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Dependence of granzyme B-mediated cell death on a pathway regulated by Bcl-2 or its viral homolog, BHRF1

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Abstract

The molecular pathways responsible for apoptosis in response to granzyme B have remained unresolved. Here we present data supporting the notion that granzyme B-mediated cell death is largely dependent on a pathway that is inhibitable by Bcl-2 or its viral analog BHRF1. We used a panel of stably transfected FDC-P1 mouse myeloid cell lines to show that overexpression of functional, wild-type Bcl-2 or BHRF1 rescued cells from granzyme B-mediated apoptosis, whereas mutated (Gly^{145}→Glu) Bcl-2, or wild-type Bcl-2 directed to the plasma membrane conferred no protection. Overexpression of Bcl-2 resulted in inhibition of multiple parameters of apoptosis in response to purified perforin and granzyme B, including DNA fragmentation, changes in light scatter profile indicating cell shrinkage and increased refractivity, loss of mitochondrial membrane potential and inhibited colony formation in clonogenic assays. Nevertheless, when exposed to cytotoxic lymphocytes, FDC-P1 and YAC-1 cells over-expressing Bcl-2 remained susceptible to death imparted by cytolytic granules, irrespective of whether the granules contained granzyme B. Thus, alternative granzyme B-independent pathways can be activated by intact lymphocytes to overcome Bcl-2-like inhibitors of apoptosis, enabling CTLs to overcome potential viral blocks to granzyme B-mediated cell death. *Cell Death and Differentiation* (2000) **7,** 973–983.

Keywords: granzyme; apoptosis; mitochondrion; Bcl-2; BHRF1

Abbreviations: z-VAD-fmk, Val-Ala-Asp-fluoromethylketone; z-FA-fmk, Phe-Ala-fluoromethylketone; CTL, cytotoxic T lymphocyte; NK, natural killer

Introduction

Cytotoxic T cells (CTL) and natural killer (NK) cells kill virus-infected and premalignant cells by two distinct contact-dependent mechanisms. The first involves the generation of a signaling complex and subsequent activation of pro-caspase-8 at the inner leaflet of the target cell membrane, following engagement of cell surface Fas (CD95) by its ligand on the effector cell.[1] This mechanism is indispensable for normal lymphoid homeostasis in mice and humans, and defects are accompanied by progressive accumulation of activated T and B cells and a strong predisposition to auto-immunity.[2] The second mechanism relies upon the release of granule-bound toxins including a membrane-disrupting agent, perforin and granzyme B from the cytoplasm of the effector cell into the target cell.[3] This mechanism plays a major physiological role in protecting the host against virus infection and cellular transformation.[4]

The granzymes are a family of granule-bound serine proteases with diverse substrate preferences.[5,6] They activate molecular pathways that result in apoptosis after accessing target cell substrates in a perforin-dependent manner.[7–13] Like the caspases, granzyme B cleaves after aspartate residues and is highly toxic to target cells[7] because it can both activate caspases directly, and under some circumstances also cleaves caspase substrates directly in the cytoplasm and nucleus.[14] A number of apparently distinct pathways contribute to a cumulative death signal generated by granzyme B. It can activate many caspases *in vitro*,[15–17] but probably initiates caspase activation *in vivo* through cleavage of procaspase-3, followed by activation of caspase-7.[18] Once free in the target cell cytosol, granzyme B also rapidly targets the nucleus[19,20] and cleaves poly(ADPribose) polymerase and nuclear matrix antigen directly, at sites different from those preferred by caspases.[14] Surprisingly, granzyme B is also capable of inducing cell death that is not reliant on caspase activation, through a mechanism that produces minimal nuclear damage, but rather is centered on damage to specific cytoskeletal elements[21,22] (also JAT *et al*, unpublished data). These additional granzyme B-mediated pathways probably act as safeguards for overcoming viruses that express anti-apoptotic molecules such as crmA[23] and p35[21,24] that can delay or even prevent programmed cell death by interfering directly with caspase function.

Certain viruses express Bcl-2-like inhibitors of apoptosis that operate by blocking mitochondrial events essential for some forms of anti-apoptotic death, such as growth factor withdrawal, irradiation and exposure to certain toxins.[25] Examples include BHRF1, a Bcl-2-like molecule expressed by Epstein-Barr virus,[26] the 19 kDa adenovirus E1B protein[27] and ORF 16 elaborated by herpesvirus Saimiri.[28] These molecules all function by suppressing the mitochondrial events of apoptosis including their release of cytochrome c and apoptosis-inducing factor, and by inhibiting mitochondrial membrane depolarization thought to result from opening of mitochondrial permeability

transition pores.[29] We have been interested for some time in whether Bcl-2-like inhibitors can block granzyme B-mediated cell death. Although granzyme B can kill cells through apparently distinct caspase-dependent and -independent pathways,[21,30,31] we have reported that Bcl-2 can completely block cell death mediated by granzyme B.[22] We also found that although Bcl-2 overexpression had no effect on granzyme B uptake into the cell cytoplasm[32] or sensitivity to perform lysis,[22] redistribution of granzyme B to the nucleus was totally inhibited despite co-addition of perforin.[32] Overall, our results strongly suggested that mitochondrial constituents are essential for efficient granzyme B-mediated apoptosis, and that this pathway can be regulated by Bcl-2 and like inhibitors. Despite this, intact cytotoxic T cells can still efficiently kill Bcl-2-expressing target cells provided perforin is present,[22,33,34] indicating that additional perforin-dependent mechanisms can be activated to bypass Bcl-2.

Despite the findings described above, the ability of Bcl-2-like inhibitors to block granzyme B-mediated cell death has remained controversial. While concurring with many of our findings, Macdonald *et al*,[35] recently reported that granzyme B-mediated cell death proceeds through two pathways, one dependent on, and the other apparently independent of, mitochondria. They showed that Bcl-2 overexpression blocked many of the nuclear events of apoptosis, however other measures of cell death such as loss of cell membrane integrity were only weakly inhibited.[35] In the present report, we used assays of clonogenic potential to show that populations of cells over-expressing functional and correctly targeted Bcl-2 or BHRF1 are resistant to granzyme B-mediated cell death. We therefore contend that Bcl-2-like inhibitors regulate an early event in granzyme B-mediated cell death, that is probably controlled at the level of the mitochondrion.

