BCR-ABL Does Not Prevent Apoptotic Death Induced by Human Natural Killer or Lymphokine-Activated Killer Cells

By Rémi Roger, Cherifa Issaad, Marc Pallardy, Marie-Chantal Léglise, Ali G. Turhan, Jacques Bertoglio, and Jacqueline Bréard

The erythromyeloid cell line, K562, the most sensitive target in human natural killer (NK) cell mediated cytotoxicity, is derived from a chronic myeloid leukemia (CML) patient and expresses the characteristic reciprocal translocation t(9;22). The resulting BCR-ABL fusion protein has been shown to mediate the unusual resistance of K562, and other BCR-ABL expressing lines, to apoptosis induced by a variety of agents (irradiation, UV light, cytotoxic drugs). Here we show that human NK and lymphokine-activated killer (LAK) cells, when tested at low effector to target ratio, can readily induce apoptotic death in K562 cells. This was accompanied with classical DNA oligonucleosomal fragmentation. Similar data were obtained with a pluripotent human hematopoietic cell line, UT-7, infected with a defective amphotropic p210 BCR-ABL retrovirus. The BCR-ABL expressing subclone UT-7/9, while being no longer sensitive to cytotoxic drugs or to tumor necrosis factor, a lytic mediator to which UT-7 cells are sensitive, underwent apoptotic death when exposed to LAK effectors to the same degree as the parental UT-7 line. With these targets, DNA oligonucleosomal fragmentation occurred concomitantly with isotope release. Results obtained with several inhibitors of exocytosis strongly suggest that cytotoxic granules mediate NK and LAK cell-induced apoptotic death. In conclusion, NK and LAK cell-induced apoptotic signals, unlike those activated by chemotherapeutic agents, are unaffected by the antiapoptotic action of BCR-ABL. This unique property may support the observed curative effect of sileionic bone marrow transplantation in CML.

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CELL-MEDIATED cytotoxicity leads to apoptotic death and DNA oligonucleosomal fragmentation of murine targets of hematopoietic origin, including YAC cells, a standard target for mouse natural killer (NK) cell activity. Inasmuch as early studies in the human system did not detect DNA digestion in several human cell lines, particularly the classical NK target K562, a species-specific difference was suggested. That apoptotic death would be solely determined by the species of origin of target cells has been refuted by a subsequent work where extensive T-cell induced DNA solubilization was evident in human lymphoid cells. However, a recent report, comparing YAC and K562 targets exposed to syngeneic or xenogeneic lymphokine-activated killer (LAK) cells concluded that both types of cytotoxic cells killed K562 without inducing the double-stranded DNA fragmentation associated with apoptosis.

The erythromyeloid K562 cell line, the most sensitive target in human NK-cell-mediated cytotoxicity, is derived from a chronic myeloid leukemia (CML) patient and characterized by the presence of the Philadelphia chromosome due to a reciprocal translocation between chromosome 22 and chromosome 9. This translocation, hallmark of CML cells, results in the creation of a chimeric bcr-abl gene and synthesis of a 210-kD fusion protein (p210 BCR-ABL) that displays uncontrolled ABL tyrosine kinase activity and induces cell transformation. Several recent studies indicate that BCR-ABL also influences clonal expansion of CML myeloid progenitors by decreasing the rate of apoptosis. Most interestingly, K562 cells have been found unusually resistant to apoptosis induced by a variety of agents (irradiation, UV light, chemical inducers, protein synthesis inhibitors). Downregulation of BCR-ABL protein in K562 by antisense oligonucleotide renders the cells susceptible to induction of apoptosis by the same agents indicating clearly that bcr-abl can act as an antiapoptosis gene.

In this report, we show that signals generated by human NK or LAK cells lead to apoptotic death of K562 targets and, therefore, seem to circumvent the antiapoptotic effect of BCR-ABL. Furthermore, and in contrast to what has been observed with other apoptosis inducers after BCR-ABL downregulation, NK and LAK cells induce fragmentation of K562 DNA into typical oligonucleosomal fragments. Most importantly, LAK cells were found to induce an equivalent level of apoptosis in a pluripotent human hematopoietic cell line, UT-7, and a BCR-ABL expressing subclone, UT-7/9, obtained by retrovirus-mediated gene transfer. However, the BCR-ABL expressing UT-7 cells were no longer susceptible to apoptosis induced by chemicals or tumor necrosis factor (TNF), as opposed to the parental UT-7 cell line. These results demonstrate that BCR-ABL antiapoptotic activity is not efficient against NK cell-induced apoptosis.

