

Natural killer resistance of a drug-resistant leukemia cell line, mediated by up-regulation of HLA class I expression

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Background and Objectives. Drug-resistant leukemia cells may exhibit cross-resistance towards immunological effector mechanisms by alterations of apoptosis pathways. This is particularly relevant in allogeneic bone marrow transplantation for leukemia, where the graft-versus-leukemia effect acts on cells pretreated with cytostatic drugs. Here, we clarify the mechanism underlying cross-resistance of drug-resistant variants of the T-leukemia cell line CEM towards natural killer cells.

Design and Methods. We determined the sensitivity of different CEM sublines to natural killer (NK) cytotoxicity, and separately analyzed the components of the killing machinery by detection of granzyme B-induced caspase cleavage and HLA class I-dependent recognition mechanisms. Furthermore, we studied regulation of HLA class I expression comparing CEM with other cell lines.

Results. We found that CEM cells resistant to cytostatic drugs or CD95 were cross-resistant towards NK cells from a variety of donors. Granzyme B-induced caspase and PARP cleavage in the sensitive and resistant cells were comparable, indicating that downstream apoptosis pathways were not altered in the drug-resistant cells. HLA class I molecules were upregulated in the resistant cells, inhibiting NK cells at the level of killer/target recognition. HLA class I upregulation was not found in other leukemia cell lines.

Interpretation and Conclusions. This is the first description of HLA class I-mediated NK cross-resistance in drug-resistant cells. This finding may have a clinical impact since it may be considered as a possible reason for resistance to a graft-versus-leukemia approach in allogeneic bone marrow transplantation.

Key words: leukemia, NK cells, HLA class I, graft-versus-leukemia.

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The sensitivity of leukemia cells towards cellular cytotoxicity is influenced by pretreatment with cytostatic drugs.¹⁻⁶ Acquired resistance of leukemic cells towards immune attack is of particular interest in the clinical setting since most immunotherapeutic approaches, especially in the context of allogeneic bone marrow transplantation, using a well established graft-versus-leukemia effect are performed after prolonged pretreatment with cytostatic drugs. Sensitivity towards cytotoxic effector cells depends on several different mechanisms, especially the CD95 receptor/ligand system.¹⁻³ For example, sensitivity to CD95-induced apoptosis has been found to increase upon short-term exposure to cytotoxic drugs.⁴ On the other hand, drug-resistance can be induced in leukemic cells by continuous incubation in the presence of low doses of cytostatic drugs. These cells may become cross-resistant to CD95-mediated cytotoxicity by down-regulation of CD95.¹⁻³ Other mechanisms leading to altered target cell sensitivity towards cytotoxic cells include modifications of the caspase cascade, expression of apoptosis inhibitors, or disturbed effector/target recognition mechanisms.³⁻¹⁰

Depending on the individual effector and target cell type, cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells destroy their targets by different mechanisms and pathways. To elucidate resistance mechanisms these pathways must be analyzed separately. Killer cells lyse their targets by death-inducing ligands such as CD95 ligand (CD95-L), tumor necrosis factor- α (TNF- α), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and/or by the lysosomal granule contents perforin and granzyme B (GrzB).^{11,12} Since degranulation is Ca⁺⁺-dependent, blocking the perforin/GrzB pathway by ethylene-glycol-bis(β -aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA)-mediated Ca⁺⁺-chelation discriminates between ligand or lysosomal enzyme-mediated cytotoxicity mechanisms. Using the T-lymphocytic leukemia cell line, CEM, and the neuroblastoma cell line, SH-EP, we have demonstrated cross-resistance of drug and CD95-resistant cells towards cytotoxicity exerted by lymphokine activated killer (LAK) cells.⁴ In this model, killing of CEM cells is mediated by the CD95 and the lysosomal (perforin/GrzB) pathways. Activated NK effectors lyse CEM cells preferentially via the lysosomal, but not the death receptor system. Therefore, this model allows target resistance mechanisms independent of the CD95 pathway to be studied. GrzB and perforin are the predominant granule constituents involved in NK-cell-induced apoptosis.¹²⁻¹⁴ GrzB is a serine protease that

induces apoptosis by cleavage of caspases, especially caspases 3, 7 and 8,¹⁵ activation of mitochondria by the Bcl-family member, Bid,¹⁶ and by direct cleavage of DNase inhibitor, ICAD (inhibitor of caspase-activated DNase).¹⁷ GrzB enters target cells via the insulin-like growth factor II/mannose-6-phosphate (IGFII/M6P) receptor¹⁸ and is stored in endosomal vesicles.¹⁹⁻²¹ In intact cells, induction of apoptosis by GrzB requires the pore-forming molecule perforin, which liberates GrzB from vesicles and allows interaction with target molecules in the cytosol, the nucleus and mitochondria.²¹⁻²³ In cell-free extracts, however, GrzB alone can induce all molecular alterations associated with apoptosis: activation of the caspase cascade poly(ADP-ribose)-polymerase (PARP) cleavage, breakdown of mitochondrial membrane potential, cytochrome C release and nuclear fragmentation.²³⁻²⁵

NK cells recognize their targets by immunoglobulin-like or lectin-type receptors, which interact with classical or non-classical MHC class I molecules on the target cell. These receptors are selectively expressed on NK cells as well as on a subset of T cells, and act predominantly as inhibitory receptors. The distribution of the receptor types is heterogeneous among different individuals. Among the killer immunoglobulin-like receptors (KIR), those receptors which inhibit NK function upon binding to HLA-C molecules seem to play the most important role.²⁶⁻²⁸ The lectin-like CD94/NKG2A receptor dimer constitutes an inhibitory receptor binding to HLA-E molecules. HLA-E, to be stabilized at the cell surface, is assembled with peptides derived from certain non-amer leader sequences of other HLA class I, especially HLA-A, allotypes.^{27,28}

Both HLA-A alleles expressed by CEM cells, HLA-A1 and HLA-A31, contain these sequences.²⁹ The aim of this study was to analyze separately the different components of NK/target interactions in a model of acquired cytotoxicity resistance of CEM sublines resistant to CD95 or anticancer drugs. Therefore, target cell lysis was studied in the presence and absence of EGTA to assess the role of death-inducing ligands and granule components. Furthermore, the function of GrzB-induced caspase metabolism was studied using cell-free extracts and streptolysin O-permeabilized cells. Finally, to assess the role of effector-target interaction on the membrane level, expression of HLA class I proteins, in particular HLA-A, -C and -E molecules, was assessed.

Design and Methods

Cell lines and culture conditions

Cells were kept in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS, Conco, Wiesbaden, Germany), 12.5 mM HEPES (Biochrom, Berlin, Germany), 100 U/mL penicillin/streptomycin

(Life Technologies) and 2.0 mM L-glutamine (Biochrom). CEM (T-cell leukemia) cells resistant to CD95 and doxorubicin, (CEM-CD95-R, CEM-doxo-R) and similar sublines of the T-cell leukemia line J16, the B-lymphoma line Nalm6 and the small lung cell cancer line P693 were prepared by continuous culture in medium containing anti-CD95 or the cytostatic drugs as described previously.^{2,5} NKL³⁰ is a NK-cell line expressing CD94 and NKG2A, which leads to inhibition by HLA-E positive target cells. It also expresses ILT2 (CD85), leading to inhibition by HLA-G. It is negative for KIRs and cannot, therefore, be inhibited by HLA-C.