Results and Discussion

In order to determine whether apoptosis mediated by granzyme B is regulated by anti-apoptotic members of the Bcl-2 family and viral Bcl-2 mimics, we generated a series of transfectant cell lines overexpressing human Bcl-2 or its viral analog BHRF1. We elected to firstly express Bcl-2, BHRF1 or mutants of Bcl-2 in factor-dependent FDC-P1 cells, so that the anti-apoptotic function of these molecules could be verified by growth-factor (IL-3) withdrawal. Non-clonal pools of stably expressing FDC-P1 cells were initially characterized by intracellular staining and flow cytometry using mAbs specific for human Bcl-2 or 'Flag' epitope-tagged BHRF1 (Figure 1A). Wild-type human Bcl-2 was expressed at high and relatively uniform levels in virtually 100% of cells in one transfectant pool (FDBcl2.wt). Very similar and uniform expression of a mutated form of Bcl-2 in which Gly^{145} was mutated to Glu was also achieved (FDBcl2.G145E). This mutation has been shown to have no effect on the localization of Bcl-2, but results in loss of anti-apoptotic function coincident with its inability to bind with Bax.[36] We also expressed a Bcl-2 mutant into which was engineered a CAAX motif for farnesylation,[37] resulting in its mis-sorting to the inner leaflet of the plasma membrane rather than to mitochondria (FDBcl2.CAAX). The levels of Bcl-2 expression in these three transfectant pools were similar, as indicated by flow cytometry (Figure 1A). Western blotting confirmed the expression of proteins of the expected size, and that mutant Bcl-2 proteins were expressed at least as efficiently as wild-type Bcl-2 (Figure 1B). We also confirmed that the overexpressed molecules were localized appropriately to the mitochondria or the cell membrane, using immunofluorescence and confocal microscopy (data not shown).

We next assessed the ability of the cell populations to withstand IL-3 withdrawal. BHRF1 and wild-type Bcl-2 bestowed virtually equivalent resistance to cell death, as over 75% of the cells were still alive more than 7 days after IL-3 withdrawal. However, as expected, G145E-mutated Bcl-2 gave no protection and the rates of cell death were equivalent to untransfected (Figure 1C) or mock transfected cells (data not shown). Cells expressing plasma membrane-associated Bcl-2 were also not protected, although cell death (assessed by dye exclusion) was delayed by approximately 24 h in most cells.

Our next experiments examined whether the transfectants were protected from granzyme B-mediated apoptosis. We[30] and others[21] have previously shown that Jurkat cells exposed to granzyme B with perforin are protected from nuclear apoptotic changes when caspase function is inhibited, although cell death is not blocked. In these circumstances, non-nuclear manifestations of cell death including membrane blebbing, release of ^{51}Cr, and changes in light scatter profiles of the cells (reduced forward scatter, increased side scatter) persisted[21] and were accurate predictors of cell death in clonogenic assays.[30] In agreement with previous findings, when FDC-P1 cells expressing Bcl-2 were exposed to granzyme B (30 nM) with sublytic quantities of perforin, we observed complete abolition of both DNA fragmentation (TUNEL staining) and the altered light scatter characteristics of the cells (Figure 2A). In contrast, both cell membrane-bound and G145E-mutated Bcl-2 were unable to prevent DNA fragmentation whereas expression of wild-type Bcl-2 or BHRF1 in mitochondria resulted in complete protection (Figure 2B). The observed resistance of Bcl-2-overexpressing cells to granzyme B was not due to acquired resistance to perforin, as ^{51}Cr release induced by increasing concentrations of perforin was no different in wild-type and Bcl-2-over-expressing cells.[22]

We next attempted to correlate these biochemical and morphological findings with the clonogenic potential of cells treated in the same manner (Figure 3). Consistent with previous findings,[30] FDC-P1 cells pre-incubated with z-VAD-fmk (100 μM) showed no DNA fragmentation after exposure to granzyme B and perforin, yet failed to form clones. By contrast, cells overexpressing wild-type Bcl-2 were able to form clones as efficiently as FDC-P1 cells that were either untreated, or treated with perforin alone. The co-addition of z-VAD-fmk to wild-type Bcl-2-expressing cells had no additional effect on the number of colonies observed, nor did it affect clonogenicity in the absence of the CTL toxins. Collectively, the above data argue strongly that granzyme B induces cell death through a pathway that can be regulated by Bcl-2 or its viral anti-apoptotic mimic BHRF1.