MATERIALS AND METHODS

Cells. Human peripheral blood mononuclear cells (PBMC) as a source of NK cells were isolated by centrifugation through Ficoll-Hypaque.

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Hypaque gradients. LAK cells were obtained by overnight culture of PBMC in RPMI 1640 medium supplemented with 25 mmol/L HEPES, L-glutamine, 10% fetal calf serum, 2% sodium pyruvate, 0.1 mg/ml streptomycin, 100 U/ml penicillin (Complete Medium, CM) with 100 U/ml of recombinant interleukin-2 (IL-2) (Cetus, France). In some experiments, NK cells were purified from PBMC by negative selection. After two rounds of complement-mediated lysis in the presence of anti-CD3 monoclonal antibody, PBMC were incubated with anti-CD3, anti-CD14, and anti-CD19 antibodies (ImmunoTech, Marseille, France), and reactive cells were eliminated with goat antimouse Ig coated immunomagnetic beads (Dynabeads M450; Dynal, Compiegne, France). This last step was performed twice and the resulting population routinely consisted of over 90% CD3+CD56+ lymphocytes. Human tumor cell lines (K562, HL60) were maintained in CM. The human pluripotent UT-7 line, established from a patient with megakaryoblastic leukemia, was routinely cultured in Dulbecco’s Minimal Essential Medium (DMEM) with 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). BCR-ABL gene transfer into UT-7 was accomplished in the laboratory of A.G.T. using a defective amphotropic p210 retrovirus. The UT-7/9 clone used in this study was selected for its high expression of BCR-ABL mRNA. As opposed to UT-7 parental line, UT-7/9 cells are no longer dependent on GM-CSF for their growth. Additional functional properties of this and several other BCR-ABL expressing clones will be presented elsewhere (A.G. Turhan et al, manuscript in preparation).

Chemicals and biologicals. 4,4'-Disothiocyanatostilbene-2,2'-Disulfonic acid (DIDS), 3,4-Dichloroisoucoumarin (DCI), camptothecin, and actinomycin-D, were purchased from Sigma (Saint Quentin-en-Yvelines, France) and dissolved in dimethyl sulfoxide (DMSO). Etoposide (Vesptide; Sandoz, Basel, Switzerland) was obtained at stock concentrations. They were used at final concentrations of 50 and 25 nM/l, respectively.

Results are expressed as percentage of specific 51Cr-release. Maximum 51Cr-radioactivity was determined by counting the entire content of quadruplicate wells containing 5 x 10^5 target cells. Spontaneous release is the amount of radioactivity found in Triton X-100 supernatants of target cells incubated alone.

Isotope release experiments were performed with six different blood donors, and perfectly concordant results were always obtained. Results are presented as representative experiments inasmuch as basic levels of NK-cell killing varies between individuals and error bars of pooled experiments would represent biologic rather than experimental variations. Moreover, it is now widely accepted that conclusions on apoptotic death should be backed up by data from a combination of assay methods. To facilitate the comparison between the various techniques that we used, the figures for isotope release, agarose gel electrophoresis, and morphological studies are derived from experiments performed with the same blood sample.

