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Decreased sensitivity of drug-resistant cells towards T cell cytotoxicity

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Killing of target cells by cytotoxic T cells is mediated by induction of apoptosis requiring functional death pathways. Kill is mediated either by the CD95 or the perforin/granzyme pathway. We found that SH-EP neuroblastoma cells are preferentially killed via CD95, while in the T leukemia cell line CEM CD95 and perforin/granzyme are involved. In both types of cell lines, cells resistant to CD95- and drug-induced apoptosis are cross-resistant to cytotoxic T cell kill. Resistant cells show decreased apoptosis and deficient activation of caspases indicated by decreased cleavage of the prototype caspase substrate PARP. Preincubation with the caspase inhibitor zVAD-fmk strongly decreased LAK cell kill in sensitive cells. Although parental CEM cells could be sensitized for LAK kill by preincubation with doxorubicin, resistance could not be reverted in doxorubicin or CD95 resistant CEM cells. These data demonstrate the crossresistance in induction of apoptosis by different cytotoxic regimens in tumor cells and may have implications for the immunotherapy of tumors in which apoptosis resistance was induced by previous chemotherapy.
Keywords: LAK cells; cytotoxicity; drug-resistance; resistance; apoptosis; APO-1

Introduction

Immunotherapy of malignant tumors using cytotoxic T cells has been considered for a long time as a promising treatment strategy in cases where conventional therapeutic regimens, eg using chemotherapeutic drugs, have failed. Thus, cytotoxic T cells have been expected to overcome resistance to anticancer treatment by conventional strategies. While the molecular mechanisms underlying T cell cytotoxicity or cytotoxicity mediated by anticancer agents have been studied as independent events leading to cell death, recent evidence suggests that the cytotoxicity of both approaches is mediated by induction of apoptosis involving the activation of evolutionary conserved pathways.[1–3] Treatment of tumor cells with cytotoxic drugs used in chemotherapy involves activation of death inducing ligand/receptor systems such as CD95 (APO-1/Fas) and activation of caspases, a cascade of proteolytic enzymes involved in the execution of cell death.[4–9] Thus, irrespective of the primary intracellular targets, intact apoptosis signalling systems ultimately contribute to effective cytotoxicity of antitumor drugs. In line with this, blockade of apoptosis pathways, eg by overexpression of anti-apoptotic Bcl-2 family proteins has been shown to block drug-induced apoptosis.[10–12] Recent experimental data from several laboratories have demonstrated that cytotoxic T cells kill the target cells also by activation of the apoptosis machinery.[13–15] The two critical pathways that determine CTL kill appear to be the CD95 system and perforin/granzyme B. Lymphokine activated killer (LAK) cells from *gld*-mice, deficient in CD95 ligand, are profoundly less cytotoxic towards CD95 receptor-positive targets.[16] In human AML, CD95-sensitive cells are more susceptible for LAK kill than CD95-resistant cells and preincubation of sensitive cells with the blocking anti-CD95-antibody strongly reduces apoptotic cell death.[2] On the other hand, the perforin/granzyme system mediates cytotoxicity by releasing perforin from granules of cytotoxic cells together with granzyme B. This leads to pore formation in targets and subsequent activation of caspases such as caspase-1 or caspase-3 by granzyme B which mediate apoptotic cell death.[17–21] Consequently, CTL from granzyme B knockout mice or perforin knockout mice show a significant reduction in cytotoxicity.[16,22] Crossing of perforin knockout mice and *gld*-mice deficient in CD95 ligand leads to a functional double knockout mouse in which short-term cytotoxicity of lymphoid cells is completely abolished.[16,22]

Based on these data we investigated the sensitivity of drug-resistant cells, in which deficient activation of the apoptosis machinery has been found, towards the cytotoxic activity exerted by lymphoid killer cell populations (LAK cells). We used neuroblastoma cells that are preferentially killed via CD95 and T leukemia cells, in which CD95 and perforin/granzyme are involved. In addition to the parental cell lines, doxorubicin-resistant derivative cell lines were used which were generated *in vitro* by continuous culture in the presence of low levels of doxorubicin as described.[4–6,23] In addition, CD95-resistant cells generated *in vitro* by continuous culture in the presence of anti-CD95 (anti-APO-1) were used in an *in vitro* kill assay.[4–6,23] Furthermore, we studied the effects of caspase inhibition and of preincubation of targets cells with anticancer drugs for LAK kill.[4–6,24,25]

Our data show that cells resistant to doxorubicin or CD95 display crossresistance for killing by cytotoxic T cells both in cells killed via CD95 or via perforin/granzyme suggesting that similar pathways are used by lymphocyte-mediated kill and cytotoxicity of anticancer treatment.

Methods

Materials and media

The cells were kept in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Conco, Wiesbaden, Germany), 12.5 mm Hepes (Biochrom, Berlin, Germany), 100 U/ml penicillin/streptomycin solution (Life Technologies) and 2.0 mm l-glutamine solution (Biochrom). Sterofundin (B Braun, Melsungen, Germany), a balanced electrolyte solution containing Ca^{2+} and Mg^{2+}, supplemented with 0.5% bovine serum albumin (Sigma, Deisenhofen, Germany) and 10 mm Hepes, was used as washing buffer.

The bispecific monoclonal antibody CD3 × CD19[26–28] was kindly provided by Dr Moldenhauer from the German Cancer Research Center (Heidelberg, Germany). The stock solution containing 1 mg/ml was diluted freshly for each experiment with PBS 1:1000 to form a solution of 1 μg/ml.

