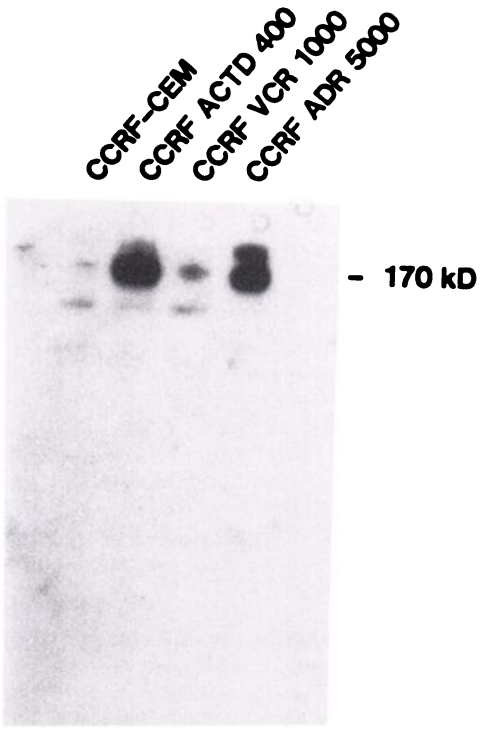


Fig. 1. P-glycoprotein expression in CCRF-CEM and three drug-resistant sublines determined by Western blot analysis. The CCRF VCR1000 signal intensity was set at setting 1. The signal intensities of the other cell lines were: CCRF-CEM, 0.6-fold; CCRF ACTD400, 27-fold; and CCRF ADR5000, 21-fold (major signal). The signal of a smaller protein (M_r 125,000) and the signal of a M_r 170,000 (*170 kD*) species in CCRF-CEM were not seen when the monoclonal antibody C219 was used.

Fig. 1 shows the expression of the P-glycoprotein of the different sublines as analyzed by immunoblotting. The Western blot as shown in Fig. 1 was probed with the polyclonal antibody mdr. CCRF-CEM and CCRF VCR1000 (arbitrarily set at setting 1.0) sublines show a very low signal while CCRF ADR5000 (2-fold) and CCRF ACTD400 (27-fold) sublines show a high and almost similar signal of P-glycoprotein in the plasma membrane fraction. A second protein with a lower molecular weight (M_r 125,000) was recognized by the polyclonal antibody mdr, but not by the monoclonal antibody C219 (Fig. 2).

Fig. 2 shows one representative analysis of several Western immunoblotting experiments using the monoclonal antibody C219. Material from CCRF ACTD (REV) cells shows low P-glycoprotein levels. The expression of P-glycoprotein after an 8-day culturing period in drug-free medium is rather stable; no

[3] V. Gekeler, G. Frese, H. Diddens, M. Neumann, E. Daumiller, and H. Probst. Multidrug-resistant human leukemia cell lines exhibiting drug mediated induction of the *mdr*1 P-glycoprotein gene, manuscript submitted for publication.

[4] E. Daumiller, V. Gekeler, S. Weger, H. Probst, D. Niethammer, and H. Diddens. Chromosome 7 aberrations in multidrug-resistant human leukemia cell lines, manuscript in preparation.