Morphological assessment of apoptosis in target cells. At the end of 4-hour cytotoxicity assays, effector and target cell pellets were collected on a glass slide by addition of 500 µl of detergent buffer (10 mmol/L Tris-HCl, pH 7.4, containing 5 mmol/L EDTA and 0.2% Triton X-100) for 30 minutes on ice. Fragmented DNA was isolated by high-speed centrifugation on a Beckman Optima TL ultracentrifuge (Beckman, Gagny, France) at 27,000 rpm for 30 minutes. Supernatants were divided in two 250 µl fractions in 1.5 ml centrifuge tubes, and 50 µl of cold 5 mol/L NaCl was added. After vortexing, DNA was precipitated by addition of 600 µl of ethanol and 150 µl of 3 mol/L sodium acetate, pH 5.2 at -80°C for 1 hour. DNA was then isolated by centrifugation at 15,000g for 20 minutes. Pellets of each identical fractions were pooled and resuspended in 400 µl of extraction buffer (10 mmol/L Tris-HCl, pH 7.4, containing 5 mmol/L EDTA). A total of 2 µl of 10 mg/ml DNAse-free RNase was added and the mixture incubated for 5 hours at 37°C. A total of 25 µl of proteinase K (20 mg/ml) and 40 µl of 10X proteinase K buffer (100 mmol/L Tris-HCl pH 8.100 mol/L EDTA and 250 mmol/L NaCl) were added and a further incubation was performed overnight at 65°C. After extraction with phenol and chloroform/isoamylalcohol (24:1) and precipitation with ethanol, purified DNA was subjected to electrophoresis in a 1.2% agarose gel containing 10 µg/ml ethidium bromide. DNA markers (Pharmacia, Saint Quentin en-Yvelines, France) were run in parallel. In some experiments, 125I labeled target cells were used and, after electrophoresis, DNA was hydrolyzed in agarose gel with 0.25 N HCl (10 minutes at room temperature), neutralized with 1 mol/L NaCl, 0.5 N NaOH,
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Fig. 1. Morphological detection of apoptosis in K562 cells. Cells were stained with acridine orange and ethidium bromide. (a) NK effector cells, E/T: 8:1; (b) LAK effector cells, E/T: 4:1. Examples of the different morphological features of target cells mentioned in the text are shown with arrows. Apoptotic cells: perinuclear chromatin condensation (arrow 1); nuclear fragmentation into spherical bodies (arrow 2). Necrotic cell: uptake of ethidium bromide by a nucleus with normal morphology (arrow 3).

RESULTS

NK cells induce apoptosis in K562 targets. NK and LAK cells kill K562 targets very efficiently, and the type of death induced was evaluated morphologically at the end of a standard 4-hour cytotoxicity assay after staining with acridine orange and ethidium bromide. Effector and target cells could easily be identified due to their relative size. These studies showed that NK and LAK cells induced typical features of apoptosis in K562 targets. An example is shown in Fig 1a, a microphotograph taken at a PBMC to target ratio of 8:1. K562 cells at various stages of the apoptotic process can be visualized: perinuclear chromatin condensation (arrow 1) seen in four cells is characteristic of the early morphologic nuclear manifestation of apoptosis, and nuclear fragmentation into spherical beads (arrow 2) presented by four additional cells is a later event. The apoptotic cells had not yet undergone secondary necrosis at this stage, as indicated by the fact that they were not permeable to ethidium bromide. In contrast, the red color in a K562 cell (due to entry of ethidium bromide) together with an intact nuclear morphology (arrow 3) is indicative of a necrotic death (secondary...
Table 1. Comparison of \( ^{51} \)Cr and \( ^{3} \)H-DNA Release at the End of a 4-Hour Cytotoxicity Assay

<table>
<thead>
<tr>
<th>E/T Ratio</th>
<th>( ^{51} )Cr-Release (%)</th>
<th>( ^{3} )H-DNA Release (%)</th>
</tr>
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<tbody>
<tr>
<td>NK (8:1)</td>
<td>27.3 ± 1.8</td>
<td>32.7 ± 1.9</td>
</tr>
<tr>
<td>LAK (4:1)</td>
<td>33.5 ± 1.7</td>
<td>27.7 ± 1.2</td>
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NK and LAK cell activity was measured on K562 targets at the indicated effector to target (E/T) ratios. Results are expressed as mean of quadruplicates ± SD. Spontaneous \( ^{51} \)Cr release: 4.5% of maximal release; spontaneous \( ^{3} \)H-DNA release: 2.7% of total \( ^{3} \)H incorporated.

cytosis seen in vitro at very late stages of apoptosis, and not shown here, is identified by a red coloration of nuclei retaining typical apoptotic features. The same morphological results were obtained with LAK cells and are illustrated in Fig 1b (effector to target ratio 4:1), where a conjugate of an apoptotic K562 cell surrounded by four effector cells can be visualized.