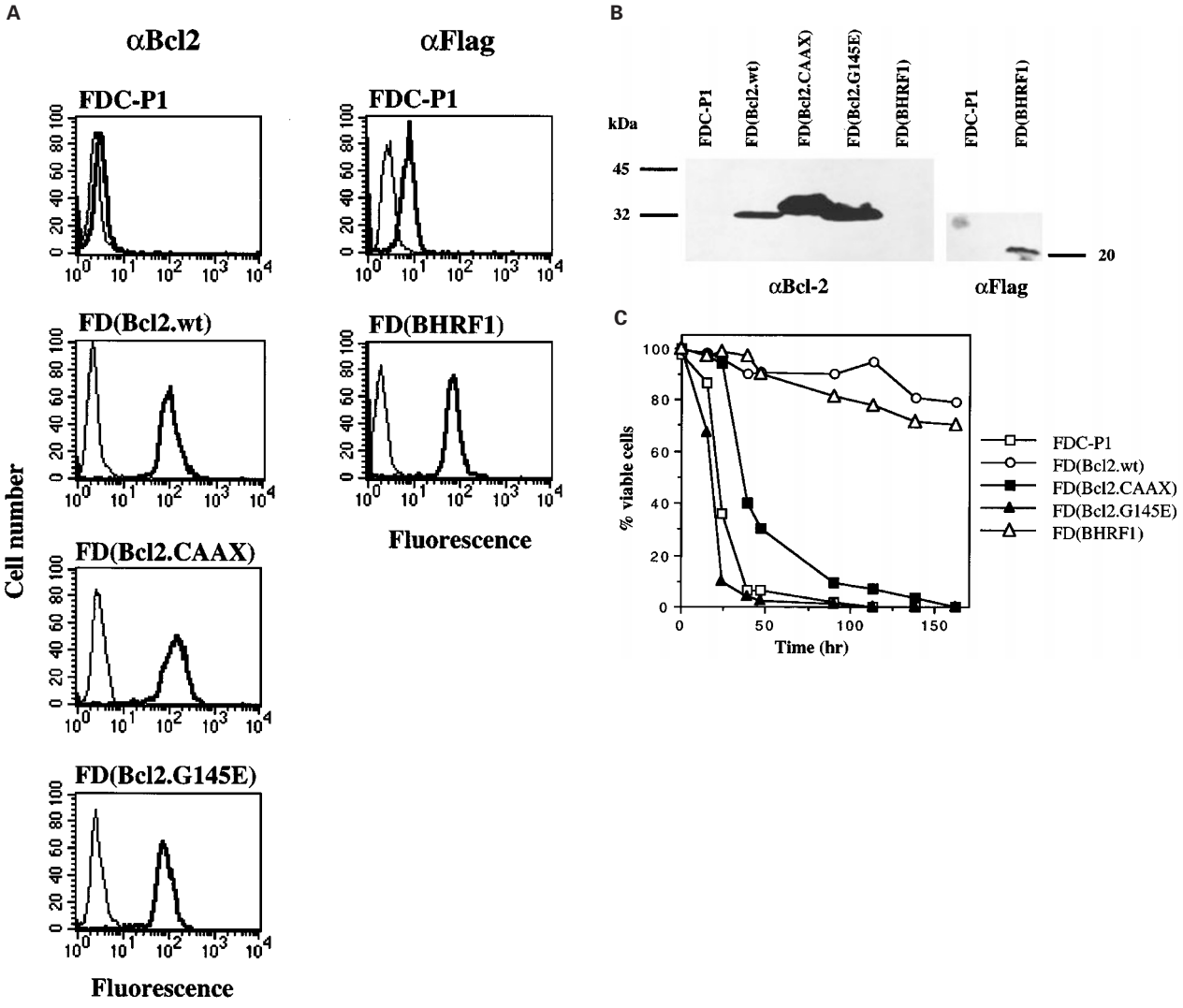

Figure 1 (**A**) Expression and function of Bcl-2, mutant Bcl-2 and BHRF1 in FDC-P1 transfectant populations. Expression of human Bcl-2 and BHRF1 (thick traces) following gene transfection and puromycin selection. Cells were permeabilized, stained and analyzed by flow cytometry. Fluorescence staining is shown in each case by comparison to that seen with the secondary FITC-conjugated anti-mouse reagent alone (thin traces). (**B**) Western blotting analysis following fractionation of cell lysates on SDS – PAGE in the presence of reducing agent. Nylon membranes were probed with the same primary mAbs as in (**A**). (**C**) Survival of transfected cells following IL-3 withdrawal. Cell viability was monitored daily by dye exclusion following resuspension of the cells in medium deficient in IL-3

If mitochondrial disruption is necessary for granzyme B-mediated killing, and granzyme B-mediated cell death is inhibitable by mitochondrial Bcl-2, it would be expected that mitochondrial and post-mitochondrial apoptotic events in response to granzyme B would also be inhibited by Bcl-2. We therefore examined Rhodamine 123 fluxes in Jurkat (Figure 4) and FDC-P1 (data not shown) cell populations expressing uniformly high levels of wild-type Bcl-2. This diffusible dye is concentrated within normal mitochondria, but is lost from mitochondria and diffuses out of cells when membrane depolarization is lost. Bcl-2 expression in Jurkat cells (Figure 4A) resulted in complete abrogation of TUNEL staining and light scatter changes in response to granzyme B (60 nM) and sublytic perforin (Figure 4B). Inhibition of these apoptotic parameters was also accompanied by the suppression of Rhodamine 123 fluxes in cells expressing Bcl-2 (Figure 4C). The release of cytochrome c from mitochondria and procaspase-3 processing were also suppressed by Bcl-2 overexpression, indicating that both events are downstream of an initial disruption of mitochondrial function induced by granzyme B (data not shown).

The ability of Bcl-2 and like inhibitors to completely block cell death in response to granzyme B raises an important issue with regard to CTL-mediated clearance of cells expressing a viral inhibitor such as BHRF1. We have previously reported that purified cytolytic granules from rat NK cells are able to overcome such a block to cell death, in a perforin-dependent manner.[22] To address this issue further and to assess the role played by granzyme B in this process, we raised alloreactive (H2b anti-H2d) anti-

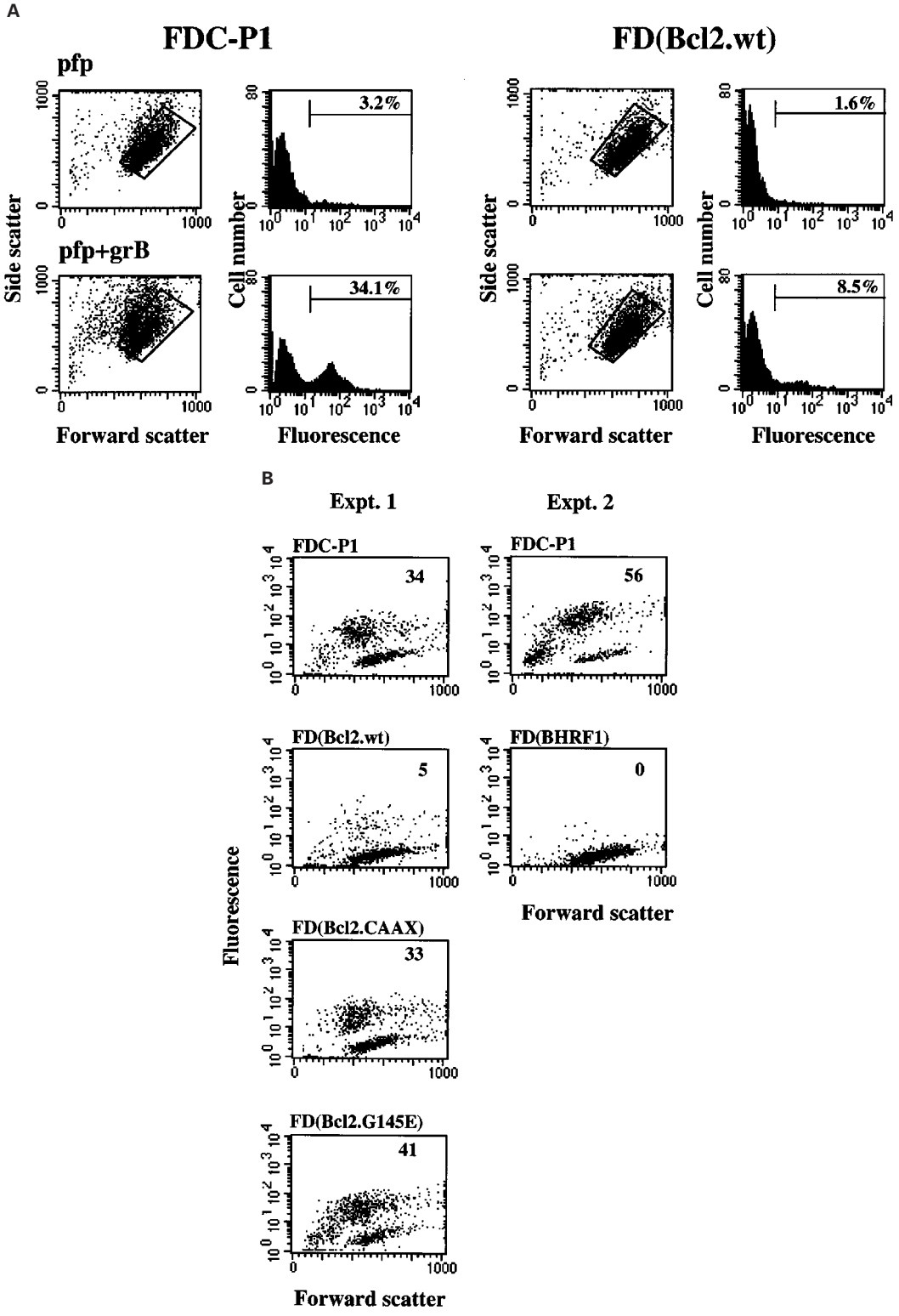

Figure 2 Bcl-2 protects FDC-P1 cells from apoptotic morphological changes and DNA fragmentation in response to granzyme B. (**A**) TUNEL analysis of FDC-P1 and FD(Bcl2.wt) cells following exposure to a sublytic quantity of purified perforin (337 U/ml) and purified granzyme B (30 nM) for 90 min. Plots of side scatter (vertical axis) *versus* forward scatter (horizontal axis), and histograms showing fluorescence incorporated during TUNEL assays (horizontal axis) are shown, in the presence of perforin alone, and in combination with granzyme B. The experiment shown is representative of six performed. (**B**) Inhibition of DNA fragmentation (fluorescence assay for TUNEL+ cells) and change in forward and scatter characteristics of untransfected FDC-P1 and transfectant cell populations exposed to the same quantities of reagents as in (**A**). The results of two separate experiments with control untransfected cells, and transfectants expressing Bcl-2 and mutants of Bcl-2 (left column) or BHRF1 (right column) are shown. The assays are representative of five similar experiments performed using various concentrations of granzyme B (15 – 60 nM) and perforin (337 – 674 U/ml). The numerical values shown indicate the percentage of TUNEL+ cells after subtraction of background cell death (< 4%) in the absence of CTL toxins