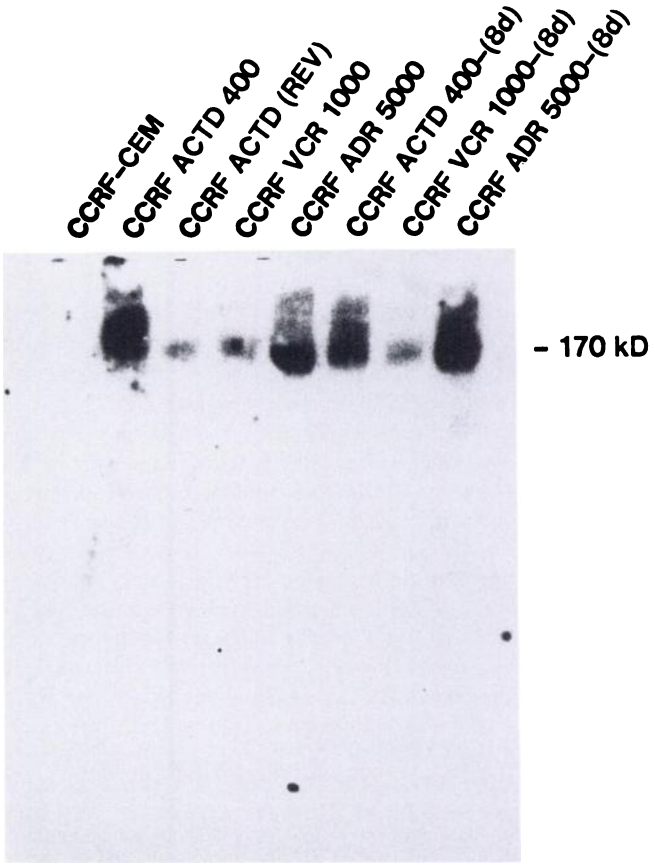


Fig. 2. P-glycoprotein expression in CCRF-CEM and the MDR sublines determined by Western blot analysis by using the monoclonal antibody C 219. No P-glycoprotein specific signal could be seen with material from the parental subline CCRF-CEM. The P-glycoprotein expression of the MDR sublines cultured continuously in the presence of the selecting drug is compared to the P-glycoprotein expression shown by the same cell lines cultured in drug-free medium for 8 days (indicated by a "−(8d)" sign). No distinct changes of the P-glycoprotein expression were seen. CCRF ACTD (REV) cells show only a weak signal for P-glycoprotein. *170 kD, M,* 170,000.

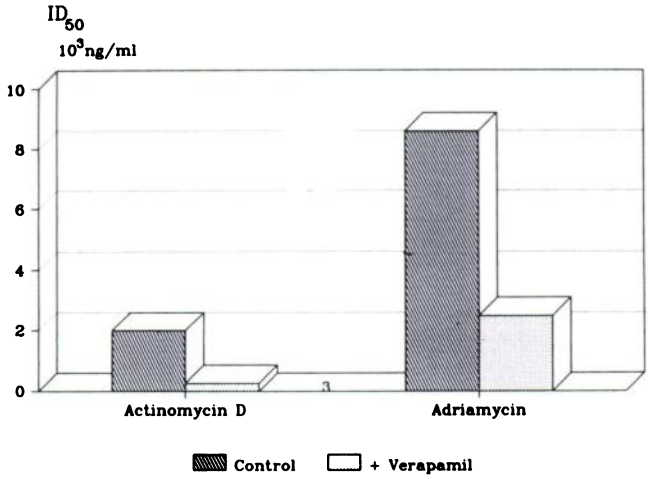


Fig. 3. Influence of verapamil on drug sensitivities of CCRF ACTD400 cells. The ID_{50} values in ng/ml with or without verapamil (1 μg/ml for 2 h) are shown for actinomycin D (determined by [³H]uridine incorporation, 10 min labeling after a 2-h incubation with various drug concentrations) and Adriamycin (determined by [³H]thymidine incorporation, methods described in Footnote 3).

distinct changes of the signal were seen.

The influence of verapamil on the sensitivity of the macromolecular synthesis to actinomycin D and Adriamycin of the CCRF ACTD400 subline is shown in Fig. 3 (indicated are the ID_{50} values). Verapamil is able to enhance the level of the drug sensitivity within 2 h.

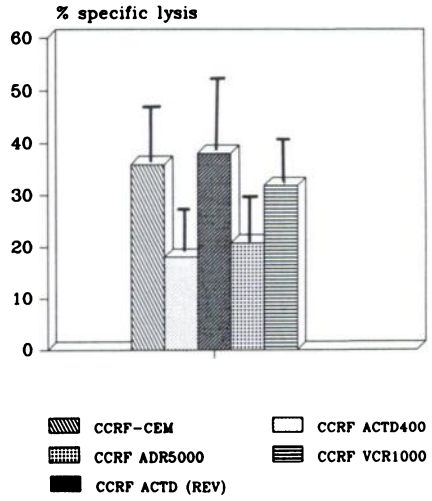


Fig. 4. Specific lysis at an effector:target ratio of 40:1 (mean ± SD; $n = 16$) of the differently selected MDR sublines in a 4-h ^{51}Cr release assay. The differences between the sublines CCRF ACTD400 and CCRF ADR5000 and the parental cell line CCRF-CEM is significant ($P < 0.001$, respectively).