By using graded effector to target ratios, we observed that necrotic death is prevalent at high ratios, whereas lower numbers of effector cells induce primarily apoptosis (not shown). As our interest lies in the apoptotic component of K562 death, all of our studies were performed at ratios of around 10:1 for NK cell cytotoxicity and 5:1 for LAK cells.

Release of \( ^{3} \)H-DNA from \( ^{125} \)IUDR prelabeled cells, as a monitor of nuclear damage, is classically used in cell-mediated cytotoxicity simultaneously with \( ^{51} \)Cr to distinguish between apoptotic and necrotic cell death. As our interest lies in the apoptotic component of K562 death, all of our studies were performed at ratios of around 10:1 for NK cell cytotoxicity and 5:1 for LAK cells.

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DNA oligonucleosomal fragmentation in K562 targets. Release of detergent-soluble DNA, as measured above, shows a loss of integrity of the nuclear matrix, most probably due to lamin proteolysis characteristic of apoptosis and evidenced by sensitivity of nuclear content to triton release (this nonionic detergent disrupts the plasma membrane, but not the inner nuclear membrane of undamaged cells). However, this technique, which does not include a high-speed centrifugation step, could not determine whether, and moreover what type of, DNA fragmentation might have occurred.

Agarose gel electrophoresis, performed at the end of a standard 4-hour cytotoxicity assay in \( ^{125} \)IUDR-labelled K562 cells incubated with NK or LAK cells from several donors showed no oligonucleosomal fragmentation of K562 DNA at this time, even though morphological features of apoptosis and \( ^{125} \)I-DNA release were readily detectable. Inasmuch as this type of DNA digestion is now described as a late event in the apoptotic cascade, electrophoretic mobility of K562 DNA was studied after prolonged incubation time, and classical DNA fragmentation was detected in all cases after an additional 3 to 4 hours coculture. The gel shown in Fig 2 was performed after 7 hours of cocoincubation of target cells and the same effector cells used in Fig 1 and Table 1. HL60 cells, known to easily fragment their DNA and that we found to undergo apoptosis when submitted to LAK cells, were run in parallel as a positive control. Staining of the agarose gel with ethidium bromide (left panel) showed a typical pattern of DNA fragmentation in K562 cells incubated with NK or LAK cells. Define proof of the target cell origin of the digested DNA was shown in the corresponding autoradiogram (Fig 2, right panel). K562 cells incubated alone displayed no chromatin digestion, whereas the known limited level of spontaneous apoptosis observed in HL60 culture was apparent on the gel. Additional extensive DNA fragmentation in HL60 was induced by LAK cells. These results, therefore, established that NK or LAK cells-induced K562 apoptotic death was accompanied by typical oligonucleosomal DNA cleavage.

Resistance of K562 cells to classical apoptosis inducers. To exclude that the K562 line used in this study might have lost its genotypic characteristics, RT-PCR for \( bcr-abl \) translocation was performed. Amplification of cDNA fragments of 579 bp, as expected from cells expressing the \( bcr-abl \) hybrid gene, was evident (not shown).

In concordance with the literature, morphological studies (not shown) confirmed the notion that K562 cells were resistant to all apoptosis inducers tested (camptothecin, \( \text{H}_{2}\text{O}_{2} \), puromycin, actinomycin-D, cycloheximide) except when cells where overgrown by the lack of culture medium renewal leading to nutrients depletion. Agarose gel electrophoresis (Fig 3) indicated that, as expected, no DNA digestion was detected in K562 cells cultured in the presence of actinomycin-D or camptothecin. Despite morphological evidence of apoptosis in the majority of K562 cells in the overcrowded cultures, no oligonucleosomal fragmentation was found. Instead, a DNA smear could be visualized, indicating that some other form of DNA degradation had probably occurred.

HL60 cells, used as a positive control, displayed a typical...
BCR-ABL does not prevent NK cell killing.