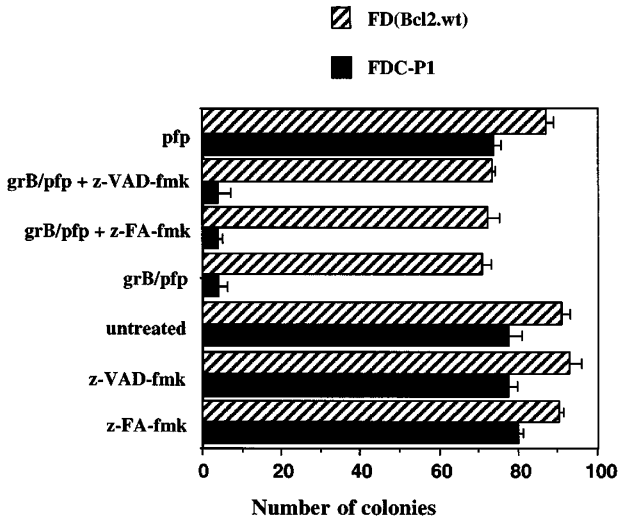

Figure 3 Clonogenic growth of FDC-P1 and FD(Bcl2.wt) cells following exposure to granzyme B (15 nM) and sublytic perforin. FDC-P1 and FD(Bcl2.wt) cells exposed to perforin and granzyme B for 60 min in the presence or absence of fmk caspase inhibitors were plated in soft agar and cultured for 12 days (see Materials and Methods). Colony numbers were estimated as the mean of triplicate assays ± standard error. The data are representative of two similar experiments

FDC-P1 CTL in C57BL/6 or (C57BL/6 × 129) mice that either expressed or were congenitally deficient in granzyme B. In order to separately assess the effects on both nuclear and non-nuclear events in the target cells, we simultaneously labeled their DNA with ^{125}I-UdR and their cytosol with ^{51}Cr. Wild-type CTL were able to induce equivalent ^{51}Cr release from FDC-P1 or cells expressing Bcl-2 at a range of E:T ratios, irrespective of prior incubation of the cells in z-VAD-fmk (200 μM) (Figure 5A). By comparison, expression of Bcl-2 had no effect on the level of DNA fragmentation, whereas pre-incubation with z-VAD-fmk resulted in virtually total inhibition (Figure 5B). These findings indicated that (i) Bcl-2 overexpression in mitochondria does not block either the nuclear or non-nuclear manifestations of cell death in response to intact CTL granules, and (ii) DNA fragmentation under these circumstances continues to be dependent on caspase activation. We were able to exclude the effects of Fas-mediated killing, as FDC-P1 cells lack expression of cell surface Fas, and confirmed the reliance on granule-mediated cell death by demonstrating that perforin-deficient effector cells generated in the same manner were totally ineffective in killing FDC-P1 cells (data not shown).

When we examined the ability of CTL deficient in granzyme B to kill the same targets (Figure 5C,D), we found that Bcl-2 overexpression resulted in a reduction in ^{51}Cr release that was not augmented by inhibition of caspases. As seen with granzyme B-expressing CTL, DNA damage was still dependent on caspase activation even in the absence of granzyme B. In these experiments, about half the release of both ^{51}Cr and ^{125}I-DNA was inhibitable by Bcl-2, while the remainder was not. This indicated that

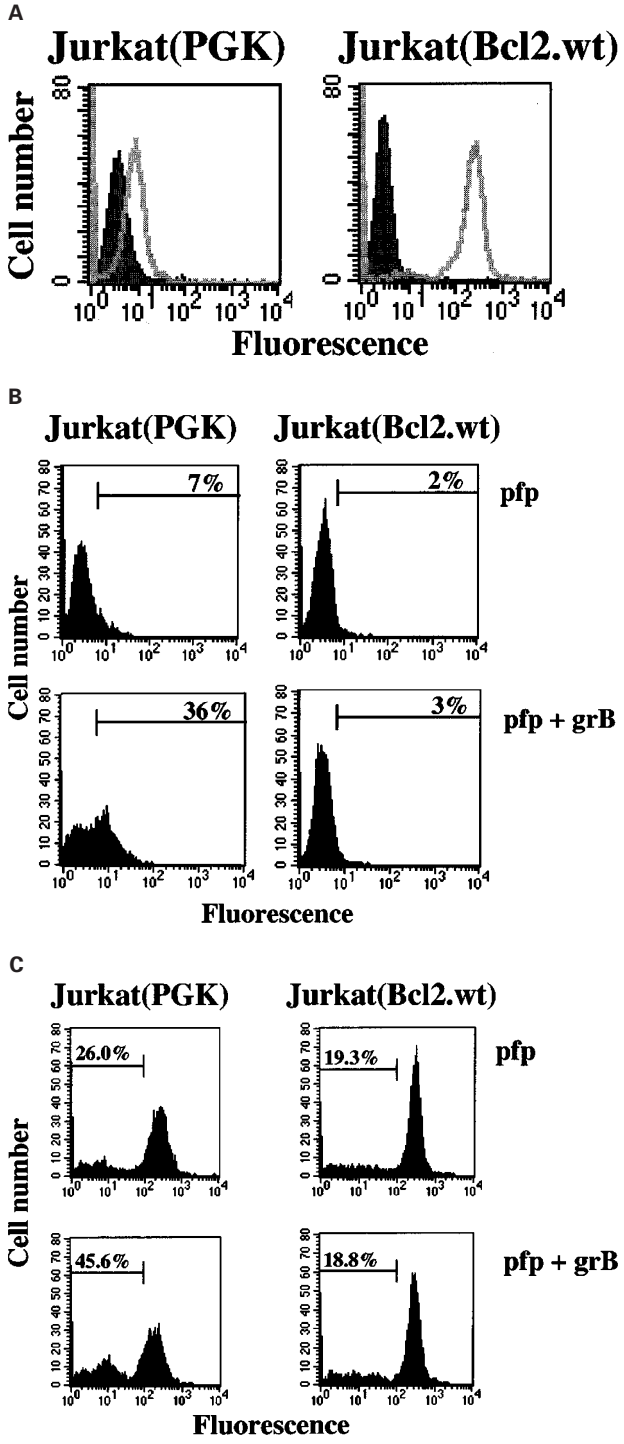

Figure 4 Inhibition of Rhodamine 123 flux by Bcl-2 expression, in Jurkat cells exposed to perforin and granzyme B. (**A**) Expression of wild-type human Bcl-2 in transfected Jurkat(Bcl2.wt) and vector alone-transfected control Jurkat cells (pgk), demonstrated with intracellular staining and flow cytometry as described in Figure 1a. (**B**) The same cell pools as in (**A**) were analyzed by flow cytometry (left panels) and TUNEL staining (right panels) following exposure to perforin and granzyme B (60 nM). The data shown is representative of experiments using three different concentrations of perforin (337–674 U/ml), and either of two concentrations of granzyme B (30 or 60 nM). (**C**) Inhibition of Rhodamine 123 fluxes indicating loss of mitochondrial membrane depolarization in the same cell pools as in (**A**), following their exposure to perforin and granzyme B (60 nM) or to perforin alone (see Materials and Methods)