Table 2 *Specific lysis of the different multidrug resistant sublines in a 4-h ^{51}Cr release assay*

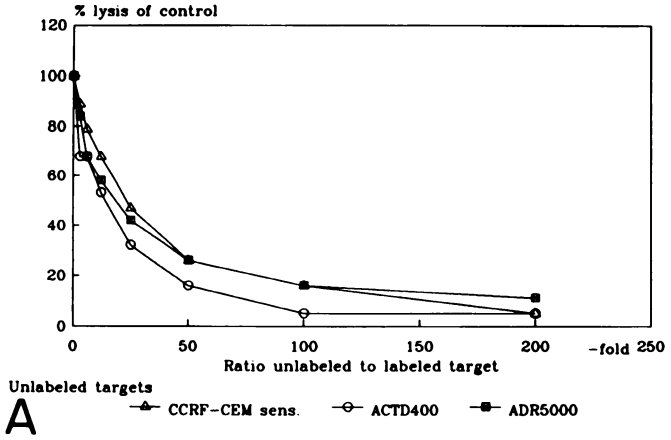
Mean ± SD of 26 experiments [effector:target (E:T) ratios = 40:1 and 20:1] and of 10 experiments (E:T = 5:1 and 2.5:1) at different E:T ratios.

Subline	% of specific lysis at effector:target ratios of			
	20:1	10:1	5:1	2.5:1
CCRF-CEM	25.8 ± 8.6	20.5 ± 9.8	11.5 ± 3.4	6.9 ± 3.4
CCRF ACTD400[a]	12.9 ± 7.1	9.1 ± 5.9	6.0 ± 3.2	3.5 ± 2.1
CCRF ACTD (REV)	28.6 ± 12.6	21.0 ± 11.1	10.2 ± 2.8	5.3 ± 2.8
CCRF ADR5000[a]	15.2 ± 9.6	10.0 ± 6.3	ND[b]	ND
CCRF VCR1000	24.4 ± 10.5	17.5 ± 6.0	11.1 ± 3.0	6.1 ± 3.0

[a] Difference between CCRF-CEM and indicated subline significant with $P < 0.001$ for each of the E:T ratios tested.
[b] ND, not determined.

Susceptibility of Different Sublines to Il-2-activated Killer Cells. With all effector:target ratios tested so far we found a marked decrease in the susceptibility of the CCRF ACTD400 and CCRF ADR5000 sublines compared to the parental cell line with each donor ($P < 0.001$). In contrast, CCRF VCR1000 cells showed nearly the same or a slightly (nonsignificant) decreased susceptibility compared to the parental cells. The revertant subline CCRF ACTD (REV) had the same characteristics. Fig. 4 shows the mean specific lysis of CCRF-CEM, CCRF ACTD400, CCRF ACTD (REV), CCRF ADR5000, and CCRF VCR1000 obtained in 6 different cytotoxic assays. The mean ± SD of the various experiments are summarized in Table 2.

Cold Target Competition Experiments. For a further investigation of the mechanism of the decreased susceptibility of the two multidrug-resistant sublines CCRF ACTD400 and CCRF ADR5000 compared with the parental CCRF-CEM cell line we performed competition experiments. Each of the three sublines was used as a labeled target in competition with each of the three other unlabeled sublines. There was no significant difference between the three cell lines in their ability to enter into competition with each other. Fig. 5 illustrates the inhibition of the specific lysis by the unlabeled targets in two experiments. Table 3 shows the cell number of unlabeled cells that cause 50% inhibition of the specific lysis of the 5×10^3 labeled target cells.