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Cell lines and culture conditions

All cells were maintained in culture medium at 37°C, in 5% CO_2 unless otherwise mentioned. Parental SH-EP neuroblastoma cells (SH-EP) were kindly provided by Prof M Schwab (German Cancer Research Center, Heidelberg, Germany). SH-EP cells resistant to CD95-mediated apoptosis (SH-EP-CD95-R) were prepared by continuous culture in medium containing agonistic anti-CD95 (anti-APO-1, 1 μg/ml); SH-EP cells resistant to doxorubicin (SH-EP-doxo-R) were prepared by continuous culture in medium containing doxorubicin (up to 0.3 μg/ml), as described elsewhere.[4–6] For experiments, SH-EP-CD95-R/SH-EP-doxo-R cells were washed in culture medium and cultured for at least 7 days without anti-CD95 or doxorubicin.

Before use in a kill assay, adherent SH-EP cells were dissolved in medium by trypsin (Life Technologies, a stock solution of trypsin was diluted 1:10 with PBS).

Parental T cell leukemia cells (CEM) and human pre-B leukemia cells (Nalm6) were used. CEM cells resistant to CD95-mediated apoptosis (CEM-CD95-R) were prepared by continuous culture in medium containing anti-CD95 (1 μg/ml); CEM cells resistant to doxorubicin (CEM-doxo-R) were prepared by continous culture in medium containing doxorubicin (up to 0.1 μg/ml) as described elsewhere.[4–6,23] For the experiments, CEM-CD95-R/CEM-doxo-R cells were washed in culture medium and cultured for at least 7 days without anti-CD95 or doxorubicin, respectively. Before use in a kill assay, CEM and Nalm6 were separated from dead cells by Ficoll separation.

Preparation of LAK cells and BAS-PBMC

Fresh heparinized blood from healthy normal donors was diluted 1:1 with sterile NaCl 0.9% solution, then mononuclear cells (PBMC) were isolated by Ficoll–Paque density gradient centrifugation. LAK cells were prepared by culture of fresh PBMC at a concentration of 1×10^6 cells/ml in culture medium containing 200 U/ml human IL-2 (Novus Molecular, San Diego, CA, USA). Medium was replaced every 5 days and cells were used for cytotoxicity assays after day 10 of culture. Bispecific antibody-stimulated PBMC (BAS-PBMC) were prepared by culture of PBMC at a concentration of 1×10^6 cells/ml in culture medium containing CD3 × CD19 at 100 ng/ml and IL-2 at 20 IU/ml. Cells stimulated for 3 days were washed and kept for 12 h in medium.[26–28] Before use in a kill assay, LAK cells and BAS-PBMC were separated by Ficoll separation.

Cytotoxicity assay

Effector cells and target cells were cocultured at 5×10^6 cells/ml in a volume of 200 μl in 96-well flat-bottomed microtiter plates (Falcon, Becton Dickinson, NJ, USA) at a 1:1 effector:target ratio. Plates were centrifugated for 8 min at 120 *g* and incubated for 4 h at 37°C, in 5% CO_2. For the experiments using anti-CD95-F(ab')$_2$ fragments, cells were prepared at 2×10^6 cells/ml at a 1:1 effector:target ratio.

Morphological assessment of apoptosis in neuroblastoma cells

In neuroblastoma cells, cell kill was assessed by morphological examination and cell counting. After the cytotoxicity assay, cytospin slides were prepared by centrifugating 150 μl of a 5×10^5 cells/ml suspension in a cytospin tube for 7 min at 600 r.p.m. Slides were dryed and stained with a conventional Pappenheim staining.

SH-EP and LAK cells could easily be differentiated from each other by shape and size. For each assay done in triplicate, 100 target cells were counted.

Flow cytometric analysis of apoptosis using calcein-AM/annexin V staining in leukemia cells

In leukemia cells, cell kill was assed by a two-color flow cytometry assay.[29–31]

Target cells were brought to a concentration of 1×10^7 cells/μl in culture medium. Calcein-AM (Molecular Probes, Eugene, OR, USA) – a green fluorescent intravital dye – was added at a concentration of 50 ng/ml. Cells were incubated for 60 min in the dark and washed with culture medium. Stained cells were viable (as shown by trypan blue exclusion) and could be maintained in culture for at least 12 h with no significant decrease in fluorescence intensity. Calcein-AM is an uncharged molecule that can permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a charged free acid from.[30] This form, when leaked in the medium, will not enter other cells. Thus the possibility of staining effectors by leakage of calcein-AM out of apoptotic targets is excluded.

After calcein-AM staining of targets, the cocultivation was performed as described. Apoptosis was measured by annexin V staining of calcein positive cells. Annexin V staining was performed as described.[29–31] Flow cytometry analysis was done in a FACScan flow cytometer (Becton Dickinson) using the 'CellQuest' software program (Becton Dickinson) by gating on calcein-AM positive cells. Per cent specific apoptosis was calculated as follows:

$$\%\ \text{specific apoptosis} = \frac{\text{annexin V positivity (kill assay)} - \text{spont. annexin V positivity}}{100 - \text{spontaneous annexin V positivity}} \times 100$$

Anti-CD95-F(ab')$_2$ fragments, EGTA, zVAD-fmk preincubation, concanamycin A preincubation, doxorubicin preincubation

F(ab')$_2$ fragments of anti-CD95 antibodies were prepared as described.[7,32] Target cell suspensions were preincubated in the 96-well microtiter plates at 4°C in the presence of 0, 25 and 50 μg/ml anti-CD95-F(ab')$_2$ fragments 1 h prior to addition of LAK cells. Targets and effectors were than cocultured at 2×10^6 cells/ml in a volume of 200 μl at a 1:1 effector:target ratio. Afterwards the kill assay was performed as described above.

Kill assays were also performed in the presence of ethylene-glycol-bis(β-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma) 0, 2.5, 5 and 10 mmol/l.

Benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoro-methyl ketone (zVAD-fmk) (Enzyme Systems Products, Dublin, CA, USA),[33] was dissolved in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) and diluted with PBS to 10 mm. Target cell suspensions were preincubated in the 96-well microtiter plates at 37°C in the presence of 0, 30 and 60 μmol/l ZVAD-fmk 12 h prior to addition of LAK.

Concanamycin A (Sigma)[34] was prepared as a 1 nm solution in DMSO. LAK cell suspensions were preincubated in the 96-well microtiter plates at 37°C in the presence of 0, 10 and 100 nmol/l concanamycin A 1 h prior to addition of target cells.

For 12 h doxorubicin preincubation, after separation from dead cells by Ficoll, target cell suspensions were preincubated in culture flasks in the presence of 0 or 0.1 μg/ml doxorubicin at 37°C. After 12 h, cells were washed in medium, prior to performing the kill assay.

CD95 staining

Anti-CD95 antibodies were prepared as described.[7,33,35] Target cell suspensions (1×10^6 cells) were incubated with the antibody for 40 min at 4°C, then washed and stained with a FITC-labelled goat-anti-mouse IgG antibody (Sigma) for 40 min at 4°C. After washing, flow cytometry analysis was done in a FACScan flow cytometer (Becton Dickinson) using the 'CellQuest' software program (Becton Dickinson).

Western blot analysis

After 4 h cocultivation of effector and target cells, proteins for Western blot analysis were extracted from cells lysed for 30 min at 4°C in PBS with 0.5% Triton X (Serva, Heidelberg, Germany) and 1 mm phenylmethylsulfonylfluoride (Sigma) followed by high-speed centrifugation. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, IL, USA). 40 μg protein per lane was separated by 12% SDS-PAGE and electroblotted on to nitrocellulose (Amersham, Braunschweig, Germany). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking for 1 h in PBS supplemented with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection of poly (ADP-ribose)polymerase (PARP) protein was done using rabbit anti-PARP polyclonal antibody (Enzyme Systems Products) and goat anti-rabbit IgG (Santa Cruz Biotechnology). Enhanced chemiluminescence system (Amersham) was used for detection.[36]

Results

LAK cell-induced apoptosis in SH-EP and CEM cells

To measure effector cell-induced apoptosis, we used a morphological technique for neuroblastoma cells and a flow cytometric technique for leukemia cells. After 4 h coincubation of SH-EP with LAK cells at an effector:target ratio of 1:1, 25% apoptotic cells were detected by morphology with a spontanous apoptosis of less than 5% (Figures 1 and 3a).

Using fluorescence analysis of calcein-AM-stained CEM cells coincubation with LAK cells resulted in specific apoptosis of 30–40% (Figures 2 and 3b).

In the LAK cells, there was no specific apoptosis visible in any of the kill assays (data not shown).

Inhibition of LAK cell-induced apoptosis in SH-EP cells and CEM cells by anti-CD95-F(ab')$_2$ fragments

Next, the involvement of the CD95 receptor in LAK cell-induced apoptosis of SH-EP and CEM cells was studied by use

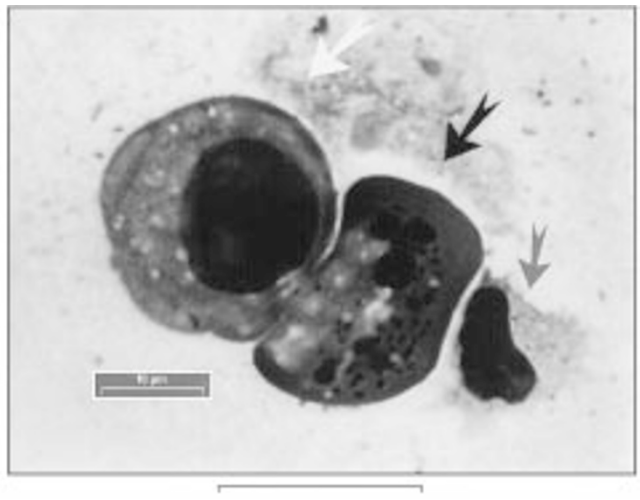

Figure 1 Light microscopic photographs of cytospin preparations showing LAK and SH-EP cells in a kill assay. Cytospin slides were prepared from LAK cells (effectors, grey arrow) and SH-EP neuroblastoma cells (targets, white arrow) after co-incubation as described. Apoptotic SH-EP cells (black arrow) were identified and counted.

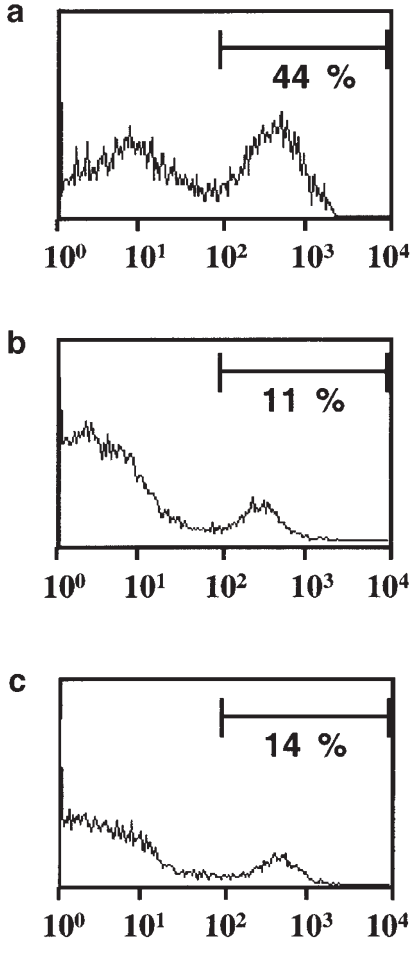

Figure 2 Calcein-AM/annexin V-based flowcytometric assay of cytotoxicity. LAK cell-induced kill of CEM cells (a), CEM-CD95-R (b), and CEM-doxo-R (c) was assessed by two-color flow cytometry as described. Flow cytometry analysis was done by gating on calcein-AM-positive cells.