The NK-cell line B.3NK has been described recently.³¹ It was generated from peripheral blood mononuclear cells (PBMC) of a normal, healthy HLA-Cw7-positive donor by allogeneic stimulation using irradiated HLA-Cw7-negative feeder cells.³¹ This was followed by enrichment using negative selection of CD3⁺ and CD4⁺ cells with magnetic beads, resulting in a >80% CD3-negative population, displaying a NK phenotype. This subline, designated as B.3NK, is strongly positive for the KIR p58.2 (CD158b), recognizing HLA-Cw7, and is negative for NKG2A. Using several transfectant cell lines, it has been shown that these NK cells are not inhibited by any HLA molecules other than HLA-Cw7.³¹ Therefore, this cell line can be used as a specific indicator of functional HLA-Cw7 expression.

Preparation and activation of random donor NK cells

Random NK cells were isolated from PBMC of healthy donors as the CD56 positive fraction (MACS system, Miltenyi Biotech, Bergisch Gladbach, Germany) as described elsewhere.³² Fresh heparinized blood from healthy normal donors was diluted 1:1 with sterile NaCl 0.9 % solution, then PBMC were isolated by Ficoll-Paque (Biochrom) density gradient centrifugation and washed in PBS buffer. Next, 10⁷ cells were resuspended in 80 µL PBS buffer, 20 µL of MACS CD56 MicroBeadsTM (Miltenyi) were added, and the cells were incubated at 4°C for 20 min. After washing, CD56 positive cells were positively selected by a MS⁺ selection column using a MiniMACS separator system (Miltenyi). The resulting cell suspension – henceforth referred to as NK cells – was >85% CD56 positive. Short-time activated donor NK cells were prepared by culture of freshly separated NK cells at a concentration of 1×10⁶ cells/mL in culture medium containing 1,000 U/mL human interleukin-2 (Novus Molecular, San Diego, CA, USA) for 24 h.

Cytotoxicity assay by two-color flow cytometry

Target cells were stained by 60 min incubation with calcein-AM (Molecular Probes, Eugene, OR, USA) – a green fluorescent intravital dye – at a

concentration of 50 ng/mL and subsequent washing with culture medium. Ten thousand target cells were mixed with effector cells in the given ratios in 96-well round-bottom microtiter plates (Falcon, Becton Dickinson, NJ, USA) in a volume of 100 μ L, centrifuged for 5 minutes at 1,000 rpm and incubated for 4 hours at 37°C, in 5 % CO₂. Cells were then stained by propidium iodide (PI, Sigma; 3 μ L per pellet of a 20 μ g/mL solution of PI in NaCl 0.9%) and cytotoxicity was assessed in a two color-flow cytometry assay as PI positivity of calcein-AM positive targets using a FACScan flow cytometer (Becton-Dickinson). The percent specific apoptosis was calculated as follows:

$$\% \text{ specific apoptosis} = \frac{\text{PI-positivity (kill assay)} - \text{spontaneous PI-positivity}}{100 - \text{spontaneous PI-positivity}} \times 100\%$$

Cytotoxicity assay by chromium release

In several experiments, cell-mediated lysis was quantified in a standard 4 h chromium-51 release assay as described elsewhere.³³ Spontaneous release was determined by incubating target cells alone in complete medium. Total release was determined by directly counting an aliquot of labeled cells. The percent cytotoxicity was calculated according to the formula: % lysis = (experimental cpm - spontaneous cpm) / (total cpm - spontaneous cpm) \times 100. Duplicate measurements of three-step titrations of effector cells were used for all experiments.

Cell-free extracts

Preparation of cell-free extracts basically followed the protocol used by Martin *et al.*²⁴ Briefly, 1 \times 10⁸ cells were washed in PBS twice, than once in cell extraction buffer (CEB), containing 50 mM PIPES (Sigma, Taufkirchen, Germany), pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol (DTT, Sigma), 10 mM cytochalasin B (Sigma) and 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma). The pellet was resuspended in 1 mL CEB, then centrifuged at 4000 rpm for 40s. The supernatant was discarded and one packed cell volume of CEB was added. Cells were resuspended and kept on ice for 20 min to swell. Then, cells were transferred to a 2 mL glass douncer (Wheaton, Millville, NJ, USA) and gently lysed by 20 strokes of the pestle. Efficacy of lysis was controlled by trypan blue staining. When most of the cells were disrupted, the probe was diluted with one volume of extract dilution buffer (EDB), containing 10 mM HEPES (Biochrom), 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA (Sigma), 1 mM DTT, and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was taken and adjusted to a concentration of 10 mg/mL protein by dilution with EDB after protein determination in a photometer at 280 nm.

In vitro granzyme B assay of cell-free extracts

For this assay, 10 or 20 μ L of cell free extracts were incubated with granzyme B (Calbiochem, La Jolla, CA, USA) at given concentrations and for the given times at 37°C, in 5 % CO₂. Afterwards, they were immediately frozen at -80°C until the Western blot analysis was performed.

Western blot

A 10% (v/v) reducing loading buffer containing TrisCl 50 mM (Roth, Karlsruhe, Germany), 1% SDS (Serva, Heidelberg, Germany), 0.05% bromophenol blue (Merck, Darmstadt, Germany), 5% glycerol (Roth), and β -mercaptoethanol (Sigma) was added to the probes, which were then heated to 95°C for 3 min. Samples of 50 to 100 μ g protein per lane were separated by 12% SDS-PAGE gel (Owl, Portsmouth, NH, USA) and electroblotted onto a nitrocellulose membrane (Amersham, Braunschweig, Germany). After blocking for 30 min in PBS supplemented with 5% (w/v) skimmed milk powder (Fluka Chemie, Buchs, Germany) and 0.1% Tween 20 (Sigma, Steinheim, Germany), for immunodetection, the following antibodies were used: rabbit anti-PARP polyclonal antibody (Boehringer Mannheim, Germany), mouse anti-caspase 3 (CPP32) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-caspase 8 (FLICE) antibody (Pharminingen, San Diego, CA, USA). As secondary antibodies, horseradish-peroxidase-coupled goat anti-rabbit or goat anti-mouse IgG (Santa Cruz Biotechnology) were used. An enhanced chemiluminescence system (Amersham) was used for detection.

Acid treatment of targets

Target cells were treated with acid as described by Avril *et al.*³⁴ Briefly, cell pellets were suspended in 50 μ L of an acid solution (0.263 M citric acid, 0.123 M Na₂HPO₄ buffer, containing 1 % BSA, pH3.0) (all from Sigma) and incubated at 4°C for 4 min. Then 300 μ L of RPMI 1640 medium containing 20% FCS and 200 mM NaHCO₃ were added and the cells were washed three times in culture medium. Cells were more than 80% viable as determined by trypan blue exclusion. After acid treatment, cells were immediately used for immunostaining or cytotoxicity assays.