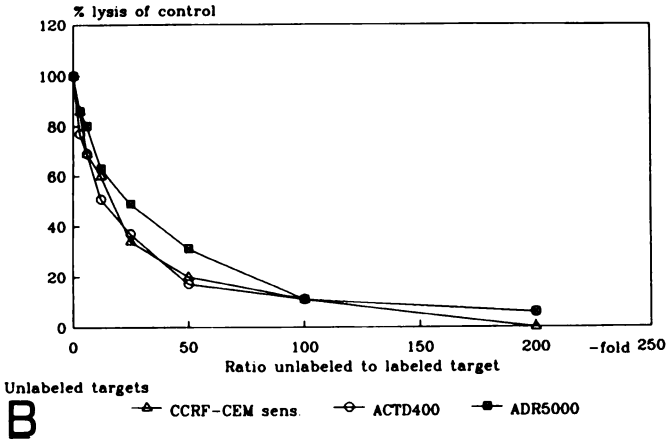


Fig. 5. The results from the cold target competitions are shown. Unlabeled targets competed with the labeled targets in a 4-h ^{51}Cr release assay with a ratio ranging from 3:1 to 200:1. The specific lysis in the absence of unlabeled target was determined as control (100%). CCRF ACTD400 cells (*A*) and CCRF-CEM cells (*B*) were used as labeled targets, as unlabeled competitors the CCRF-CEM, CCRF ACTD400, and CCRF ADR5000 cells were used, respectively (effector:target ratio, 40:1).

Table 3 *Cold target competition*

Indicated is the number of unlabeled competitors (×10,000) that cause a 50% reduction of the specific lysis of the 5000 labeled target cells in a 4-h ^{51}Cr release assay. Labeled target:CCRF-CEM. Effector:target ratio = 40:1.

Donor	Unlabeled target		
	CCRF-CEM	CCRF ACTD400	CCFR ADR5000
34	11	22	12
35	2.5	3	3
36	10	4	5
37	8	6	12
Mean ± SD	7.9 ± 3.8	8.8 ± 8.9	8.0 ± 4.7

Influence of Verapamil on Susceptibility of Drug-resistant Sublines. Since verapamil is known to reduce the drug resistance of MDR cells (Fig. 3; Ref. 31), we examined whether verapamil might also be able to reduce the increased resistance of multidrug-resistant CCRF-CEM sublines to Il-2-activated killer cells. No influence of verapamil (5 μM) during the 4-h ^{51}Cr release assay on the specific lysis of CCRF-CEM cells was seen in the control (data not shown). Then, we incubated the CCRF ACTD400 cells during the cytotoxicity assay (4 h) with 5 μM verapamil, and again no difference of the specific lysis of the treated cells and the untreated control was found. Moreover, CCRF-CEM and CCRF-ACTD400 cells were subcultivated with 5 μM verapamil for 3 days prior to the cytotoxicity assay, with verapamil present during the assay. No difference between the pretreated and untreated CCRF-CEM cells and CCRF-ACTD400 cells, respectively, was noted (Table 4).

Immunophenotyping Results. The results are summarized in Table 5. All cell lines express the CD45, CD15, and CD7 antigen, demonstrating their generation from a T-cell leukemia. CD3 is negative or only weakly expressed on this cell lines. CD4 is only expressed on the parental cell line and CD5 only on the parental and the CCRF ACTD400 cell line. The differences between the expression of surface markers of the differently selected sublines do not correlate with the susceptibility to LAK cells. We also investigated the stability of the cell surface antigens after a period of culturing in drug-free medium for 7 days. No significant differences were seen.

Correlation between P-Glycoprotein Gene Induction and Specific Cell Lysis in CCRF ACTD400 Cells. The expression of the P-glycoprotein gene at the mRNA level is reduced after a 4-week period of growth in drug-free medium and can be induced again after a short period (24–72 h) of reexposure to the drug[3] (23) concomitant with an increased resistance to actinomycin D and Adriamycin. We investigated differences in the susceptibility of the CCRF ACTD400− cells grown in drug-free medium (5 weeks) or reexposed to actinomycin D (3 days; cells are then designated CCRF ACTD400−+) according to the protocol described (23). For this experiment the CCRF ACTD400 and the CCRF ACTD400−+ cells were washed 1 day before starting the cytotoxicity assay and were allowed to grow in drug-free medium for 1 day to minimize any influence of the drug itself (29, 30). The results of the cytotoxicity assay are shown in Fig. 6. In summary, the parental CCRF-CEM cells and the revertant CCRF ACTD (REV) subline showed a similar susceptibility to the LAK cells, while the permanently with 400 ng/ml cultured CCRF ACTD400 cells and the reexposed CCRF ACTD400−+ cells (no drug for 5 weeks, drug reexposure for 3 days) both showed a similar and marked decrease of the LAK susceptibility in comparison to the parental cell line. ($P < 0.001$). The difference between the susceptibility of the CCRF ACTD400 cells (drug present continuously) and the CCRF ACTD400-cells (5 weeks grown without the drug) was significant ($P < 0.01$) as well as the difference between the CCRF ACTD400− and the reexposed CCFR ACTD400−+ cells ($P < 0.01$).