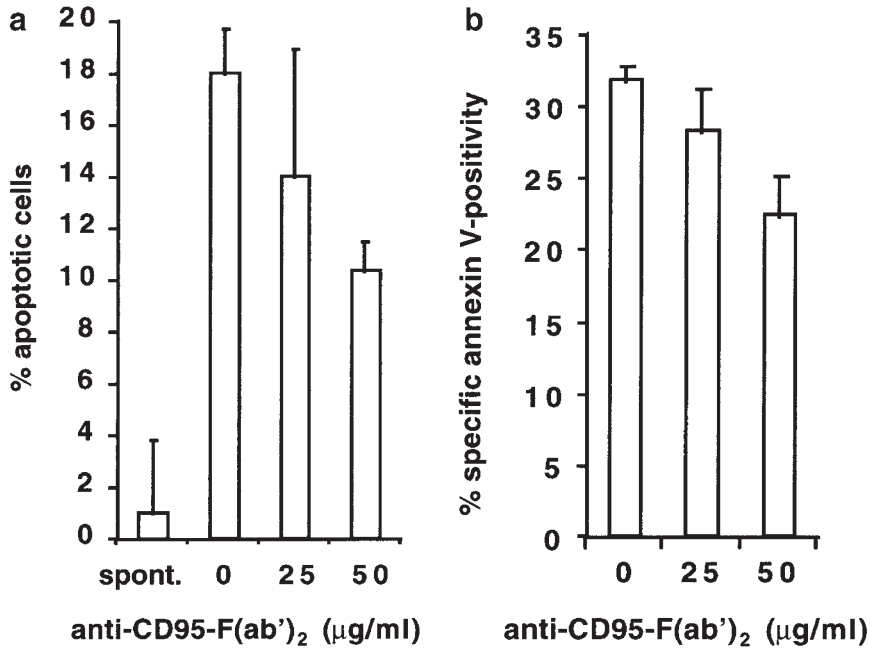

Figure 3 Inhibition of LAK cell-induced apoptosis in SH-EP and CEM cell lines by anti-CD95-F $(ab')_2$ fragments. SH-EP (a) and CEM (b) cells were preincubated with different concentrations of anti-CD95-F$(ab')_2$ fragments for 1 h prior to addition of LAK cells. Apoptosis was assessed by morphology (a) or annexin V-staining (b). Data are given as mean value and s.d. of three separate experiments.

of blocking anti-CD95-F$(ab')_2$ fragments (Figure 3a and b). At the concentrations of anti-CD95-F$(ab')_2$ fragments used, optimal inhibition was found with 2×10^6 cells/ml at a 1:1 effector:target ratio. Under these conditions, apoptosis in both target cells was markedly reduced, indicating a substantial role of the CD95 ligand/receptor system in the killing.

Inhibition of LAK cell-induced apoptosis in SH-EP cells and CEM cells by EGTA or concanamycin A

To assess the role of the perforin/granzyme system in LAK cell-induced apoptosis of SH-EP and CEM cells we used the calcium chelator EGTA to block the degranulation of killer cells (Figure 4a and c). In SH-EP cells, no significant reduction in LAK cell kill was seen. In CEM cells, however, target cell apoptosis was markedly reduced. To confirm these results, we used concanamycin A which is known to inhibit the function of perforin by inhibition of vacuolar H^+-ATPase.[34] Preincubation of LAK cells with concanamycin A had no influence on apoptosis in SH-EP cells, but inhibited LAK cell-induced apoptosis of CEM cells (Figure 4b and d). Thus, LAK kill in SH-EP cells is mediated by the CD-95 ligand/receptor system, while in CEM cells both the CD-95 ligand/receptor and the perforin/granzyme system are involved.

CD95- or drug-resistant SH-EP or CEM cells are crossresistant to LAK cells

We next studied whether drug-resistant and CD95-resistant cell lines would exhibit similar LAK sensitivity compared to parental cells. Drug-resistant and CD95-resistant cell lines (SH-EP-CD95-R and SH-EP-doxo-R or CEM-CD95-R and CEM-doxo-R) have been found to be resistant towards apoptosis induced by anti-CD95.[4,23]

Spontaneous apoptosis of SH-EP-CD95-R and SH-EP-doxo-R was similar to that of SH-EP. However, SH-EP-CD95-R and SH-EP-doxo-R were completely resistant towards LAK-mediated kill (Figure 5a). Similar results were obtained in CD95-sensitive and -resistant CEM cell lines. However, doxorubicin-resistant cells showed a higher degree of residual sensitivity compared to CD95-resistant cells (Figure 5b).

LAK cell-induced apoptosis involves activation of caspases in sensitive cells

To see whether killer cell resistance was the result of deficient caspase activation we analyzed cleavage of the prototype caspase substrate PARP by Western blot in sensitive and resistant targets co-incubated with LAK cells.

Co-incubation of SH-EP (Figure 6a) or CEM (Figure 6b) with LAK cells resulted in PARP cleavage. However, no PARP cleavage was detected when LAK cells where co-incubated with SH-EP-CD95-R, SH-EP-doxo-R, CEM-CD95-R or CEM-doxo-R.

The PARP assay does not give information whether PARP is cleaved in targets or in effectors. However, since there was no spontaneous PARP cleavage in effector cells and morphological changes and specific annexin V positivity occurred only in the sensitive targets, the PARP cleavage detected can be attributed to the targets.