HL60 cells treated with several apoptosis inducers. Ethidium bromide stain, H2O2 (1 pmol/L, overnight culture); HL60 with camptothecin; empty lane; HL60 after culture overcrowding for 1 week; cells in each sample. From left to right: DNA markers; HL60 alone; HL60 cultured with actinomycin-D (5 μg/mL, overnight culture); HL60 with H2O2 (1 μmol/L, overnight culture); HL60 with camptothecin (5 μg/mL, 6-hour culture); HL60 after culture overcrowding for 1 week; empty lane; K562 alone; K562 with actinomycin; K562 with camptothecin; K562 after culture overcrowding.

Fig 3. DNA agarose gel electrophoresis of K562 and HL60 cells treated with several apoptosis inducers. Ethidium bromide stain, 10⁶ cells in each sample. From left to right: DNA markers; HL60 alone; HL60 cultured with actinomycin-D (5 μg/mL, overnight culture); HL60 with H2O2 (1 μmol/L, overnight culture); HL60 with camptothecin (5 μg/mL, 6-hour culture); HL60 after culture overcrowding for 1 week; empty lane; K562 alone; K562 with actinomycin; K562 with camptothecin; K562 after culture overcrowding.

Fig 4. Effect of specific inhibitors on %Cr and 125I-DNA release by K562 targets. (A) NK effector cells (E/T: 8:1); (B) LAK effector cells (E/T: 4:1). DIDS: 200 μmol/L, 1 hour preincubation of effector cells and presence during the cytotoxicity assay; EGTA: 1 mmol/L EGTA, 1.5 mmol/L MgCl₂ during the assay; DCI-E: 50 μmol/L, 1 hour preincubation of effector cells; DCI-T: 50 μmol/L, 1 hour preincubation of target cells—in both cases, no DCI during the assay. Results are expressed as mean of quadruplicate wells ± standard deviation (SD).

DNA ladder under the influence of the three chemical inducers used, as well as in overcrowded cultures.

Lytic pathway involved in NK and LAK cell-induced apoptosis of K562 targets. K562 cells are insensitive to TNF-mediated lysis and do not express Fas antigen, and (our own unpublished data), a recently discovered surface molecule that, via interaction with its ligand on effector cells, can transduce an apoptotic signal in cell-mediated cytotoxicity. Therefore, experiments were designed to assess the possible role of granule exocytosis in K562 apoptotic death. As shown in Fig 4, DIDS, known to inhibit degranulation by its blocking effect on Cl⁻ channels, strongly reduced the release of both ⁵¹Cr and ¹²⁵I-DNA by K562 cells submitted to either NK or LAK effectors. Incubation of the same cell mixture in the presence of 1 mmol/L MgEGTA, which blocks degranulation and prevents perforin polymerization, resulted in total abrogation of release of both isotopes. DCI, a broadly reactive serine esterase inhibitor, which has been shown to neutralize granzyme enzymatic activity and is appropriate for use in intact cells, was also strongly inhibitory. Its action was at the level of effector cells as inasmuch as preincubation of NK or LAK cells with DCI was sufficient to block lysis, with no DCI added during the cytotoxicity assay. In contrast, preincubation of target K562 cells with DCI had no effect on cell lysis. Disappearance of all morphological features of apoptosis with these three agents (not shown) was in perfect correlation with inhibition of isotope release. These results were also in agreement with those obtained by agarose gel electrophoresis (Fig 5), where DNA fragmentation was totally abrogated in the presence of EGTA and barely detectable under the influence of DIDS or DCI.