DISCUSSION

We investigated the susceptibility to LAK cells of three independently selected multidrug-resistant T-lymphoblastoid sublines in comparison to the parental cell line. Two sublines, CCRF ACTD400 and CCRF ADR5000 showing a substantial expression of the M_r 170,000 P-glycoprotein which is known to cause MDR (32, 33) showed a significantly decreased susceptibility to LAK cells. To our knowledge this effect was not shown before with LAK cells. The third subline, CCRF VCR1000, exhibits a similar degree of multidrug resistance, but a relatively low expression of the P-glycoprotein. This subline was as susceptible as the drug sensitive parental CCRF-CEM cell line to the LAK cell-mediated lysis. Furthermore, concomitantly with the decrease of P-glycoprotein expression in CCRF ACTD400 cells grown 5 weeks in drug-free medium, the susceptibility to LAK cells was enhanced, and a redecrease was seen after reinduction of the P-glycoprotein gene expression by exposure to the drug actinomycin D. Therefore our results suggest that there is an inverse relationship between the expres-

Table 4 *Specific lysis of verapamil-treated and untreated CCRF-CEM and CCRF ACTD400 cells*

Mean ± SD for the specific lysis in a 4-h ^{51}Cr release assay.

Effector:target ratio	% of specific lysis			
	CCRF-CEM	CCRF-CEM + verapamil	CCRF ACTD400	CCRF ACTD400 + verapamil
40:1[a]	33.9 ± 7.3[b]	ND[c]	20.9 ± 7.0	22.2 ± 6.2
20:1[a]	29.8 ± 6.3	ND	16.8 ± 5.8	17.0 ± 5.4
10:1[a]	23.3 ± 6.4	ND	11.4 ± 5.2	12.4 ± 4.1
40:1[d]	30.5 ± 18.9	30.5 ± 16.9	15.5 ± 10.6	15.5 ± 9.2
20:1[d]	22.8 ± 14.2	21.3 ± 13.9	9.0 ± 6.3	9.5 ± 4.5
10:1[d]	16.0 ± 14.1	14.0 ± 11.6	5.0 ± 4.9	5.8 ± 4.5

[a] Eight experiments, 4-h incubation of 5 μM verapamil.
[b] Mean ± SD.
[c] ND, not done.
[d] Four experiments, 3-day incubation of 5 μM verapamil.

Table 5 *Immunophenotype analysis of the differently selected MDR sublines*[a]

For immunophenotyping directly fluorescence marked antibodies purchased from Becton Dickinson were used, data were analyzed with a FACS scanner.

Antigen	% of positive cells in following cell lines				
	CCRF-CEM	ACTD400	ACTD (REV)	ADR5000	VCR1000
CD3	6	10 (13)[b]	7	0 (0)	0 (0)
CD4	71	0 (0)	0	0 (0)	0 (0)
CD5	95	82 (74)	3	0 (6)	0 (0)
CD7	97	68 (71)	87	93 (90)	97 (99)
CD15	75	97 (93)	89	90 (93)	97 (97)
CD45	99	84 (93)	96	91 (90)	98 (95)

[a] The following antigens were negative for all sublines: CD2, CD8, CD16, CD25, CD56, HLA-DR. Also we could not find the T-cell receptor $T_{\alpha/\beta}$ and $T_{\gamma/\delta}$ on the cell surface with this method.
[b] The numbers in parentheses indicate the number of positive cells after a drug-free culturing period of 7 days.

Table 6 *P-Glycoprotein expression and specific lysis of the differently selected CCRF-CEM sublines*

For details see Fig. 1 (the polyclonal antibody mdr was used, similar values were obtained with the monoclonal antibody C 219).