These data indicate a block of the apoptosis pathway upstream of PARP cleavage in the resistant cell lines.

LAK cell-induced apoptosis is inhibited by zVAD-fmk in SH-EP and CEM, but not in resistant cells

To further investigate the role of caspases in LAK cell-induced apoptosis, we pretreated target cells with the broad spectrum caspase inhibitor zVAD-fmk prior to addition of LAK cells. In SH-EP and in CEM cells we saw a concentration-dependent

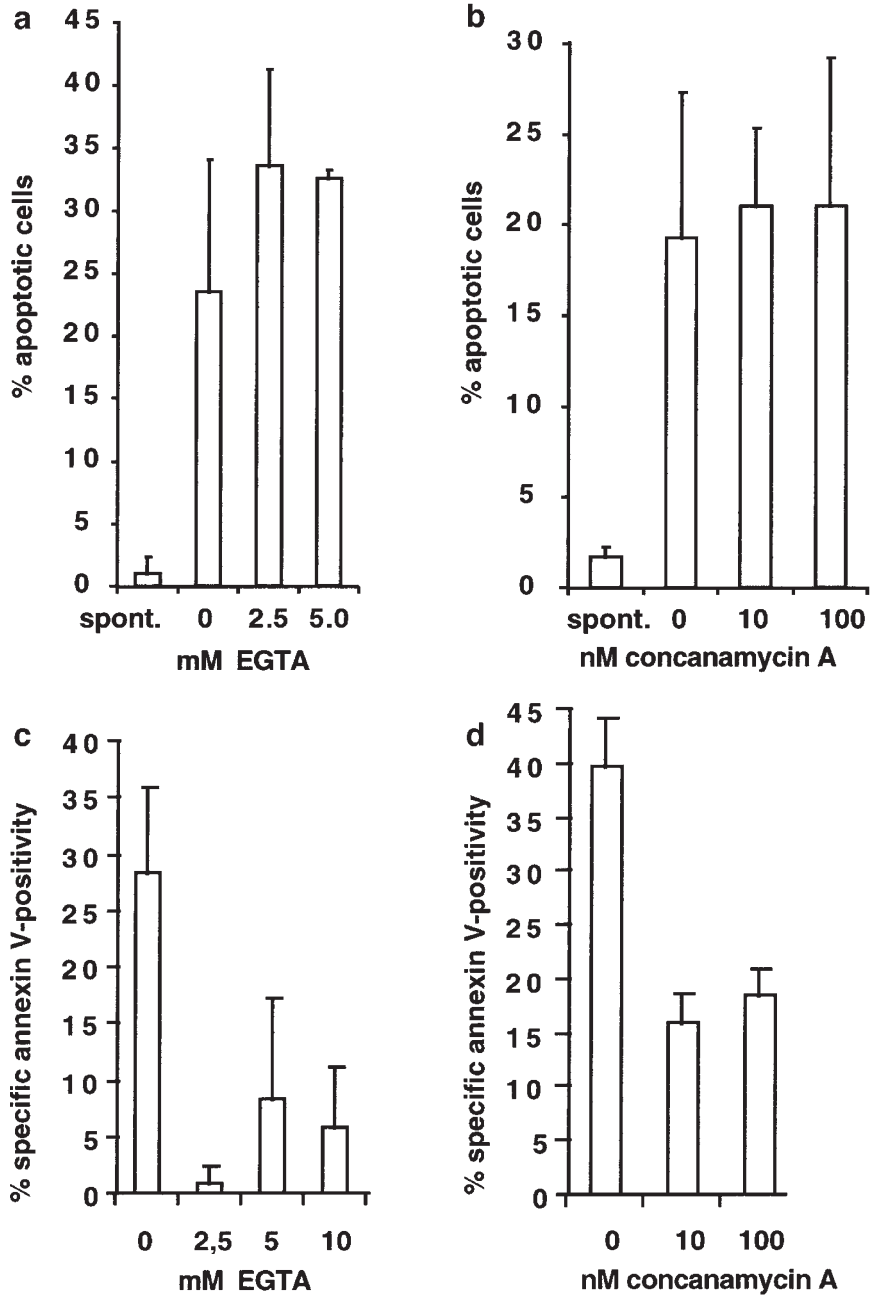

Figure 4 LAK cell-induced apoptosis of SH-EP and CEM cells in the presence of EGTA or after preincubation of LAK cells with concanamycin A. (a, c) SH-EP cells (a) or CEM cells (c) were incubated with LAK cells in the presence of EGTA at concentrations indicated. (b, d) LAK cells were preincubated with concanamycin A 1 h prior to addition to SH-EP (b) or CEM (d) cells. Cytotoxicity was measured as described. Data are given as mean value and s.d. of three separate experiments.

reduction of LAK cell-induced apoptosis (Figure 7a and b), reaching very low levels at 60 μmol/l zVAD-fmk.

These data suggest that activation of caspases in the target cell is an essential requirement for LAK-mediated target kill.

zVAD-fmk inhibits T cell receptor-mediated kill

To study a model of cytotoxicity involving the T cell receptor, we next investigated bispecific antibody (CD3 × CD19)-mediated kill of Nalm6 cells by BAS-PBMC in the presence of CD3 × CD19.[26–28] After preincubation of targets with zVAD-fmk a concentration-dependent decline of apoptosis could be demonstrated (Figure 7c).

This indicates that caspase-dependent apoptosis in target cells is not restricted to LAK cell kill but rather involved more generally in T cell cytotoxicity.