Streptolysin O/granzyme B assay

Cells were washed 3 times in PBS, once in RPMI without supplements, and then 5 \times 10⁵ cells per probe were suspended in 10 μ L pure RPMI. Reduced Streptolysin O (SLO)23 was prepared by incubation of 25,000 U of fresh SLO (Sigma), dissolved in 250 μ L dithiothreitol (DTT) 10 mM for 2 hours at 4°C. SLO was then further diluted 1:10 in DTT 10 mM, aliquot-

ed and kept frozen at -80°C until use. To induce fragmentation, 10 U/ μL reduced SLO and 5 ng/ μL GrzB were added to the cell suspensions. As negative controls untreated cells or cells treated with SLO or GrzB only were used whereas 2 μM (Sigma) staurosporin served as the positive control. Cells were vortexed and incubated for 8 hours at 37°C , in 5% CO_2 . Nuclear fragmentation was assessed by a modified Nicoletti assay:³⁵ 200 μL lysis-PI-staining-solution (1 mL 0.1 % tris(hydroxymethyl)aminomethane [100 mg/100 mL H_2O], 1 mL 0.1 % Triton X [100 mg/100 mL H_2O], 100 μL propidium iodide [1 mg/1 mL H_2O] and 200 μL formaldehyde [37%]) were added per probe. Cells were vortexed vigorously and incubated for 12 hours at 4°C . Using flow cytometry analysis, the PI-stained nuclear particles could be visualized as G_1/G_2 and sub- G_1 -peaks. Specific nuclear fragmentation/apoptosis was calculated from the relative size of the sub- G_1 -peaks using the following formula:

$$\% \text{ specific nuclear fragmentation} = \frac{\text{sub-G}_1\text{-peak (assay) minus spontaneous sub-G}_1\text{-peak}}{100 \text{ minus spontaneous sub-G}_1\text{-peak}} \times 100\%$$

RT-PCR for classical and non-classical HLA class I molecules

RNA was isolated using the Qiagen kit (Qiagen, Hilden, Germany). For RT-PCR, the RNA PCR Kit from Perkin-Elmer (Perkin-Elmer Foster City, CA, USA) was used, according to the manufacturer's instructions. The following primer pairs were used: B-actin (Stratagene), HLA-A and HLA-B,³⁶ HLA-C,³⁷ HLA-E and HLA-F³⁸ and HLA-G.³⁹ Primers were synthesized by Thermo-Hybaid™, Ulm, Germany. The PCR programs were chosen according to the indicated protocols. Electrophoresis was done using 100 V and 400 mA for 40 min on a 0.5 % agarose gel; for detection, we used SybrGreen™ (FMC Bio-products, Rockland, ME, USA) and an ultraviolet camera.

Analysis of surface HLA expression by flow cytometry

For the analysis of HLA class I expression, first, the monoclonal pan-HLA class I-antibody W6/32 (Mouse monoclonal IgG2a, Dianova, Hamburg, Germany) was used, with phycoerythrin-conjugated goat-anti-mouse-IgG (Serotec, Oxford, UK) as the secondary antibody. PBMC from healthy donors served as a positive control, and K562 cells as a negative control.

For analysis of HLA-A expression we used a biotinylated HLA-A1/36 and HLA-A30/31 monoclonal antibody (One Lambda, Canoga Park, CA, USA) with Streptavidin-Quantum Red (Sigma) as the secondary antibody. For HLA-B, we used a FITC-labeled HLA-B8 monoclonal antibody (One Lambda). For

analysis of HLA-C and HLA-E we used the antibody MEM-E/06 (a generous gift from V. Horejsi, University of Prague, Czechia, and E. Weiss, University of Munich, Germany), which recognizes most HLA-C molecules and HLA-E. Goat-anti-mouse (Serotec) was used as the secondary antibody.

After washing in PBS, cells were stained by incubation for 30 min at 4°C in the presence of the given antibodies and washed. Analysis was then done by flow cytometry using a FACScan flow cytometer (Becton-Dickinson).

Doxorubicin/BSO treatment of cells

Doxorubicin (Farmitalia, Milan, Italy) and L-buthionine-(S,R)-sulfoximine (BSO) (Sigma) were freshly dissolved in sterile distilled water prior to the experiments. Cell suspensions were then incubated for the given times in the presence of doxorubicin or BSO. Flow cytometric data were corrected for the autofluorescence of doxorubicin.

Results

NK cytotoxicity to CEM cells is mediated by the lysosomal pathway, CD95-resistant and drug-resistant CEM cells are cross-resistant to NK cells

NK cells lyse their targets by death-inducing ligands or by the lysosomal (mainly perforin/GrzB) pathway. Calcium chelation by EGTA can be used to discriminate the two pathways, since this chelation inhibits degranulation, but not activation of death-inducing ligands.^{13,21} We found that cytotoxic death of CEM cells (CEM-S) by short-term activated NK cells is nearly completely inhibited by EGTA (Figure 1A), indicating the predominant action of the lysosomal pathway. Interestingly, CD95-resistant (CEM-CD95-R) and doxorubicin-resistant (CEM-doxo-R) CEM cells were equally lysed by NK cells of some donors, while NK cells of other donors lysed only CEM-S, but just marginally affected CEM-CD95-R and CEM-doxo-R cells (Figures 1B-D), indicating that the targets were cross-resistant to these NK cells.

GrzB-induced nuclear fragmentation is similar in sensitive and resistant CEM cells

Since GrzB, cytotoxic drugs and CD95-L utilize the caspase-dependent apoptosis machinery in target cells, we wondered whether cross-resistance of doxorubicin-resistant and CD95-resistant CEM cells towards NK-cell cytotoxicity was due to alterations of apoptosis pathways. Since perforin can be substituted by streptolysin O (SLO) to promote GrzB-induced apoptosis,²³ we established an assay demonstrating nuclear fragmentation induced by GrzB in SLO-permeabilized cells (Figure 2A).^{23,35} G_1/G_2 peaks of intact nuclei and the sub- G_1 peak (see marker M) of fragmented nuclei could be iden-

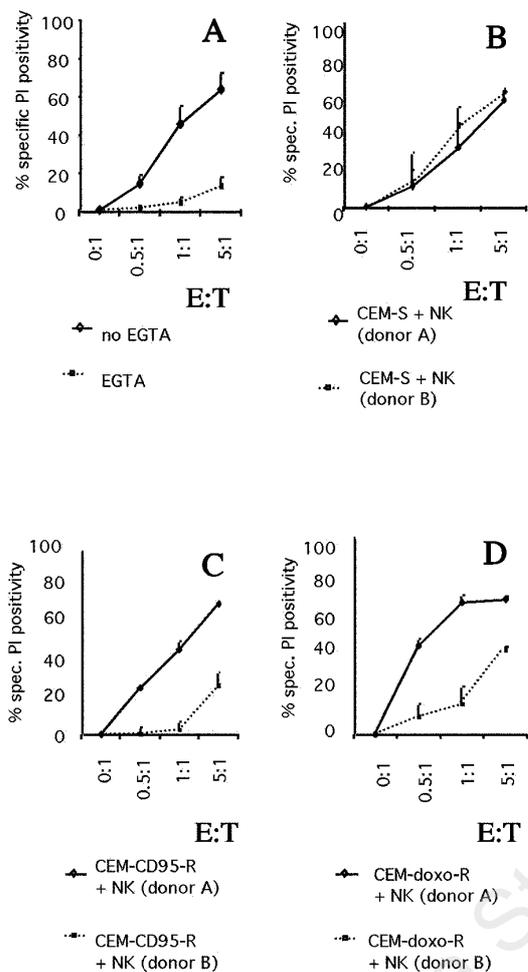


Figure 1. NK cytotoxicity to parental CEM cells (CEM-S) can be inhibited by EGTA. CD95-resistant (CEM-CD95-R) and doxorubicin-resistant (CEM-doxo-R) CEM cells are cross-resistant towards cytotoxicity by NK cells from some donors. (A) NK cytotoxicity to CEM-S at different effector:target (E:T) ratios was measured as specific propidium iodide positivity in a two-color flow cytometric assay in the absence (black line) or presence (dotted line) of EGTA (5 mM). (B-D) By testing polyclonal NK cells from several healthy donors, NK cells were identified in which cytotoxicity towards parental CEM-S was comparable to that to CD95-resistant (CEM-CD95-R) and doxorubicin-resistant (CEM-doxo-R) CEM cells. NK cells from other donors displayed substantial differences in their cytotoxicity to the two targets. Prototypic data for NK cells from a donor A and donor B are shown. Cytotoxicity was determined as described in Figure 1A. Data are given as mean value and standard deviation of a typical experiment out of several triplicate ones.