Bcl-2 can also affect the efficiency of granzyme B-independent death pathways. The fact that this inhibition was seen only in the absence of granzyme B suggests that granzyme B may play a facilitatory role in these alternative pathways. The results obtained with double-labeled target cells were next compared with the clonogenic capacity of cells treated in the same fashion (Figure 6). Cell death was dependent upon perforin, as perforin-deficient CTL were unable to significantly reduce the numbers of surviving FDC-P1 cells following 4 h exposure to the killer cells. Killer cells expressing perforin were able to reduce the number of FDC-P1 clones, irrespective of whether granzyme B was also expressed. Furthermore, overexpression of Bcl-2 or BHRF1 afforded no protection against either wild-type or granzyme B-deficient CTL. Interestingly, the cloning data also confirmed that although granzyme B-deficient effectors induce nuclear damage more slowly than wild-type cells, they kill FDC-P1 targets as efficiently as wild-type effector cells. As it has recently been proposed that grA might contribute to apoptotic pathways in the absence of grB or caspase activity,[38] we also raised grA/B doubly-deficient CTL. We found that the defect in cell membrane damage in response to these CTL was not further augmented, compared with grB-deficient effector cells (Figure 7), indicating that non-nuclear granule pathways responsible for bypassing the block to apoptosis mediated by BHRF1 or Bcl-2 operate independently of both these granzymes.

When we performed similar experiments using IL-2-activated naive splenocytes (H2b) as effector cells and YAC-1 target cells (also deficient in Fas expression, and resistant to perforin-deficient effector cells, data not shown), we observed very similar results. Once again, Bcl-2 overexpression and inactivation of caspases had no effect on ^{51}Cr release, while DNA fragmentation was totally

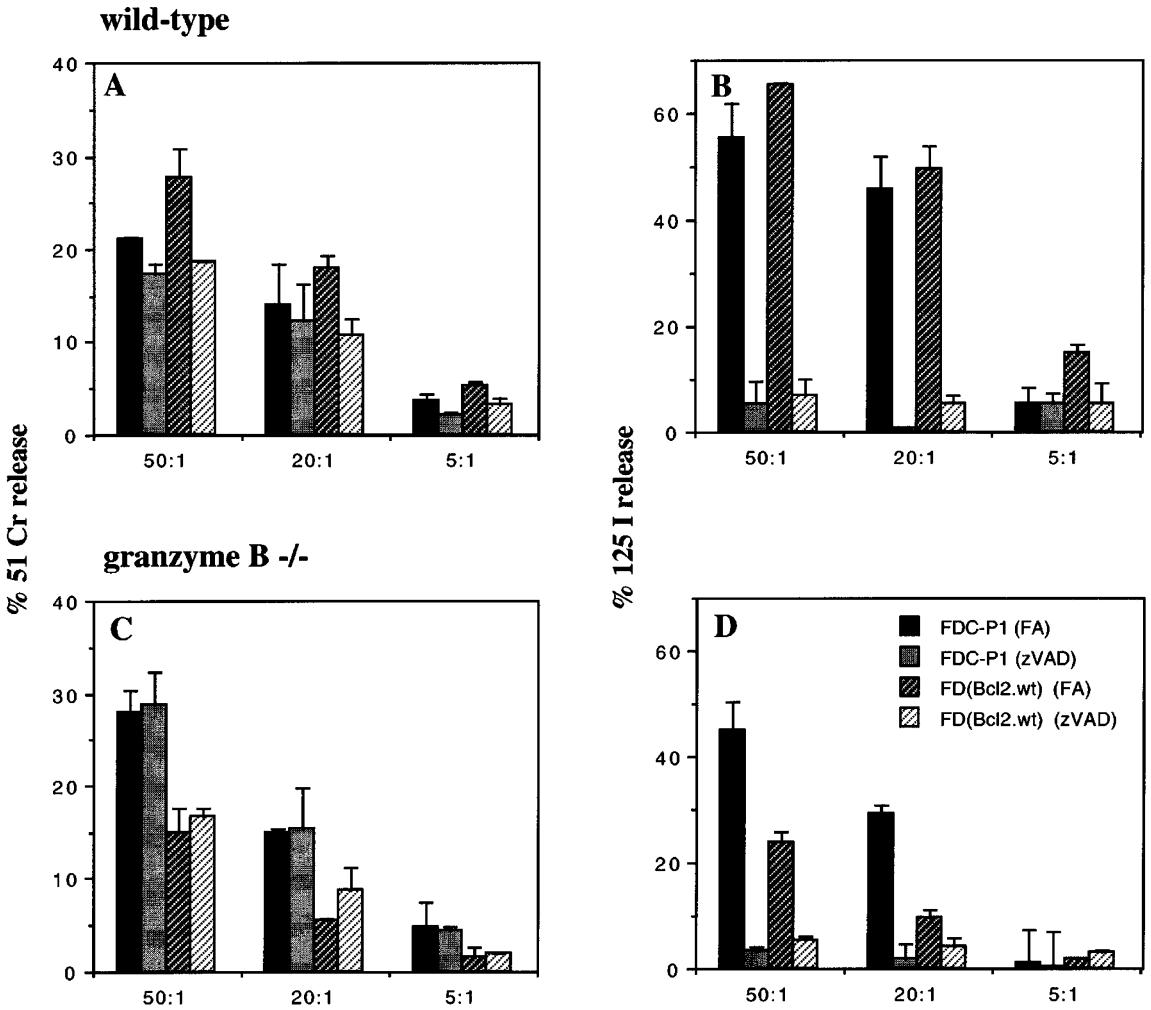

Figure 5 Killing of FDC-P1 and FD(Bcl2.wt) cells in 4 h cytotoxicity assays, by alloreactive CTLs raised in H2b mice. FDC-P1 or FD(Bcl2.wt) target cells were incubated for 30 min in medium containing either z-FA-fmk or z-VAD-fmk (200 μM) prior to addition of effector cells. The fmk compounds were also present throughout the 4 h assays. Effector cells were derived from the popliteal lymph nodes of either wild type C57BL/6 (**A** and **B**) or granzyme B-deficient H2b mice (**C** and **D**) immunized three times into both hind footpads with DBA/2 splenocytes, and added at the E : T ratios indicated. At the end of the assay, target cells were assayed for release of both ^{51}Cr (indicating cell membrane damage) and ^{125}I-DNA (indicating DNA fragmentation). The data points shown are the means of triplicate assays $\pm$ standard errors. A representative assay of four similar experiments is shown

dependent on caspases (Figure 8A,B). In the absence of granzyme B, DNA fragmentation was virtually abolished in 4 h assays, but ^{51}Cr release was not reduced (Figure 8C,D). Consistent with previous reports,[39] the delayed DNA fragmentation observed with granzyme B deficiency was completely rescued by prolonged incubation (18 h), indicating that granzyme B is the most efficient, but not the sole mediator of this type of cell damage (data not shown).