Sensitization for LAK cell-induced apoptosis in CEM cells by doxorubicin preincubation

Pre-incubation with cytostatic drugs may enhance killer cell sensitivity in tumour cell lines.[37,38] We studied LAK sensitivity

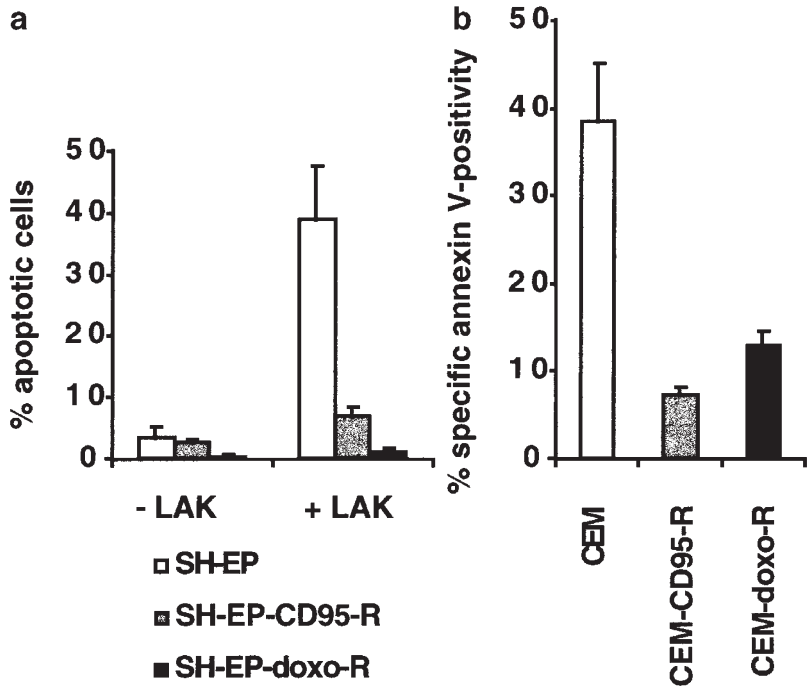

Figure 5 LAK cell-induced apoptosis of sensitive *vs* resistant SH-EP and CEM cell lines. SH-EP, SH-EP-CD95-R and SH-EP-doxo-R cells (a) and CEM, CEM-CD95-R and CEM-doxo-R cells (b) were incubated with LAK. Cytotoxicity was measured as described. Data are given as mean value and s.d. of three separate experiments.

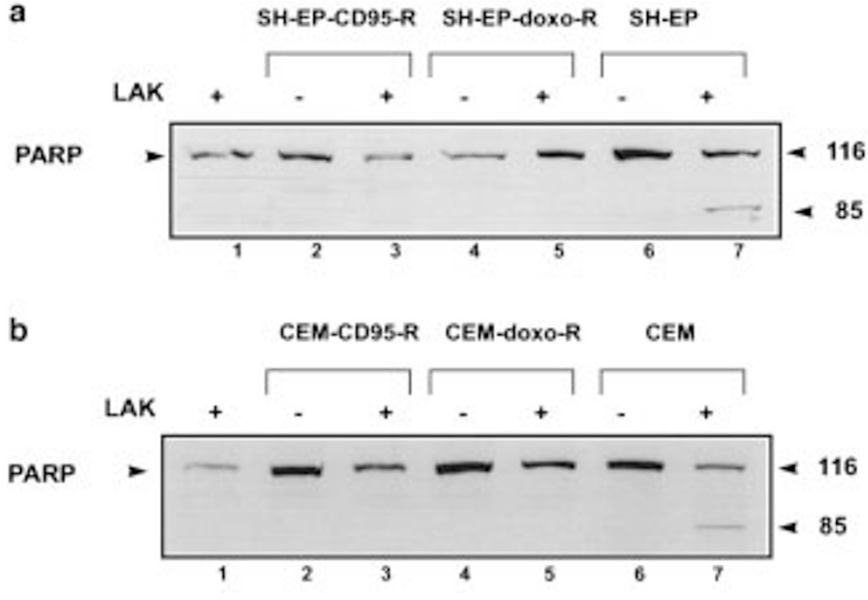

Figure 6 LAK cell-induced PARP cleavage in sensitive, but not resistant SH-EP and CEM cell lines. PARP cleavage was studied by Western blot analysis as described in lysates from SH-EP (a) and CEM (b) cells co-incubated with LAK cells in a kill assay. No spontaneous PARP cleavage was seen in LAK cells or any of the target cells (lanes 1, 2, 4 and 6).

of parental CEM cells and of CEM-CD95-R and CEM-doxoR after 12 h preincubation with or without doxorubicin (0.1 μg/ml). While LAK sensitivity of CEM cells was significantly enhanced, no increase could be seen in CEM-CD95-R or CEM-doxo-R cells (Figure 8). Spontaneous apoptosis was not higher than in cells incubated without doxorubicin (data not shown). Interestingly, CD95 expression of all cell types was unchanged after 12 h preincubation with doxorubicin (0.1 μg/ml) (Figure 9). CEM-CD95-R cells exhibited a lower level of CD95 expression compared to parental CEM or CEM-doxo-R cells.