tified. The derived specific nuclear fragmentation suggested that SLO or GrzB alone did not induce nuclear fragmentation, while the combination of both compounds was highly effective (Figure 2A).

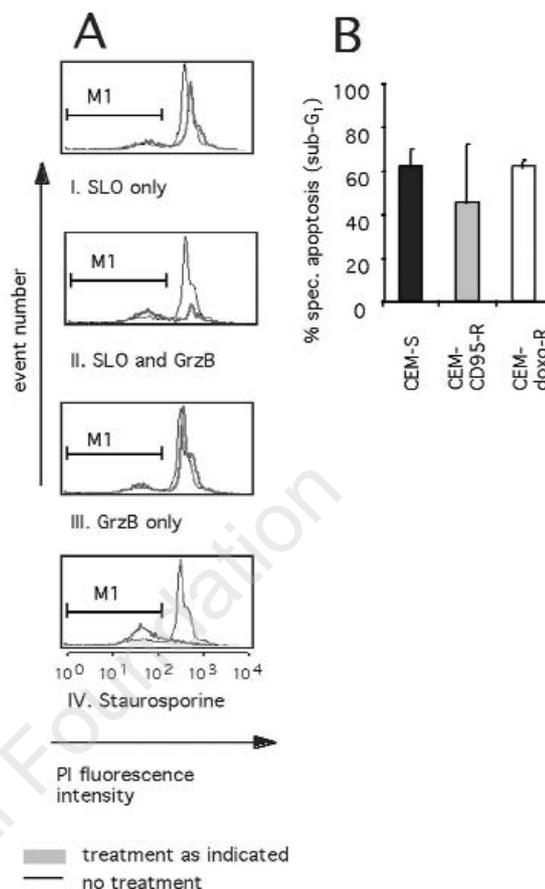


Figure 2. Nuclear fragmentation by GrzB (3 mg/mL) in streptolysin O SLO-permeabilized CEM-S, CEM-CD95-R and CEM-doxo-R cells. (A) In CEM-S cells, modified *Nicoletti* staining of nuclei is shown after 4 hours' incubation in medium (slim black background curve in each histogram). The G₁/G₂ peaks can be seen on the right side, while the low peak on the left, indicated by the marker M, represents the sub-G₁ peak. After 4 hours' incubation with SLO only (overlaid curve, Figure 2A-I) the sub-G₁ peak shows no change, just as after incubation with GrzB alone (3 µg/mL, Figure 2A-III). After incubation with SLO and GrzB (II), however, there is an increase of the sub-G₁ peak, and loss of the G₁/G₂ peaks, indicating fragmentation of nuclei (see marker). With staurosporin (IV) as the positive apoptosis control, all nuclei were fragmented (in the sub-G₁ peak). One typical experiment out of several repeated experiments with similar results is shown. (B) Using this assay, GrzB-induced nuclear fragmentation was then studied in permeabilized CEM-S, CEM-CD95-R and CEM-doxo-R cells using the assay described in Figure 2A. Sub-G₁ peaks were the same in the sensitive and resistant sublines. Data are given as mean value and standard deviation of triplicate experiments.

Staurosporin-treated cells served as positive controls for apoptosis. Applying this assay on CEM-CD95-R and CEM-doxo-R cells (Figure 2B), we found that the pattern of nuclear fragmentation