Subline	P-glycoprotein expression	Specific lysis[a] (effector:target ratio, 40:1)
CCRF-CEM	0.6	35.9 ± 10.0
CCRF ACTD400	27	18.1 ± 8.6[b]
CCRF ACTD (REV)	ND[c]	38.0 ± 13.0
CCRF ADR5000	21	20.9 ± 10.4[b]
CCRF VCR1000	set = 1	31.8 ± 9.1

[a] Mean ± SD of 16 experiments in a 4-h ^{51}Cr release assay.
[b] $P < 0.001$ in comparison to the specific lysis of CCRF-CEM cell line.
[c] ND, not done; the P-glycoprotein expression of the CCRF ACTD (REV) cell line determined by Western blot analysis with the monoclonal antibody C219 is about 50% of the expression shown by the CCRF VCR1000 cells (see Fig. 2).

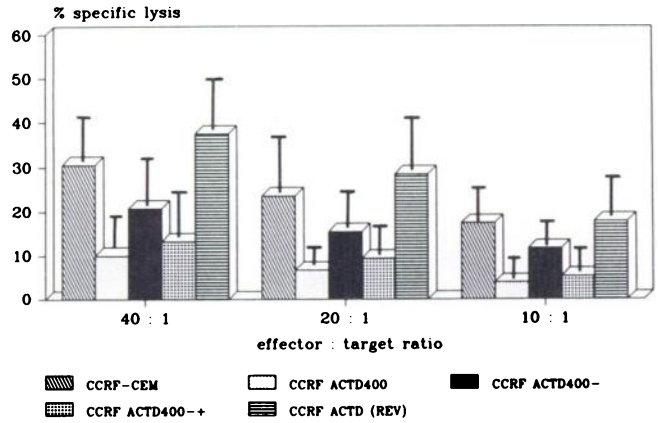


Fig. 6. Specific lysis of differently treated CCRF ACTD400 cells in a 4-h ^{51}Cr release assay. CCRF-CEM indicates the parental cell line, CCRF ACTD400 cells grew in continuous presence of actinomycin D (400 ng/ml). CCRF ACTD400− cells were allowed to grow in a drug-free medium for 5 weeks while the P-glycoprotein expression was reduced (23).[3] CCRF ACTD400−+ cells were reexposed to the drug (400 ng/ml) for 3 days. The decrease of the LAK susceptibility shown here was combined with increases of the P-glycoprotein mRNA levels and actinomycin D resistance (data not shown, comparable to the results presented elsewhere (23)[3] CCRF ACTD (REV) is a revertant MDR cell line. The differences between the CCRF ACTD400, ACTD400− and ACTD400−+, respectively, and the CCRF-CEM cells are significant ($P < 0.001$), as well as the difference between ACTD400 and ACTD400− cells ($P < 0.001$). Also the difference between ACTD400− and ACTD400−+ cells is significant ($P < 0.01$).

sion of P-glycoprotein and the susceptibility of MDR cells to LAK cell-mediated lysis (Table 6). Evidence for a negative correlation of the P-glycoprotein expression and the susceptibility to natural killer-like cell-mediated cytotoxicity was reported by Woods *et al.* (18). They found a decreased susceptibility of two MDR cell lines to natural killer-like cytotoxicity. Both cell lines showed a high expression of the P-glycoprotein gene. Another study showing a decreased susceptibility of pleiotropic drug-resistant K 562 cells to natural killer cells as well as to interferon-activated killer cells was published by Yanovich *et al.* (19). In contrast, however, Allavena *et al.* (20, 21) observed no difference of the susceptibility between a human colon carcinoma cell line (LoVo) and their multidrug-resistant subline (LoVo/DX) to LAK cell- and monocyte-mediated cytotoxicity. Gambacorti-Passerini *et al.* (22) examined the same MDR subline LoVo/DX, and found a tendency toward an even higher susceptibility of the resistant subline to LAK cell-mediated lysis. However, the P-glycoprotein expression of the LoVo/DX cell line was not specifically determined. One explanation for the different findings could be that the effect of decreased susceptibility is only visible beyond an as yet unknown threshold of P-glycoprotein expression in the plasma membrane. This might be actually true in case of the LoVo/DX cell line and the CCRF VCR1000 cell line examined in this work.