Discussion

Cytotoxic T cells kill their targets by induction of apoptosis. This requires functional death pathways, ie death inducing ligand/receptor systems such as CD95, and activation of casp-

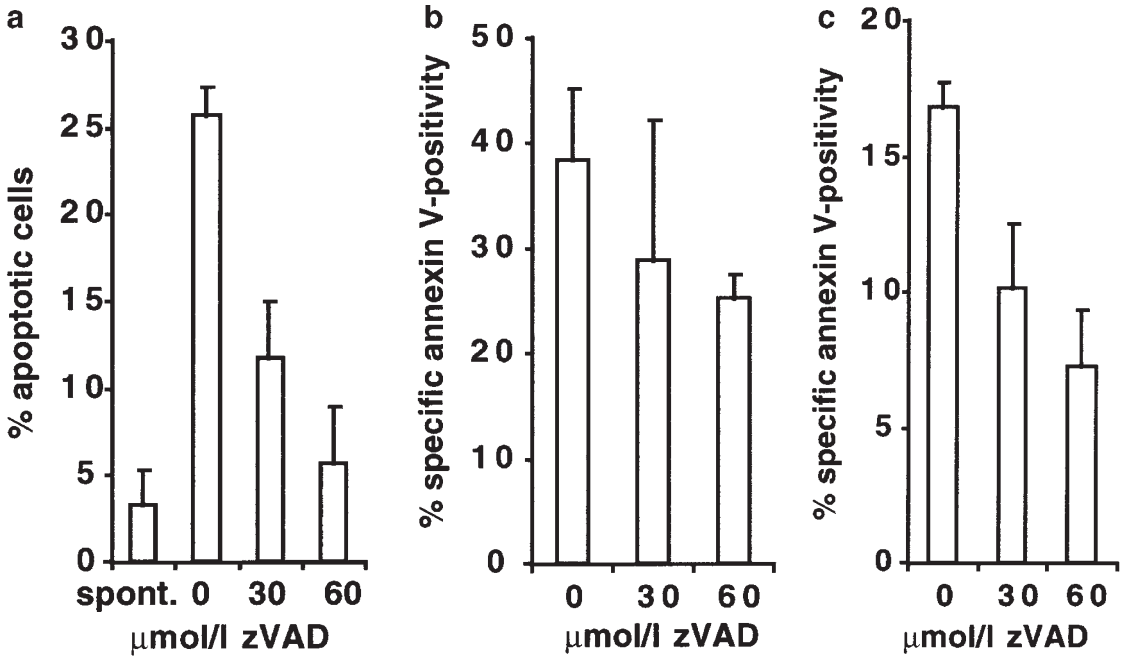

Figure 7 LAK kill of SH-EP and CEM cells and bispecific antibody CD3 × CD19-mediated kill of Nalm6 cells are inhibited by zVAD-fmk. Parental SH-EP (a) and CEM (b) cells were incubated with LAK cells after preincubation with zVAD-fmk at concentrations indicated. Nalm6 cells (c) were studied in a BAS-PBMC kill assay in the presence of CD3 × CD19 (100 ng/ml) after preincubation with zVAD-fmk at concentrations indicated. Cytotoxicity was measured as described. Data are given as mean value and s.d. of three separate experiments.

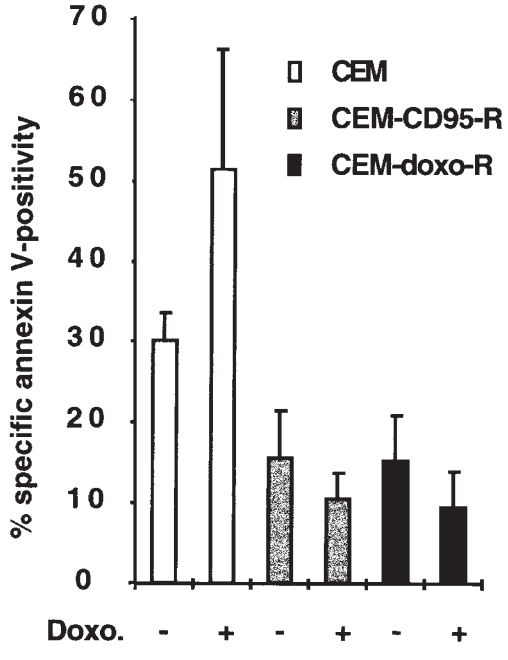

Figure 8 LAK cell-induced apoptosis of sensitive and resitant CEM cells after doxorubicin pre-incubation. Sensitive and resistant CEM cells were incubated with LAK cells after preincubation with (+) or without (–) doxorubicin at 0.1 μg/ml for 12 h. Cytotoxicity was measured as described. Data are given as mean value and s.d. of three separate experiments.

ases.[1–3] The cytotoxic effect of anticancer drugs used for chemotherapy has also been shown to involve caspase activation in tumor cells. Caspase activation may result from a dysbalance of Bcl-2 family proteins, eg in response to p53, or may result from triggering of the CD95 system as found in several cell systems.[4–9] Thus, common apoptosis pathways are required for both the cytotoxicity of anticancer drugs and CTL. In fatal malignant disease – where the problem of drug resistance is common – immunotherapy through cytotoxic T cells may be considered as a promising treatment strategy to eliminate cells resistant to other modes of treatment. Therefore it is important to know whether or not such drug-resistant cells would be sensitive to T cell cytotoxicity. In this work, we demonstrate in two cell line models the crossresistance between drug-resistant cells and T cell cytotoxicity due to alterations in apoptosis-inducing signalling pathways.

Until recently, *in vitro* drug resistance has been attributed mainly to membrane-bound transport systems like MDR1 which function by pumping drugs out of the cell. MDR1 gene expressing cells have been shown to be equally or even more sensitive to LAK cells than non-MDR1 gene expressing cells.[37] Yet, growing evidence is found for the involvement of apoptosis pathways,[9,12,37] such as alterations of the p53 system, deregulated expression of Bcl-2 family members, or other modulations of apoptosis signalling elements in drug resistance.