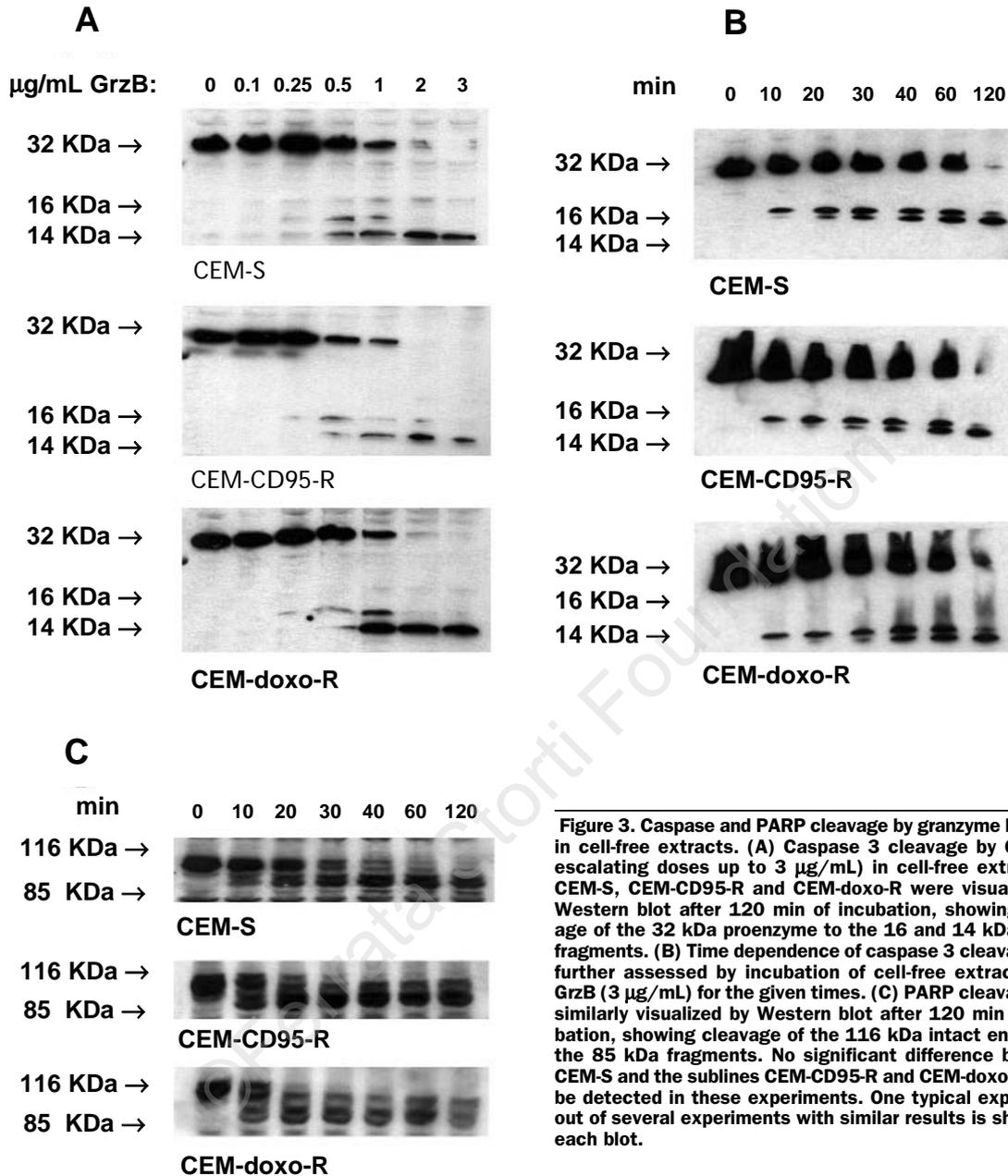


Figure 3. Caspase and PARP cleavage by granzyme B (GrzB) in cell-free extracts. (A) Caspase 3 cleavage by GrzB (in escalating doses up to 3 µg/mL) in cell-free extracts of CEM-S, CEM-CD95-R and CEM-doxo-R were visualized by Western blot after 120 min of incubation, showing cleavage of the 32 kDa proenzyme to the 16 and 14 kDa active fragments. (B) Time dependence of caspase 3 cleavage was further assessed by incubation of cell-free extracts with GrzB (3 µg/mL) for the given times. (C) PARP cleavage was similarly visualized by Western blot after 120 min of incubation, showing cleavage of the 116 kDa intact enzyme to the 85 kDa fragments. No significant difference between CEM-S and the sublines CEM-CD95-R and CEM-doxo-R could be detected in these experiments. One typical experiment out of several experiments with similar results is shown for each blot.

was identical in sensitive and resistant cells. This indicated that the apoptosis-mediating signaling pathway downstream of GrzB in the resistant cell lines was intact.

GrzB-induced caspase 3, caspase 8 and PARP cleavage is similar in sensitive and resistant CEM cell extracts

We next asked whether the effect of GrzB was similar in the different sublines at the level of cas-

pase activation, using cell free extracts. GrzB-induced cleavage of caspase 3 and caspase 8, as well as cleavage of the prototype caspase substrate poly(ADP-ribose)polymerase (PARP) were detected by immunoblotting.^{15,24} Cleavage of caspase 3 (Figures 3A,B), caspase 8 (*data not shown*) and PARP (Figures 3C) by GrzB (up to 3 µg/mL) was complete within 2 hours of incubation in a concentration- and time-dependent manner. Interestingly, there appeared to be no differences between CEM-S and

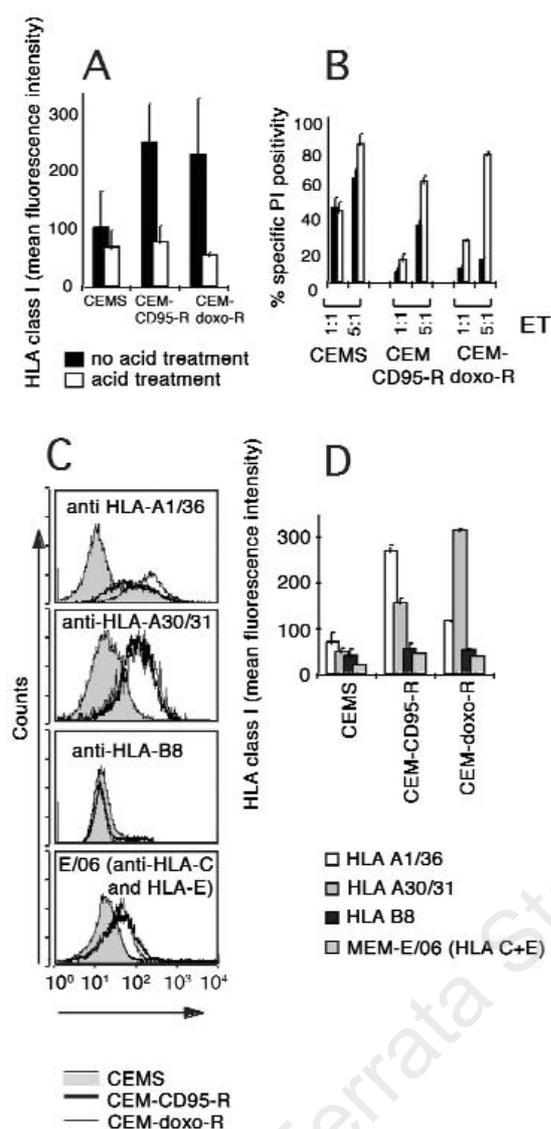


Figure 4. HLA class I-expression in sensitive and resistant CEM cells before and after acid treatment. Sensitization of resistant CEM cells to NK cytotoxicity by acid treatment. HLA-class I allelic expression on different CEM cells. (A) HLA class I expression of CEM-S, as detected by flow cytometry using the antibody W6/32, is much lower than that of CEM-CD95-R and CEM-doxo-R cells, and is downregulated in all cell lines after acid treatment. (B) Cytotoxicity towards CEM-S, CEM-CD95-R, and CEM-doxo-R cells by activated NK cells (donor type B) was measured after downregulation of HLA-class I by acid treatment and compared to cytotoxicity to untreated cells. Cytotoxicity was determined as described in Figure 1, showing sensitization by acid treatment in CEM-CD95-R, and in particular, CEM-doxo-R cells, while cytotoxicity was not altered in CEM-S cells. (C) Surface expression of HLA-A1 (shown by an HLA-A1/36 antibody), HLA-A31 (HLA-A30/31 antibody), HLA-B8 (HLA-B8 antibody), HLA-C and HLA-E (by the MEM-E/06 antibody) of CEM-S, CEM-CD95-R and CEM-doxo-R cells was determined by staining with the respective antibody and flow cytometry. One typical histogram is given. (D) Mean fluorescence intensity of the data shown in (C). Data are given as mean value and standard deviation of triplicate experiments in panels A, B and D.

the resistant sublines CEM-CD95-R and CEM-doxo-R (Figures 3A-C). Thus, no difference in GrzB-induced caspase activation could be identified in the resistant CEM cells. Therefore cross-resistance cannot be attributed to differences in intracellular molecules or events downstream of GrzB.

Resistance of CEM-CD95-R and CEM-doxo-R cells is due to increased HLA class I expression; downregulation leads to sensitization

Since we found no difference in the apoptosis machinery, we asked whether resistance to NK cells was mediated by alterations in target recognition. Since the activity of NK cells is negatively regulated by inhibitory receptors which interact with HLA class I molecules, we studied HLA class I expression on sensitive and resistant CEM cells. Using the pan-HLA class I-antibody W6/32, we found that CEM-S expressed substantially lower levels of HLA class I molecules than did either resistant subline (Figure 4A, black bars). To study the role of HLA class I expression in sensitivity to lysis, we used short-time acid treatment (4 min, pH 4),³⁴ which destroys HLA class I molecules, as detected by the W6/32 antibody (Figure 4A). There was no evidence of toxic cell damage, as demonstrated by unchanged trypan blue exclusion of the cells (*data not shown*). Interestingly, acid treatment reversed resistance to NK cells completely in CEM-doxo-R, and partly in CEM-CD95-R cells. Cytotoxicity towards CEM-S cells, however, was unaffected (Figure 4B). This suggested an important role of HLA class I expression in drug/NK cross-resistance of CEM cells.