The fact that the revertant subline CCRF ACTD (REV) showing a low P-glycoprotein expression also reverted to the same susceptibility to LAK cells than the parental drug-sensitive cell line CCRF-CEM is a further suggestion for a role of the P-glycoprotein for the susceptibility to LAK cell-mediated lysis. However, also other as yet unknown genes might play a role, if their expression might be regulated in combination with the expression of the P-glycoprotein gene. It appears worth mentioning at this point that only amplification of the *mdr*3 gene (34) was found in our MDR sublines, but expression of this second member of the human *mdr* gene family was absent here.[3] Transfection of a functional *mdr*1 gene into a drug-sensitive cell line might be helpful to clarify the role of P-glycoprotein in LAK cell-mediated cytotoxicity.

To further evaluate the functional role of the P-glycoprotein in LAK cell-mediated lysis we examined the influence of vera-

pamil (5 μM) which is known to reduce drug resistance of many MDR cell lines (see Fig. 3; Refs 31 and 35) possibly by inhibiting the P-glycoprotein pump (35, 36). P-glycoprotein is supposed to act as an energy-dependent drug efflux pump (36–39), and one might speculate that it could also affect the accumulation of cytotoxic factors produced by NK or LAK cells. Nevertheless, verapamil did not enhance the susceptibility of the CCRF ACTD400 cells after a 4-h or 3-day incubation period. Since P-glycoprotein is a very large protein with possibly more than a single drug-binding site (36, 39) one may assume that verapamil either blocks a different binding site than the one used for (hypothetic) cytotoxic factors produced by LAK cells, or other qualities of the P-glycoprotein (dissected from substrate-binding and transport properties) might be involved. However, P-glycoprotein-independent mechanisms could be involved; for example, as yet unknown factors that are coexpressed with P-glycoprotein.

In our experiments with cold target competition each of the three sublines, CCRF-CEM, CCRF ACTD400, and CCRF ADR5000, were as efficient as the others in competing with each other, suggesting that the resistance to LAK cell-mediated lysis is due to a postbinding event. Yanovich *et al.* (19) reported the same results. They found that drug-resistant and sensitive K 562 cells exhibited the same ability to bind to NK cells in a direct conjugate-forming assay. In contrast Woods *et al.* (18) observed that the drug-resistant cells were less effective in forming conjugates with NK-like cells than the sensitive cells, and they postulated that a reduced recognition of the target cells plays a role in the reduced susceptibility of MDR cells to NK cell-mediated lysis. These different results suggest that there might be various mechanisms involved in the resistance of MDR cells to NK or LAK cell-mediated cytotoxicity. Immunophenotyping of the cell lines examined here did not show differences which might be linked to the observed differences of LAK cell susceptibility.

The observation that an enhanced resistance to LAK cell-mediated lysis is qualitatively correlated with the P-glycoprotein expression encourages further research, and may help to clarify the largely unknown mechanism of target cell recognition and lysis of NK and LAK cells. In looking for possibilities to overcome multidrug resistance this observation should be considered. The development of MDR associated with P-glycoprotein expression has been reported in human cancers (15–17, 40, 41), and possibly our observation has clinical relevance. The induction of drug resistance together with P-glycoprotein gene expression[3] and a reduced susceptibility to LAK cell-mediated lysis *in vitro* after a longer drug-free interval by reexposure to the drug might be important *in vivo* as well. Further studies are under way to investigate this phenomenon.

From this point of view, there is another reason to have a longer drug-free interval between chemotherapy and immunotherapy, not only in order to give the immune system a chance to recover from chemotherapy but also to perhaps reduce the P-glycoprotein expression of MDR tumor cells. Hence, the MDR cells would become more susceptible targets for immunocompetent cells. Not all multidrug-resistant cells are resistant to LAK cells (CCRF VCR1000; Refs. 20–22), and it could be shown that the less susceptible MDR cells are still killed by LAk cells but to a smaller extent. Several experimental studies show that NK and LAK cells are able to kill leukemic blasts or cell lines (8, 10, 42), and to inhibit clonogeneic growth of myeloid and lymphoid leukemic cell lines or of fresh leukemic blasts (43, 44). Furthermore, it is possible to activate and expand killer cells from patients with acute leukemia in remission or even during active disease (7, 8, 11, 45). On the basis of these data immunotherapy could be of value in the treatment of drug-resistant leukemia despite the reduced LAK cell susceptibility of MDR leukemic cell lines with high P-glycoprotein expression.

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