In CEM and SH-EP cells, it has been shown that incubation with drugs like doxorubicin leads to up-regulation of CD95 ligand synthesis, and, by interaction with CD95, to apoptosis.[4–6] CD95 is up-regulated in some, but not all kinds of malignant cells.[25,38] In doxorubicin-resistant cells, up-regulation of CD95 ligand is lacking. These cells are not only resistant to doxorubicin, but also to agonistic anti-CD95-antibodies.[4–6] On the other hand, CD95-resistant cells are drug resistant irrespective of the levels of Bcl-2 or Bcl-xL expression.[4–6,23]

However, the precise role of the CD95 system in chemotherapy-induced apoptosis is controversial since drug-induced apoptosis could not always be inhibited by blocking anti-CD95 antibodies.[39,40] In the present study we investigated the

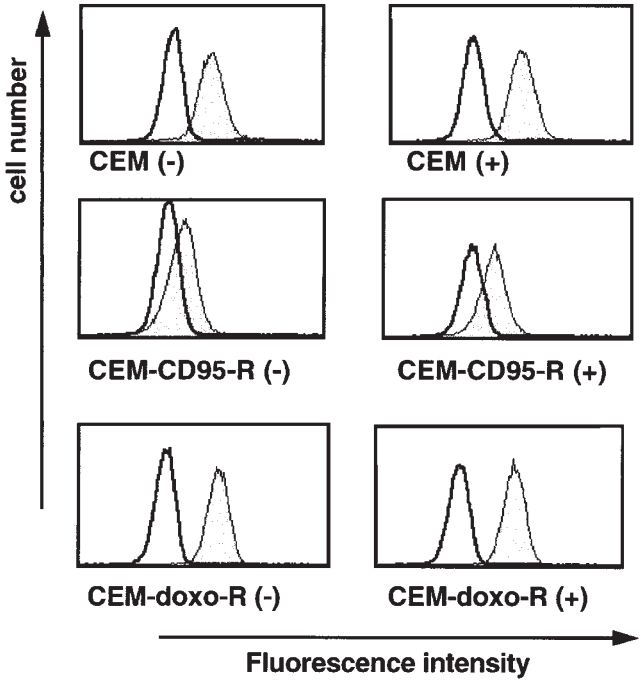

Figure 9 CD95 expression of sensitive and resistant CEM cells after pre-incubation with doxorubicin. Sensitive and resistant CEM cells were incubated with (+) or without (–) doxorubicin 0.1 μg/ml for 12 h. CD95 expression was detected by anti-CD95-staining as described (filled curve). Goat anti-mouse IgG alone served as control (empty curve). One typical out of three experiments is shown.

susceptibility of different cell lines sensitive or resistant towards anticancer drug-induced cell death for killing by cytotoxic lymphocytes (LAK).

LAK cell kill of targets in a 4 h assay was differentially mediated by the CD95 and the perforin/granzyme system in the target cells used. While SH-EP cells were only killed via the CD95 receptor/ligand system, CEM cells were killed by both the perforin/granzyme pathway and via CD95. However, both CEM and SH-EP cells resistant to CD95- and drug-induced apoptosis were crossresistant to LAK cell kill. Resistant cells showed decreased apoptosis and deficient activation of caspases as demonstrated by decreased cleavage of the prototype caspase substrate PARP,[36] indicating a block of apoptosis signalling upstream of PARP. Komada *et al*[2] established a CD95-resistant clone from a sensitive AML cell line which showed no apoptotic cell death upon cocultivation with interleukin-2-activated T cells, while apoptosis was present in the CD95-sensitive parental cells. This corresponds to our results obtained with the CD95-resistant variants of SH-EP and CEM cells. However, in addition we used cells that were made apoptosis resistant by exposure to cytostatic drugs – a model resembling the clinical situation of malignant cells becoming refractory to therapy after drug treatment. These cells were also found crossresistant to LAK cell kill.

Sensitivity of target cells for both drug-induced apoptosis and T cell cytotoxicity seems to critically depend on activation of caspases. Preincubation with the caspase inhibitor zVAD-fmk[33] conferred a resistant phenotype to killer-sensitive cells. In addition, no caspase activation as reflected by the lack of PARP cleavage could be detected in CD95-resistant and drug-resistant cells. These experiments suggest that blockade of the apoptosis executing pathway would allow tumor cells to escape immune control and T cell cytotoxicity.

Pre-incubation of tumor cells with cytostatic drugs has been shown to enhance killer cell sensitivity in tumor cell lines which involves up-regulation of CD95.[25,38] We found that CEM cells, which are killed via perforin/granzyme and CD95, can be sensitized for LAK kill by doxorubicin preincubation (0.1 μg/ml for 12 h) without further up-regulation of CD95. In contrast, CD95- or drug-resistant cells could not be rendered LAK sensitive by doxorubicin preincubation. Since CEM parental cells and CEM-doxo-R cells exhibit comparable levels of CD95 expression the resistance of CEM-doxo-R cells for LAK kill is caused by a downstream blockade at the level of caspase activation.

Taken together, our results indicate that cells induced to become drug resistant by different ways of blockade of apoptosis programs – continuous culture in the presence of drugs, CD95 down-regulation, or zVAD treatment – concomitantly acquire resistance to T-lymphocytic cytotoxicity. Resistance towards LAK kill most probably involves lack of caspase activation since crossresistance is induced in cells that are killed via CD95 only or via perforin/granzyme and CD95. It cannot be attributed to up-regulation of CD95 but probably involves regulation of the intracellular caspases.

Our findings may have implications for the immunotherapy of tumors. So far, the inability of the immune system to effectively eliminate established tumors or metastases has been mainly attributed to deficient recognition of tumours by, for example, T cells. Consequently, current strategies, eg. in gene therapy of malignancies, are aimed at increasing the immunogenicity of tumour cells. Our data indicate that immune escape of tumors may also be caused by apoptosis resistance of malignant cells – which may be induced by cytostatic drugs.

Therefore, new approaches to increase the efficacy of immunotherapy should be aimed at enhancing apoptosis sensitivity of tumour cells.

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