HLA class I upregulation in resistant CEM cells consists of HLA-A, C and E surface expression

We analyzed the pattern of HLA class I molecules in NK resistant CEM-doxo-R and CEM-CD95-R cells, using monoclonal antibodies specific for classical HLA class I epitopes. According to the HLA typing of CEM cells,²⁹ we examined surface expression of HLA-A and HLA-B by staining with the monoclonal antibodies HLA-A1/36, HLA-A30/31, and HLA-B8, and flow cytometry. Compared to in CEM-S, HLA-A1 was strongly upregulated in CEM-CD95-R and to a lesser extent in CEM-doxo-R cells, while HLA-A31 expression was enhanced both in CEM-CD95-R and in CEM-doxo-R. However, HLA-B8 expression was identical in CEM-S, CEM-CD95-R and CEM-doxo-R cells (Figures 4C-D). To study HLA-C and HLA-E expression, we used the monoclonal mouse antibody MEM-E/06, which stains both HLA-C and HLA-E molecules. Compared to expression in the parental cells, a substantial upregulation was found in the CEM-CD95-R and CEM-doxo-R cells (Figures 4C-D). By RT-PCR, we found that all CEM variants were negative for both

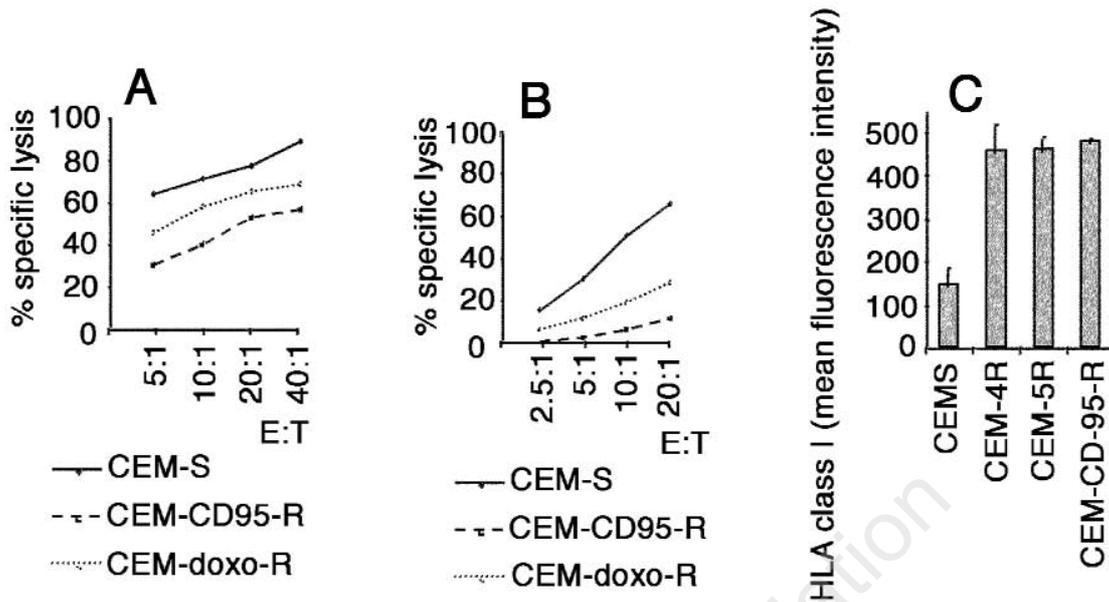


Figure 5. CEM-CD95-R and CEM-doxo-R are partially resistant to the NK cell line NKL and almost completely resistant to the NK cell line B.3NK, while CEM-S are highly sensitive to both. Gradual upregulation of HLA class I upon continuous incubation in the presence of anti-CD95-antibodies. Cytotoxicity assays of CEM-S, CEM-CD95-R and CEM-doxo-R cells using the NK cell lines NKL (A) and B.3NK (B) as effector cells. These assays were performed using a chromium release protocol. While CEM-S are highly sensitive to both NK lines, CEM-doxo-R and, to an even greater extent, CEM-CD-95-R displayed reduced sensitivity towards NKL cells. This cross-resistance is even more pronounced in B.3NK cells. One typical experiment out of three experiments with similar results is shown for each assay. (C) CEM cells treated with only four or five rounds of anti-CD95-antibody to induce CD95-resistance (CEM-CD95-4R, CEM-CD95-5R) showed gradual increase of HLA-class I-expression, indicating that it was indeed the process of anti-CD95-treatment which induced HLA-class I upregulation. Data are given as mean value and standard deviation of triplicate experiments.

HLA-F and HLA-G, whereas the RNA levels of HLA-A, HLA-B and HLA-C were comparable to the flow cytometric data (*data not shown*). Using functional assays we next analyzed whether cross-resistance of the CEM sublines to activated random donor NK cells was mediated by HLA-C or HLA-E upregulation, using two NK cell lines with well defined inhibitory patterns (Figures 5A-B). The cell line NKL³⁰ expressing CD94/NKG2A and CD85, is inhibited by HLA-E and HLA-G, but not by HLA-C. Since CEM cells do not express HLA-G, this cell line can be used as a specific indicator of functional HLA-E expression. B.3NK cells³¹ only express KIR p58.2 (CD158b), which recognizes HLA-Cw7. This cell line is negative for NKG2A, and cannot be inhibited by other HLA epitopes.³¹ Therefore, this cell line can be used as a specific indicator of functional HLA-Cw7 expression in our system. Using these NK cell lines, we found some cross-resistance of CEM-CD-95-R and CEM-doxo-R in NKL cells, and strong cross-resistance in the B.3NK cells (Fig-

ures 5A-B). These data show that HLA-A, C and E epitopes are involved in mediating cross-resistance.

Resistance induction by continuous incubation with anti-CD95 antibodies is accompanied by gradual upregulation of HLA class I

We then investigated whether upregulation of HLA class I was directly accompanied by resistance induction or whether it is a phenomenon accidentally correlated with long-term cultivation of resistant sublines, which had been cultivated separately from the parental cells for >6 months. We, therefore, studied CEM cells which had undergone only 4 or 5 rounds of continuous treatment with anti-CD95-antibody (CEM-CD95-4R, CEM-CD95-5R). By W6/32 staining, we found that these cells did indeed show gradually elevated HLA class I-expression as compared to the parental cells (Figure 5C), suggesting that the level of upregulated HLA class I directly corresponds to the increase in resistance.

Short-time incubation with doxorubicin does not induce HLA class I upregulation in sensitive cells; glutathione depletion does not revert this in resistant cells

The next question was whether HLA upregulation in the doxorubicin-resistant cells was a direct effect of the cytotoxic drugs, or whether it was acquired during the long-term selective pressure applied by continuous incubation of the cells in the presence of low doxorubicin concentrations. We, therefore, studied HLA class I expression (HLA-A31) in sensitive and resistant cells treated with doxorubicin (0.01 µg/mL) for 24 and 48 hours. No significant alteration of HLA class I expression was observed in CEM-S cells. CEM-CD95-R and, to some extent, CEM-doxo-R cells even showed further upregulation of HLA class I (Figure 6A).