Our findings are interesting for several reasons. Firstly, they indicate that caspase activation can be induced in target cells by CTL granules lacking granzyme B and that cell death cannot be prevented by Bcl-2. It can therefore be concluded that caspase activation in response to CTL granules can be induced both through a granzyme B-dependent pathway regulated by Bcl-2 (presumably at the mitochondrion), and by an alternative mechanism not requiring granzyme B. It has recently been reported that serine proteases such as cathepsin G (expressed in myeloid cells and with a preference for basic or uncharged P1 substrate residues) can activate pro-caspase-7 at sites adjacent to Asp-containing motifs normally recognized by caspases.[40] Moreover, other granzymes, for example the chymases granzyme H[41] and granzyme M, and the tryptases grA and tryptase-2[7] might perform a similar function, although direct evidence for such a mechanism is lacking to date. Secondly, granzyme B- and caspase-independent death pathways can also be mobilized during granule-mediated cytolysis, consistent either with direct lysis by perforin[42] or via hitherto unrecognized pro-apoptotic granule-bound pathways operating independently of caspases. Interestingly, the co-expression of granzyme B was required for the optimal ^{51}Cr release in response to allo-reactive CTL, but not for IL-2-activated splenocytes, possibly indicating that IL-2-activated, antigen non-specific CTL are less dependent on granzyme B and more dependent on perforin for inflicting membrane damage. Thus, subtle differences in the usage of various granule-bound effector mechanisms can be detected, possibly depending on the nature of the

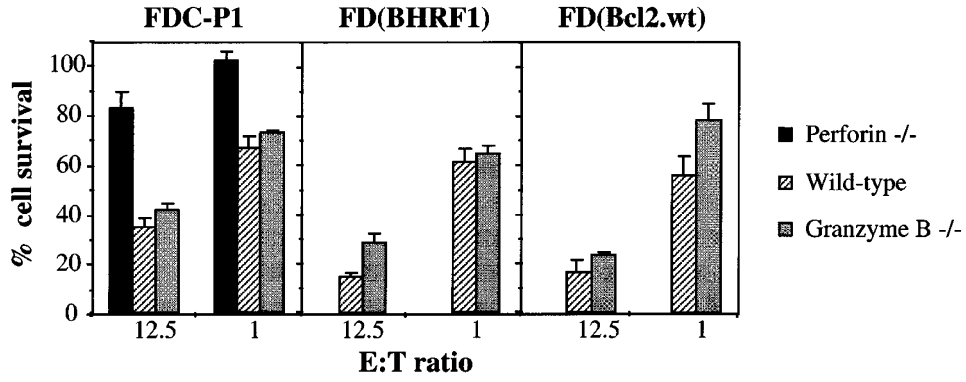

Figure 6 Clonogenic growth of FDC-P1, FD(BHRF1) and FD(Bcl2.wt) cells following exposure to alloreactive CTLs raised in H2b mice. CTL derived from the popliteal lymph nodes of either wild-type C57BL/6, perforin-deficient, or granzyme B-deficient mice were gamma-irradiated (3 Gy), and incubated for 4 h with target cells at the E : T ratios indicated. The cells were then plated in soft agar, then cultured for 5 days (see Materials and Methods); colonies were then counted. The values shown are the mean of quadruplicate wells ± standard error, and are plotted as a percentage of colony numbers arising when target cells were incubated without effector cells. Colony numbers per well for each cell line in the absence of effector cells were in the range 150 – 200 in each experiment. The data shown are representative of two experiments. Wells seeded with effector cells alone produced no colonies (data not shown)

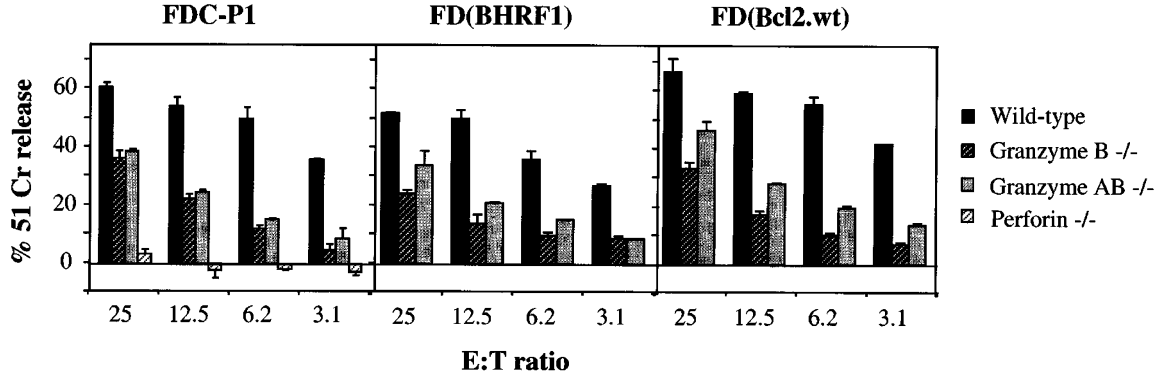

Figure 7 Killing of FDC-P1, FD(BHRF1) and FD(Bcl2.wt) cells in 4 h cytotoxicity assays, by alloreactive CTLs raised in H2b mice. Effector cells were derived from the popliteal lymph nodes of wild-type C57BL/6, granzyme B-deficient or grA/B double deficient H2b mice immunized three times into both hind footpads with DBA/2 splenocytes, and added at the E : T ratios indicated. At the end of the assay, target cells were assayed for release of ^{51}Cr (indicating cell membrane damage). The data points shown are the means of triplicate assays ± standard errors. A representative assay of three similar experiments is shown