Sensitivity to LAK cell-mediated apoptosis of UT-7 cell line and a BCR-ABL expressing subclone UT-7/9. K562 cells have genetic lesions in addition to BCR-ABL, which could be suspected of underlying their particular sensitivity to cell-mediated cytotoxicity. To test directly the effect of BCR-ABL on susceptibility to NK cell-mediated apoptosis, we compared two cell lines that are identical except for the presence of BCR-ABL. A pluripotent cell line UT-7/9 and a BCR-ABL expressing subclone, UT-7/9, obtained by retrovirus-mediated gene transfer were studied in parallel. Neither cell line was found to be significantly killed by resting NK cells, and cell-mediated cytotoxicity assays were thus performed with LAK cells. As shown in the representative experiment presented in Fig 6, where triton-soluble ¹²⁵I-DNA values are reported, both parental UT-7 and BCR-ABL expressing subclone UT-7/9 were equally sensitive to LAK cell-induced apoptosis. Most interestingly, UT-7/9 was constantly found to be slightly better killed than UT-7 in all of the experiments performed with six different normal blood donors, whatever the basic level of LAK-cell cytotoxicity for each individual. The data of this representative experiment are presented for the sake of uniformity with Fig 8 as the corresponding experiments were performed the same day and with the same LAK-cell preparation.

In opposition to LAK-cell induced apoptosis, BCR-ABL activity had a dramatic effect on apoptosis induced by chemicals, as shown in Fig 7, where the respective sensitivity of both

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Fig 6. DNA agarose gel electrophoresis of K562 and HL60 cells treated with several apoptosis inducers. Ethidium bromide stain, 10⁶ cells in each sample. From left to right: DNA markers; HL60 alone; HL60 cultured with actinomycin-D (5 μg/mL, overnight culture); HL60 with H2O2 (1 μmol/L, overnight culture); HL60 with camptothecin (5 μg/mL, 6-hour culture); HL60 after culture overcrowding for 1 week; empty lane; K562 alone; K562 with actinomycin; K562 with camptothecin; K562 after culture overcrowding.

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sensitive to specific inhibitors. DNA analysis performed after 7 hours of NK and K562 cells coincubation, E/T ratio: 8:1. Ethidium bromide staining. Lane 1 and 2: K562 and NK cells alone, respectively; lane 3: EGTA/MgCl₂, 1 mmol/L, during the coincubation; lane 4: DCI, 50 μmol/L, 1 hour preincubation of effector cells; lane 5: DIDS, 200 μmol/L, 1 hour preincubation of effector cells, presence during the test; lane 6: empty lane; lane 7: K562 and NK cells, coincubation; lane 8: DNA markers.

Fig 6. LAK cell-induced apoptotic death of UT-7 and UT-7/9 cells. Specific [%DNA release from UT-7 and UT-7/9 targets after a 4-hour coincubation with LAK cells, E/T ratio: 5:1. Results are expressed as mean of quadruplicate wells ± SD.

DISCUSSION

In this report, we show that human NK and LAK cells trigger apoptotic cell death with DNA oligonucleosomal fragmentation in K562 targets. The extensive necrotic target death seen at high effector to target ratios, which are commonly used in human NK cell-mediated cytotoxicity, might have contributed to the lack of detection of K562 apoptotic death in previous reports. If NK cell activity, as measured in in vitro systems has any relevance to the in vivo situation, it seems that low effector to target ratio would be a better model for what may happen in the whole organism, where high ratios are unlikely to occur. Furthermore, target cell elimination by apoptosis in vivo would have little detrimental secondary effect, in contrast to the intense inflammatory response caused by necrosis.

Apoptotic death of K562 was assessed by several means: morphological examination of the dying cells, release of [%DNA-
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Fig 8. DNA agarose gel electrophoresis of UT-7 and UT-7/9 cells submitted to LAK effector cells, cytotoxic drugs, or TNF. UT-7 and UT-7/9: 10⁵ cells per sample, ethidium bromide stain. From left to right for each target: LAK effector cells and targets, E/T ratio: 5:1, 4 hours incubation; targets alone 4 hours; camptothecin, 5 μg/mL, 6 hours; TNF, 25 ng/mL, 18 hours; targets alone, 18 hours; LAK alone, 4 hours.