Resistance of targets towards cytotoxic drugs and CD95 may be further influenced by the redox status of cells. Drug-resistant CEM cells have been shown to express higher amounts of the radical scavenger, glutathione, than do the parental cells,^{5,40} and to become apoptosis-sensitive upon downregulation of glutathione. Therefore, we asked whether HLA upregulation could be reversed by glutathione depletion using buthionine-(S,R)-sulfoximine (BSO), which downregulates glutathione levels. However, after incubation with BSO for 24 or 48 hours, no significant downregulation of HLA class I was observed (Figure 6B).

Therefore, it can be excluded that glutathione plays a relevant role in the upregulation of HLA class I, and both glutathione and HLA class I upregulation must be induced independently upon continuous incubation in the presence of low concentrations of doxorubicin.

Screening of other CD95-resistant and drug-resistant cell lines for HLA class I expression and NK sensitivity

To examine whether HLA class I upregulation in resistant CEM cells might be a general phenomenon of apoptosis-resistant cells, we screened several parental, CD95-resistant and drug-resistant leukemia cell lines for HLA class I expression, and for resistance to NK cells. In CD95-resistant and doxorubicin-resistant sublines of the T-leukemia cell line J16 (J16-CD95-R and J16-doxo-R), HLA class I upregulation, as observed in CEM cells, was not found, and the sensitivity towards activated NK cells was identical in parental and resistant lines (Figure 6C). In the B-lymphoma cell line, Nalm6, we studied parental cells and cyclophosphamide-resistant or doxorubicin-resistant sublines (Nalm6-cyclo-R, Nalm6-doxo-R). As in the J16 cells, no HLA class I upregulation was found and parental as well as apoptosis-resistant cells displayed comparable sensitivity towards activated NK cells (Figure 6C). In the non-small cell lung cancer line, P693, HLA class

I expression was the same in wild-type cells and in a cisplatin-resistant subline (Figure 6D). These findings suggest that the effect of NK cross-resistance by HLA-class I upregulation present in CEM cells may be restricted to subsets of malignant cells.

Discussion

Here, we report a novel mechanism of cross-resistance to NK-cell cytotoxicity in apoptosis-resistant leukemia cells. We investigated the sensitivity of drug- and CD95-resistant CEM cells to NK cytotoxicity, demonstrating cross-resistance to NK cells from several donors. This was not due to alterations in the apoptosis pathway, but to a modified pattern of HLA class I expression which was acquired in parallel with induction of apoptosis resistance. Since NK cells lyse CEM cells exclusively by the EGTA-inhibitable perforin and GrzB lysosomal pathways, NK resistance is independent from the CD95 system. Thus, it appeared most likely that the cross-resistance should be due to factors that drug-, CD95-, and GrzB-induced cell death have in common, i.e. the downstream apoptosis cascade. Unexpectedly, we found no alterations in this pathway, as shown by identical GrzB-induced cleavage of caspases and PARP and identical nuclear fragmentation in sensitive and resistant cells. Apoptosis-inhibiting agents acting downstream of GrzB, such as Bcl-2 family members,⁴¹ IAPs (inhibitors of apoptosis),¹⁰ and PI-9 (proteinase inhibitor 9),⁹ could therefore be excluded from playing a role.

By studying mechanisms upstream of perforin/GrzB, i.e. at the level of NK/target recognition, we found a substantial upregulation of HLA class I expression in the resistant sublines. This phenomenon correlates with NK resistance, since lytic activation against target cells strongly depends on engagement of inhibitory receptors, which bind HLA class I molecules.²⁶⁻²⁸ This is further highlighted by the fact that degradation of HLA class I epitopes restores NK sensitivity in resistant CEM cells. In particular, we show that resistance is due to upregulation of HLA-A as well as HLA-C and HLA-E molecules, leading to NK inhibition by binding to the appropriate inhibitory receptor p58.2 and CD94/NKG2A, respectively. Upregulation of HLA-E is probably due to stabilization by HLA-A1- and HLA-A31-derived signal peptides.⁴²⁻⁴⁴ These mechanisms explain both the cross-resistance and the diversity in NK cells from various donors, since expression of CD158a/b and CD94/NKG2A has been described to vary widely within the normal population.

HLA class I expression is regulated by a number of transcription factors, such as the STAT1/ISRE pathway, which acts in a short-term manner, e.g. transmitting cytokine signals within hours,^{45,46} and others, such as the NFκB (nuclear factor κ-B) path-