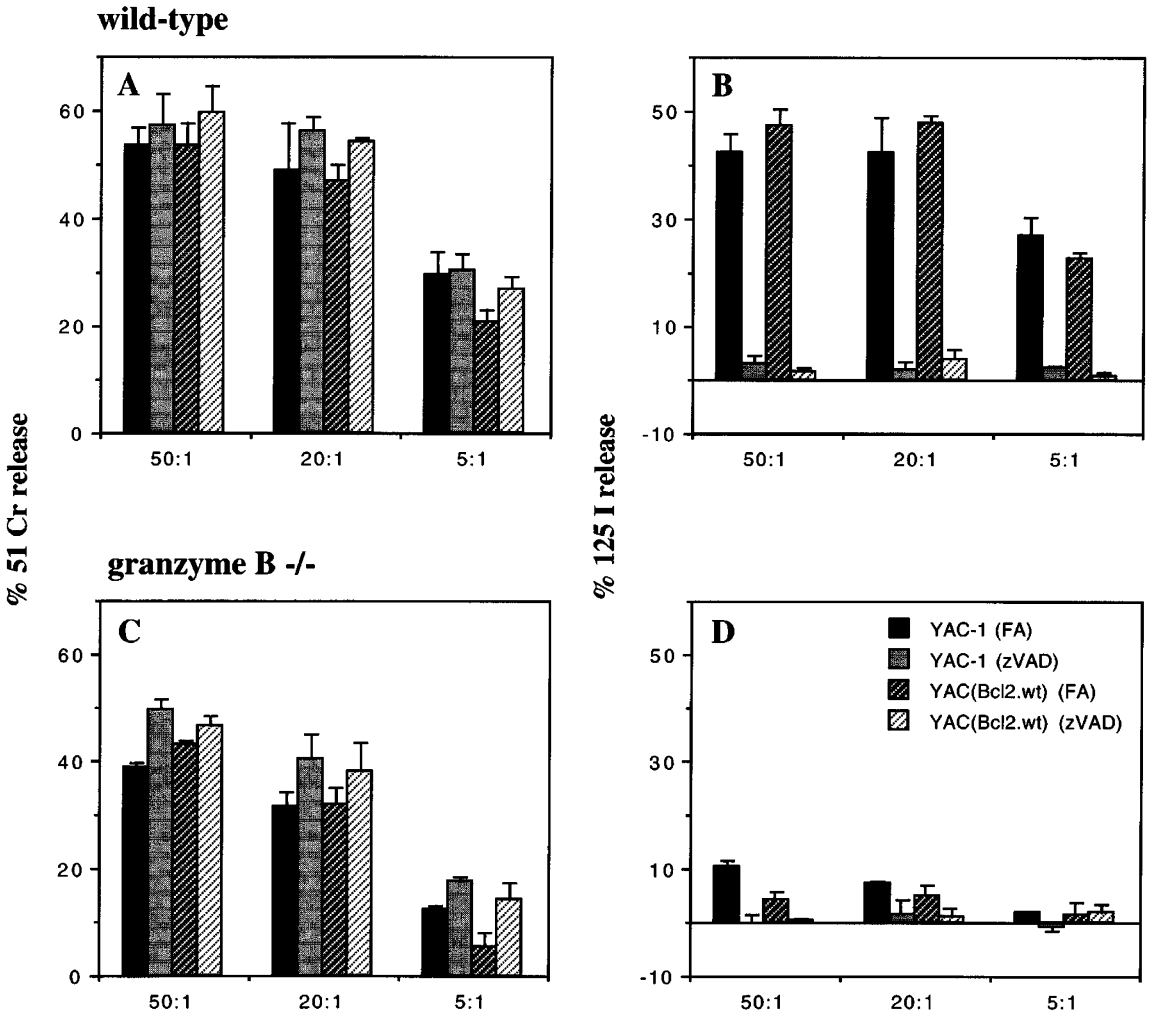

Figure 8 Killing of YAC-1 and YAC(Bcl2.wt) cells in 4 h cytotoxicity assays, by IL-2 activated cytolytic lymphocytes raised in C57BL/6 mice. YAC-1 or YAC(Bcl2.wt) target cells were incubated for 30 min in medium containing either z-FA-fmk or z-VAD-fmk (50 μM) prior to addition of effector cells. The fmk compounds were also present throughout the 4 h assays. Effector cells were derived from the spleens of C57BL/6 (**A** and **B**) or granzyme B-deficient H2b mice (**C** and **D**), and added at the E : T ratios indicated. At the end of the assay, target cells were assayed for release of both ^{51}Cr (indicating cell membrane damage) and ^{125}I-DNA (indicating DNA fragmentation). The data points shown are the means of triplicate assays $\pm$ standard errors. A representative assay of four similar experiments is shown

target cell, the presence of viral anti-apoptotic molecules, and the cytokine milieu of the effector cells.

Our studies also demonstrate the importance of using multiple short-term parameters to confirm lethal target cell damage if cell survival studies are not also performed. In this study, clonogenic assays indicated that granzyme B-deficient CTL could kill target cells as efficiently as wild-type CTL, although they were significantly defective in their ability to induce ^{51}Cr and ^{125}I-DNA release after short incubation times. The clonogenic assays confirm that although Bcl-2 overexpression completely protects FDC-P1 cells from cell death mediated by purified sublytic doses of granzyme B, it provides no protection against attack by intact CTL.

In conclusion, the experiments presented in this report clearly show that granzyme B-mediated cell death is regulated predominantly through a Bcl-2-inhibitable pathway. Our present findings are consistent with those previously reported by us[22] but only partially in agreement with those of MacDonald *et al*.[35] The present findings indicate that granzyme B-mediated pathways to cell death that are independent of mitochondria are activated inefficiently, if at all. It is feasible that the extra-mitochondrial pathways previously reported[35] may be explained on the basis of low Bcl-2 or Bcl-X_L expression in a subpopulation of the transfectant cells. In the present study, we avoided this possibility by using cell lines in which the levels of Bcl-2 were verifiable and it was possible to demonstrate protection against an apoptotic stimulus other than granzyme B. Moreover, the finding that non-mitochondrial apoptotic pathways could be greatly amplified from very low levels in cell-free systems by the addition of

mitochondrial extracts[35] is also consistent with a requirement for mitochondrial involvement.

Our findings for intact CTL killing through the perforin-dependent pathway both confirm previous reports that granzyme B is not essential for many forms of granule-mediated cell death, and strongly support the notion that a multiplicity of granule-bound pathways are unleashed on target cells following CTL degranulation. There have been recent reports that individual granzymes or combinations of granzymes may be required for an optimal immune response in specific pathophysiological situations. While some such examples could not have been selected through evolution (e.g., graft-*versus*-host disease arising from bone marrow transplantation),[43] others such as the potent role played by grA in the recovery from the poxvirus ectromelia, a natural mouse pathogen,[44,45] indicate that the absence or inhibition of a single granzyme can sometimes make the difference between life and death following certain infectious diseases. Interestingly in the latter case, the absence of one trypsin-like granzyme could not be compensated by the apparent availability of a second, tryptase-2. This indicates either that specific granzymes can be recruited preferentially in certain pathological situations, or perhaps that fine differences in the substrate preferences of similar granzymes can be responsible for major differences in immunological outcome. Nevertheless, it is clear from this study and from others that an impressive array of granule-mediated pathways, variously dependent or independent of granzymes, and variously dependent or independent of specific components of the target cell's programmed cell death pathways can be activated collectively by CTL and NK cells. We contend that this impressive and functional redundancy probably arose as a result of the co-evolution of the immune system of higher animals with viral and other intracellular pathogens collectively expressing a plethora of anti-apoptotic molecules.[6] Such intricate molecular diversity can go a long way to endowing the organism with an effective defence against many intracellular infections.