DNA, and DNA agarose gel electrophoresis. These three methods consistently led to perfectly concordant results with, however, a delayed kinetics for DNA oligonucleosomal fragmentation. It has been recently demonstrated in several studies on apoptosis that formation of large molecular weight DNA fragments occurs before internucleosomal cleavage and is sufficient to cause chromatin condensation. It is possible that, at the end of a standard 4-hour cytotoxicity assay, K562 cells have only undergone a first stage digestion of this kind and then proceed to activate the final stage of fragmentation detected after 7 hours of incubation. In any case, our results clearly show that K562 cells possess and are able to activate the endonuclease responsible for internucleosomal fragmentation and disprove suggestions that the cells may have lost this enzyme. In addition, we found that similar to what has been reported in mouse NK cell activity, DNA and Cr release occur simultaneously, starting at 1 hour (not shown) and increasing in parallel during the 4-hour assay. Inasmuch as nuclear lesions are detectable within 15 minutes of cell contact in mouse T-cell cytotoxicity, a slower kinetics of nuclear damage may represent a general characteristic of NK versus T-cell cytotoxicity.

Our observations and those of others that K562 cells are resistant to other apoptosis inducers single out NK cell activity as unique in its ability to trigger apoptotic death in these cells. Inasmuch as resistance to apoptosis of K562 and other BCR-ABL expressing cells has been ascribed to the action of BCR-ABL chimeric protein, NK cells would thus appear able to circumvent this antiapoptotic activity.

However, the potential protective role of bcl-2 in cell-mediated killing is a controversial issue at the present time. In any case and to clearly assess whether BCR-ABL activity is the sole mediator of the observations reported above, we studied two cell lines that are identical except for the presence of BCR-ABL: UT-7 and a BCR-ABL expressing subclone UT-7/9, generated by retrovirus-mediated gene transfer. All studies performed with these two lines confirmed the results obtained with K562. In particular, UT-7/9 cells were found resistant to apoptosis induced by chemical agents to which the parental UT-7 line was sensitive. In contrast, the level of LAK cell-induced apoptosis was of the same magnitude in both lines, UT-7/9 cells being even slightly more efficiently killed. These results definitely established that BCR-ABL, while displaying a potent antiapoptotic activity against most inducers, is unable to inhibit apoptotic signals generated by cytotoxic NK cells.

In light of these findings, several potential inhibitors of NK cell activity were tested as a preliminary attempt to define the lytic pathway involved. Cell-mediated cytotoxicity is a complex process mediated by several independent and not mutually exclusive mechanisms, such as triggering of the target cell-surface Fas or TNF-receptor proteins, secretion of adenosine triphosphate (ATP) by effector cells, or exocytosis of perforin and granzymes containing cytotoxic granules, the latter pathway only being dependent on the presence of extracellular Ca²⁺. K562 cells are Fas negative, insensitive to TNF, and cytotoxic activity of NK and LAK cells on K562 targets was totally abrogated by the Ca²⁺ chelator Mg²⁺/EGTA. Additional support to the notion that K562 apoptotic death might be mediated by granule exocytosis was provided by the inhibitory effect on cytolyis of the anion channel blocker DIDS, which shuts down degranulation. Furthermore, preincubation of NK or LAK cells with DCL, an efficient blocker of serine esterases and particularly granzymes, inhibited all features of apoptosis in K562 cells, whereas similar preincubation of targets had no effect. Interestingly, both DNA fragmentation and cytoplasmic membrane damage were suppressed, probably due to the involvement of granzymes in the processing of perforin.
as suggested by Hudig et al.\textsuperscript{46} Most importantly, whereas UT-7 are sensitive to TNF, resistance to apoptosis induced by BCR-ABL in UT-7/9 clone was found to apply to TNF, in addition to cytotoxic drugs. These findings indicate that insensitivity to BCR-ABL antiapoptotic activity is not a property of all cytotoxic mechanisms potentially used by NK cells, but rather a specific property of the lytic pathway triggered by granule exocytosis. A recent report, published during the completion of this study, shows that cytotoxic T-cell activity is insensitive to BCR-ABL in a mouse system.\textsuperscript{47} Even though the nature of the lytic pathway involved was not investigated in this particular work, these results indicate that resistance to BCR-ABL antiapoptotic activity must be a property of a cytotoxic mechanism used by both T and NK effector cells, as is the case for granule exocytosis.