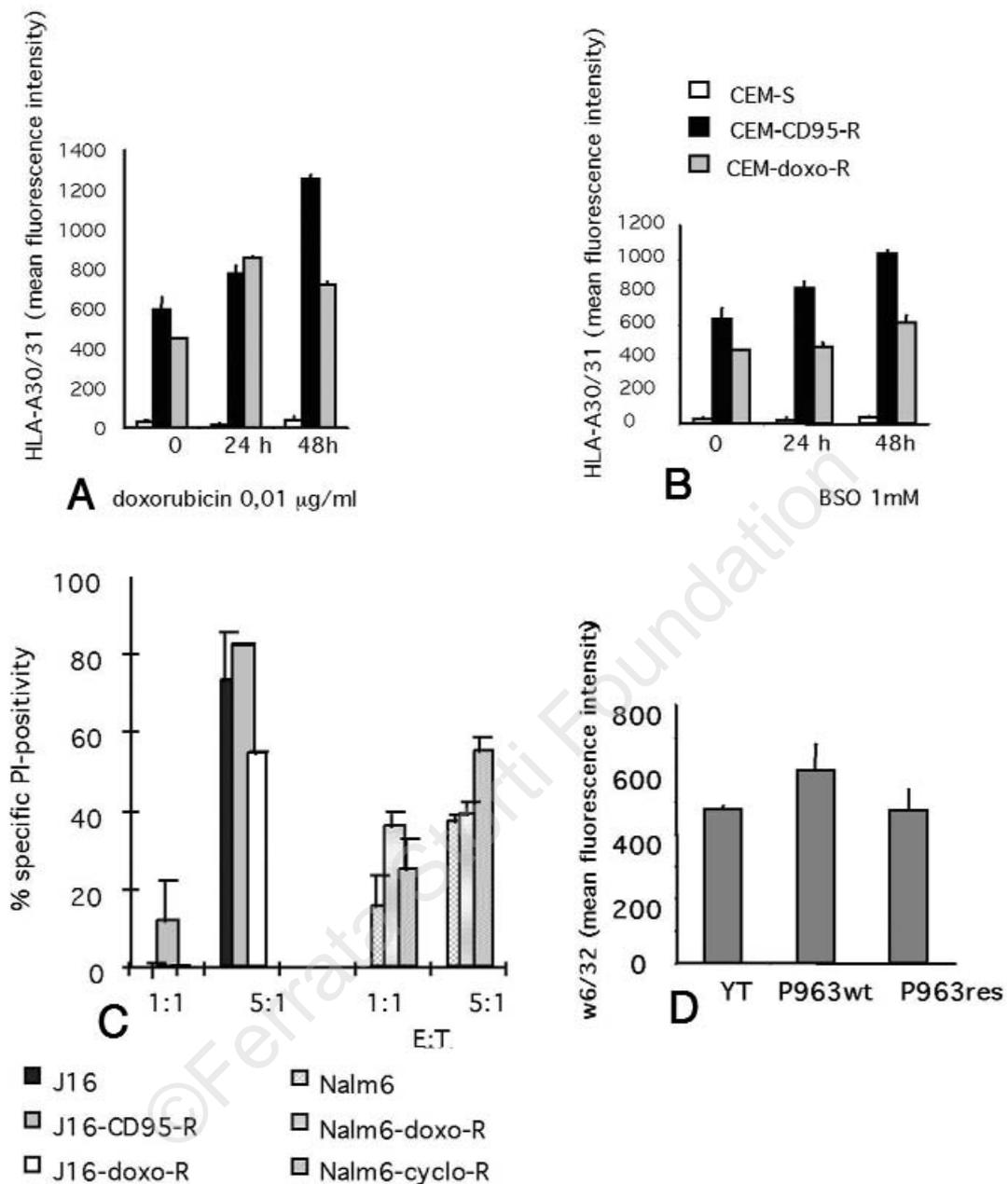


Figure 6. Short-time incubation in the presence of doxorubicin does not induce HLA class I upregulation in CEM-S. Glutathione depletion by BSO does not revert HLA class I upregulation in resistant cells. NK sensitivity of other resistant leukemia cell sublines is the same as in parental cells. (A) HLA class I expression (HLA A31) in sensitive and resistant cells was studied after short-term doxorubicin treatment (0.01 µg/mL). The data were corrected for the autofluorescence of doxorubicin. HLA class I expression in CEM-S did not change significantly after 24 or 48 hours, while CEM-CD95-R and, to some extent, CEM-doxo-R cells showed even further upregulation of HLA class I. (B) Treatment with BSO, to deplete glutathione, did not revert HLA class I upregulation in the resistant cells, but led to further upregulation. (C) NK sensitivity was also studied in other leukemia cell lines and their resistant sublines: J16 cells, the CD95-resistant subline, J16-CD95-R and the doxorubicin-resistant subline, J16-doxo-R, showed NK sensitivity equal to that of the parental cells. Nalm6 cells, the cyclophosphamide-resistant subline, Nalm6-cyclo-R, and the doxorubicin-resistant subline, Nalm6-doxo-R, showed NK sensitivity equal to that of the parental cells. Cytotoxicity assays were performed as in Figure 1, using NK cells of donor type B. (D) HLA class I expression (mean fluorescence intensity in W6/32 staining) was studied in the non-small cell lung cancer line, P693 and its cisplatin-resistant subline: compared to the YT control cell line, both wild-type P693 (P693wt) cells, and its cisplatin-resistant subline, P693res, showed equal HLA class I expression. Mean values and standard deviations of triplicate experiments are given in Figures 6A-D.

way,⁴⁷ which may also be constitutively activated in certain cells. We found that upregulation of HLA class I is not an immediate effect of doxorubicin in short-term assays, but is acquired in the slow process of selection pressure acting over weeks upon continuous incubation in the presence of doxorubicin or anti-CD95-antibodies. Furthermore, it is not reversed even after weeks of incubation in the absence of these agents (*data not shown*), suggesting a mechanism of constitutive upregulation. Intracellular glutathione content, which has been shown to be upregulated in the resistant CEM sublines^{5,40,48} is not involved, since glutathione depletion by BSO failed to reverse HLA upregulation.

Compared to other studies showing constitutive alterations of HLA expression, our model is quite unique. Viral infections may lead to either downregulation or upregulation of HLA class I.^{49,50} Cisplatin induces HLA class I upregulation in gastric cancer cells⁵¹ and cisplatin-resistant ovarian carcinoma cells show higher responsiveness to interferon- γ than do parental control cells.⁵¹ HLA downregulation in tumor progression, for example in melanoma metastases, has been described by several groups.⁵²⁻⁵⁵ HLA class I expression in acute myeloid and lymphoblastic leukemias has only been addressed by one recent study,⁵⁶ in which the expression was evaluated at primary presentation, during remission, and at relapse. In most patients, HLA class I expression was no different from that of normal cells, although in individual patients, loss or downregulation was seen. Since all these observations are individual and not defined by a molecular mechanism, a general pattern regulating HLA class I modulation in cell lines does not seem to exist. Upregulation of individual HLA loci, in parallel with the acquired resistance towards CD95 or doxorubicin, which we found in CEM cells, has not so far been described.

Since downregulation of HLA class I antigens can act as a mechanism of immune escape from T-cell cytotoxicity, e.g. in virus infections,^{49,50} the same mechanism has been considered in malignant cells, and, for years, attempts have been made to enhance antigen presentation by the HLA system in order to improve cytotoxic responses. Upregulation of HLA class I expression on resistant leukemic cells could render such cells more easily recognizable by cytotoxic T-lymphocytes. This has not been analyzed in our work. However, especially in leukemias, tumor-associated antigens are often not very immunogenic. Furthermore, as shown recently, graft-versus-leukemia effects may largely depend on NK cells, since the lack of expression of the KIR ligand on mismatched allogeneic cells was shown to trigger NK cell alloreactivity.⁵⁷⁻⁵⁹ In an analogous way, downregulation of HLA class I may trigger NK cells by the lack expression of the KIR ligand.

If NK cells play a crucial role in antileukemic cytotoxicity, the role of HLA class I-expression in malignant cells may need to be redefined. Our finding of HLA class I upregulation in a resistant cell line may reflect a relevant mechanism counteracting antileukemic immune responses. Obviously, this phenomenon cannot be generalized, since cells of the resistant sublines of J16 and Nalm6, to give only two examples, showed neither HLA class I expression alterations nor cross-resistance towards NK cells. To estimate the role that this phenomenon could play in antileukemic therapy *in vivo* is difficult. On the one hand, cell lines growing *in vitro* represent only a selected subgroup of the leukemias occurring in patients, on the other hand, generation of drug resistance *in vitro* may differ from the processes leading to drug resistance *in vivo*. We believe that, to understand antileukemic cytotoxicity in individual leukemias, the components that contribute to sensitivity or resistance, recognition molecules, lysosomal enzymes or death inducing ligands, and downstream apoptosis execution must be assessed separately. Using this approach, we demonstrate a novel resistance mechanism in CEM cells, which is drug-induced upregulation of HLA class I subtypes, leading to resistance towards NK cells. This mechanism may be an important factor in drug-pretreated leukemias, inducing resistance to NK-mediated graft-versus-leukemia effects after bone marrow transplantation.

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Pre-publication Report & Outcomes of Peer Review

Contributions

CFCl: basic conception and design of the study, performance of most experiments, interpretation of data, drafting of the article, final approval. CSF: conception of subsets of the study concerning HLA/NK interaction, analysis and interpretation of data, performance of experiments shown in Figures 5 a and b, final approval. CF: conception of some subsets of the study concerning drug resistance, analysis and interpretation of data, generation of resistant cells, final approval. SF: conception of some subsets of the study concerning molecular analysis of resistance, design of assays, analysis and interpretation of data, critical revision of the paper, final approval. KMD: basic conception of the study, critical revision with substantial contribution to its content, final approval.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received September 10, 2002; accepted March 11, 2003.

In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

HLA class I upregulation in drug-resistant CEM cells has not been reported so far. Also, NK resistance in leukemia cells, induced by HLA class I upregulation in the context of drug resistance, has not been described.

What this study adds

This is the first description of NK resistance mediated by upregulation of HLA class I expression in drug-resistant cells.