Materials and Methods

Cell culture and gene transfection

FDC-P1 IL-3-dependent mouse myeloid leukemia cells were cultured in DMEM supplemented with 10% FCS and IL-3 in a humidified CO_2 incubator. YAC-1 mouse lymphoma cells were cultured in the same medium without added growth factors. Jurkat human T leukemia cells were maintained in RPMI medium supplemented with 10% FCS.

Populations of FDC-P1 cells expressing human Bcl-2, two different Bcl-2 mutants or full length, 'Flag'-epitope-tagged BHRF1 were generated by gene transfection, using electroporation of plasmid DNA subcloned in the sense orientation into the plasmid vector pEFpuro, as described.[46] One construct encoded human Bcl-2 cDNA which was re-engineered to encode a CAAX motif for farnesylation near its amino-terminus,[37] resulting in targeting of the expressed Bcl-2 to the inner leaflet of the plasma membrane. Another Bcl-2 expression construct was generated in which a single base change was introduced, resulting in mutation of Gly^{145} to Glu. The above transfectants were generously provided by Dr. David Huang, Walter and Eliza Hall Institute, Melbourne, Australia. The same cDNA constructs were used to produce Jurkat cell populations expressing wild-type Bcl-2 in mitochondria. Following electroporation of Jurkat cells (310 V, 960 μF, using 2.5×10^6 cells in 0.5 ml RPMI) with linearized, reprecipitated plasmid DNA (10 μg), cells were plated into RPMI medium supplemented with 10% FCS. Twenty-four hours later, puromycin (5 μg/ml) was added to the cultures to select for potential Bcl-2 expressing cells. Fourteen days later, cell cultures were examined for Bcl-2 expression using intracellular staining with anti-Bcl-2 mAb (Transduction Laboratories) and flow cytometry. Control puromycin-resistant transfectant populations were also generated using pEFpuro vector DNA encoding glycerolphosphokinase (pEF.PGKpuro).

Characterization of FDC-P1 cells expressing Bcl-2-like molecules

Intracellular staining of transfected cell populations was performed following washing and permeabilization of the cells in buffer containing saponin, as described.[47] Primary antibodies were mouse mAbs detecting human Bcl-2 (culture supernatant used at 1/10) or the 'Flag' epitope expressed on BHRF1-expressing constructs (used at $1/10^4$; Sigma). Specific binding of mouse Ig was detected with FITC-conjugated anti-mouse Ig (Silenius, Hawthorn, Australia). Stained and fixed cells were analyzed on a cytofluorograph (Becton-Dickinson). For Western blotting, lysates of washed, unfractionated cells (10^6) were fractionated on SDS–PAGE in the presence of reducing agent, transferred to nylon membranes and probed with the above mAbs. Signals were detected by exposure to X-ray film following chemiluminescent amplification. For growth factor withdrawal assays, populations of cells in exponential growth were washed four times in PBS, then recultured in their usual culture medium deficient in IL-3. Cells were plated in 24-well tissue culture dishes (2×10^5/ml) and cell viability determined daily by dye exclusion.

Apoptosis assays

Assays for the release of ^{51}Cr and ^{125}I-DNA from apoptotic cells were performed as described, as were TUNEL assays.[22] Human perforin was purified as described.[48] A sublytic dose of perforin was defined as that producing $<10\%$ specific release of ^{51}Cr in a 4 h assay at 37°C. Immunoaffinity purification of human granzyme B from nuclear lysates of YT cells was performed as described.[49] The granzyme B was free of grA and Met-ase activities and perforin, as demonstrated by Western blotting and functional assays.[10,49] The oligopeptide caspase inhibitors z-val-ala-asp-fluoromethylketone (z-VAD-fmk) and phe-ala-fluoromethylketone (z-FA-fmk) were purchased from Enzyme Systems Products (San Diego, CA, USA) dissolved in DMSO and stored in aliquots at -20°C. Final concentrations of Me_2SO did not exceed 0.5% in any of the assays.

Rhodamine 123 assay

Changes in mitochondrial membrane potential were measured by flow cytometry with the mitochondrion-specific fluorescent dye, rhodamine 123. The dye was added for the final 20 min of cell death assays in response to granzyme B and perforin.

Cell cloning assays

FDC-P1 cells in logarithmic growth phase were pre-incubated with 100 μM z-VAD-fmk or z-FA-fmk for 30 min at 37°C prior to cell death/survival assays. To induce cell death, cells (4×10^5 in 0.2 ml) were

seeded into 24-well plates and incubated for 1 h with a sublytic quantity of perforin alone (300–400 U/ml) and/or granzyme B (0.5 μg/ml) in the presence or absence of z-VAD-fmk or z-FA-fmk (100 μM). Following the incubation, 0.8 ml of growth medium containing IL-3 was added, and cells (5 μl from a stock of 4×10^4 cells/ml) treated with various apoptotic stimuli were plated out in triplicate on soft agar. Cells were diluted in 5 ml RPMI containing 20% fetal calf serum and 0.3% noble agar (Difco) and plated in 60-mm dishes. Once set, the dishes were overlaid with 2.5 ml of media, incubated at 37°C for 12 days and the colonies counted under an inverted microscope as described.[30] In other experiments, allo-reactive anti-FDC-P1 CTL derived from perforin-deficient, C57BL/6 wild-type, or granzyme B-deficient mice were gamma-irradiated (3 Gy) prior to incubation with FDC-P1 targets for 4 h at a range of E:T ratios. Cells were then diluted in 2 ml RPMI agar, and 0.5 ml was plated in 15 mm dishes in quadruplicate. These were overlaid with 1 ml medium containing IL-3 but lacking IL-2, and colony numbers were counted after culture for 5 days at 37°C. Allo-reactive effector cells were also plated in the absence of target cells, to demonstrate that they did not grow as colonies.

Generation of cytolytic lymphocytes *in vivo*

All mice used in this study were maintained in the Biological Research Facility at the Austin Research Institute, and handled in accordance with Institutional Ethics guidelines. For generation of allo-reactive anti-FDC-P1 CTL, C57BL/6 or (129 × C57BL/6) mice deficient in granzyme B expression (H2b)[39] were immunized three times at 14 day intervals by injection of DBA/2 spleen cells (H2d) into both hind footpads (2×10^6 cells in 0.05 ml PBS, per injection). Four days after the third immunization, the mice were sacrificed and the popliteal lymph nodes were harvested, teased, and the lymphocytes cultured for a further 4 days in dishes containing DMEM supplemented with 10% FCS without added cytokines. In other experiments, naive spleen cells from C57BL/6 mice were harvested and cultured in the same medium supplemented with IL-2 (100 U/ml) for 4 days. Both types of effector cells were used in standard 4 and 18 h cytolytic assays in which target cells (2×10^4) were double-labeled with ^{51}Cr and ^{125}I-UdR and exposed to effector cells at various E:T ratios. In some experiments, target cells were pre-incubated in medium containing z-VAD-fmk (200 μM) or z-FA-fmk for 30 min prior to mixing with effector cells. The medium in which the 4 h cytolytic assays were performed was also supplemented with the fmk inhibitors at the same concentrations. The percentage of specific cytotoxicity was calculated in the standard way, and expressed as the mean of triplicate assays $\pm$ standard error of the mean.

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