On a technical basis, we were intrigued by the fact that \textsuperscript{51}Cr-release was detected in LAK-cell mediated apoptosis despite membrane integrity as judged by the lack of uptake of ethidium bromide. In fact, we found secondary necrosis in apoptotic human cells to be a very late phenomenon, which can take from 1 to 2 or 3 days in vitro, depending on the cell tested and the inducer used. We have investigated this point further, both for granule exocytosis-mediated and for EGTA-resistant LAK-cell cytotoxicity on a variety of targets. These data, which will be presented in a subsequent paper, lead to the conclusion that apoptotic death due to cytotoxic granules is unique in its inducing \textsuperscript{51}Cr release through an intact cytoplasmic membrane in human targets. These results are in contradiction with the current assumption that, in this setting, \textsuperscript{51}Cr release reflects membrane damage due to perforin. At low effector to target ratios, the target seems able to repair the pores formed by sublytic doses of perforin.\textsuperscript{48} A hypothesis is put forward that could explain the concomitant presence of these two apparently incompatible phenomena: \textsuperscript{51}Cr release and cytoplasmic membrane integrity (R. Roger et al, submitted).

Identification of the biochemical events underlying the resistance of NK and LAK-cell induced cytotoxicity rests on a better definition of the key mediators of BCR-ABL antiapoptotic action. However, the likely involvement of cytotoxic granules components in bypassing this inhibitory effect leads to a working hypothesis for further investigations. Proteolytic activity of endogenous enzymes is involved in signal transduction of the apoptotic cascade and appears to play a major role in activation of the nuclease(s) responsible for DNA oligonucleosomal fragmentation.\textsuperscript{49} BCR-ABL-induced defenses in expression or activation of targets endogenous proteases, while interfering with signal transduction triggered by most apoptosis inducers, could potentially be compensated for by the enzymatic activity of granzymes injected in these cells during the cytolytic process.\textsuperscript{50,51} Alternatively, expression of the chimeric BCR-ABL protein is now known to lead to Ras activation\textsuperscript{52,53} and increased expression of p21ras in fibroblast transfectants has been shown to inhibit apoptosis and diminish the availability of a nuclear endonuclease responsible for chromatin cleavage.\textsuperscript{54} However, inasmuch as multiple proteins involved in signal transduction appear to be activated by the BCR-ABL chimeric protein,\textsuperscript{55,56} it remains possible that the inhibitory effect of BCR-ABL on apoptosis is the result of its action at numerous levels rather than on a single substrate.

Standard chemotherapy regimens are largely inefficient in CML and, despite the recent demonstration of an increased survival pattern of patients treated with \textalpha-interferon versus hydroxyurea,\textsuperscript{57} allogeneic bone marrow transplantation during the chronic phase remains the only curative approach for over 50% of patients; this response being related to both the conditioning regimen and the graft-versus-leukemia (GVL) effect.\textsuperscript{58} Moreover, donor lymphocyte transfusions, which also induce a GVL effect, are used with success for adoptive immunotherapy of CML cases relapsing after bone marrow transplantation.\textsuperscript{59} The cellular mechanisms underlying this effect are not yet fully elucidated, and both T and NK effector cells are potential effectors of the antileukemic reaction. Most interestingly, in posttransplant CML patients, the risk of relapse is significantly lower in patients whose IL-2-stimulated PBMC display a significant lytic activity in vitro against host-derived CML targets, and this cytotoxic activity has been ascribed to CD16\textsuperscript{+} and CD56\textsuperscript{+} cells, but not CD3\textsuperscript{+} lymphocytes.\textsuperscript{60} In addition, low-dose IL-2 therapy after T-cell depleted allogeneic bone marrow transplantation, which leads to a major increase in the number of circulating NK cells, can reduce the incidence of disease recurrence without any of the toxic effects due to associated T-cell mediated graft-versus-host disease.\textsuperscript{61} Immunologic intervention is, therefore, the most efficient approach to treat CML patients, and our results showing that NK and LAK cells are able to offset BCR-ABL antiapoptotic action, whereas chemotherapeutic agents do not, may provide a biological explanation for these clinical observations.

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BCR-ABL does not prevent apoptotic death induced by human natural killer or lymphokine-activated killer cells

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