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Research Article

An anti-Bcl-2 antibody prevents 2-deoxy-D-ribose-induced apoptosis in the IPLB-LdFB insect cell line

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Abstract. Confocal microscopy reveals that the anti-Bcl-2 antibody (pAb) is able to diffuse across the plasma membrane of the fat body cell line IPLB-LdFB from the insect *Lymantria dispar,* demonstrating the presence of Bcl-2-like molecules in the cytoplasm. Immunoperoxidase procedure confirms the cellular localization. Furthermore, an immunoprecipitation corresponding to a molecular weight of 29 KDa is observed with western blot analysis using the anti-Bcl-2 pAb. Cytofluorimetric experiments show that anti-Bcl-2 pAb counteracts 2-deoxy-D-ribose-induced apoptosis and provokes morphological changes in the insect cell line, i.e. a reduction in cell size, the disappearance of the vacuola and changes in shape. At the same time, the antibody provokes mitochondrial membrane depolarization, and *N*-acetyl-L-cysteine is unable to reconstitute the physiological conditions. The present findings suggest that Bcl-2-like proteins play a main role in maintaining of the integrity of cellular components, e.g. mitochondria, rather than in controlling programmed cell death.

Key words. IPLB-LdFB insect cell line; anti-Bcl-2 antibody; 2-deoxy-D-ribose; *N*-acetyl-L-cysteine; valinomycin; apoptosis; mitochondrial membrane potential; confocal scanning laser microscopy; immunocytochemistry; western blot; flow cytometry.

Introduction

Cell death is mediated by a family of intracellular cysteine proteases known as caspases [1], whereas Bcl-2 is the founder of a family of genes that includes members with anti- and pro-apoptotic activities [2, 3]. The discovery of the localization of Bcl-2 in the inner and outer mitochondrial membranes [4, 5] and the need for cytochrome c [6] to activate caspases suggests that this cytoplasmic organelle plays an important role in cell life. Moreover, other localizations of Bcl-2 have also been reported, in particular, the nuclear envelope, the endoplasmic reticulum and the plasma membrane [5, 7–9]. Schendel et al. [10] have found that Bcl-2 forms channels in membranes, and that their three-dimensional structure, homologous to Bcl-X_L, shows an extraordinary similarity to the pore-forming properties seen in bacterial toxins.

Tsujimoto et al. assert that there is no evidence that ion channels formed by Bcl-2 and other members of the Bcl-2 family in synthetic lipid membranes [10–13] are also present in vivo nor does it appear that these proteins regulate apoptosis by means of the formation of ion channels [14]. The authors have also demonstrated that Bcl-2 family proteins interact with voltage-dependent anion channels (VDACs) [15], a protein component of the mitochondrial permeability transition (PT) pore [16, 17], in regulating apoptotic mitochondrial changes such as the release of cytochrome c or apoptosis-inducing factors (AIFs) or mitochondrial membrane depolarization [14, 15]. Recently, it has also been shown that the disruption

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of mitochondrial membrane potential is not directly correlated with apoptosis, whereas its effect on cell death is related to the model used [18].

In the present paper, we have studied the role of Bcl-2 in fat body cell line (IPBL-LdFB) survival in the insect *Lymantria dispar*. In the same cell line, we have found that the sugar 2-deoxy-D-ribose (dRib) induces apoptosis by oxidative stress in a concentration- and time-dependent way [19, 20].

Materials and Methods

Cell culture

The IPLB-LdFB cell line from the fat body of the insect *Lymantria dispar* (Lepidoptera) was used [21]. The cells were cultured in Ex-Cell 405 medium at 26 °C.

Confocal microscopy

Three-hundred-µl cell suspensions (10^6 cells/ml) were placed into two 35-mm plastic dishes. Anti-Bcl-2 polyclonal antibody (pAb) (1:100) was added to the first sample and Ex-cell 405 medium (control) reaching a final volume of 1000 µl to the other. The cells were incubated overnight at 26 °C. The mixture was cytocentrifuged (Cytospin 2 cytocentrifuge, Shandon, UK) on slides at 800 rpm for 3 min and air-dried. The cells were permeabilized with PBS-Triton X-100 (10 min), washed in phosphate-buffered saline (PBS), incubated in a humidified chamber with fluorescein isothiocyanate (FITC)-labelled-anti-rabbit-IgGs (1:20) for 1 h at 37 °C, washed again with PBS and mounted in DABCO anti-fading. Controls were performed by omitting the primary antibody. The cells were examined by confocal scanning laser microscopy (Omnichrome, Leica, Germany).

Immunoperoxidase procedure

The immunocytochemical reaction was performed on unfixed cytocentrifuged IPLB-LdFB cells using avidin-biotin-peroxidase complex, as previously described [19]. Anti-Bcl-2 pAb (1:500) was used as the primary antibody. Negative control was performed by substituting the primary antibody with nonimmune sera.

Western blot

The insect cell line was centrifuged at 1200 rpm for 5 min, resuspended in PBS three times and then lysated in lysis buffer (50 mM tris-HCl contains 2 mM EDTA, 1% β-mercaptoethanol, 5% glycerol, 2% SDS and 0.01% bromophenol blue) at pH 7.4. The homogenate was frozen at –20 °C until it was required for western blot analysis. After boiling (5 min), 10 µl of sample was loaded per line. The proteins were separated on 12% SDS-polyacrylamide gel (minigel) electrophoresis and electrotransferred to polyvinylidine difluoride (PVDF) membranes. Membranes were blocked in tris-buffered saline at pH 7.5, 0.1% Tween-20, 5% mother blocking solution for 1 h at room temperature (RT) and incubated with the primary antibody. The mother blocking solution was prepared as follows: 5 g of blocking reagent was diluted in 100 ml of 0.1 M maleic acid and 0.15 M NaCl buffer at pH 7.5, and autoclaved at 121 °C for 20 min. The following primary antibody used was anti-Bcl-2 pAb. The antiserum was diluted 1:1000 in blocking solution and incubated for 1 h at RT. After washing in tris-buffered saline at pH 7.5, 0.1% Tween-20, the membranes were incubated for 1 h at RT with a secondary antibody diluted 1:2000. Immunoreactive bands were visualized using a nitroblue tetrazolium 5-bromo-4-chloro-3-indolylphosphate-toluidinium (NBT/BCIP) detection system.

Preparation of the cells for cytofluorimetric analysis

The role of Bcl-2 in insect cell line survival was evaluated by incubating the cells as previously described [19, 20] for 48 h at 26 °C with 50 mM dRib, anti-Bcl-2 pAb (1:100) or 10 mM *N*-acetyl-L-cysteine (NAC). The samples were then analyzed by light microscopy and flow cytometry to detect apoptosis and mitochondrial membrane potential. K^+ ionophore valinomycin at concentrations of 10, 30 and 50 nM was also tested either alone or in combination with dRib. All the valinomycin experiments were performed for 24 h.

Sodium azide experiments

The anti-Bcl-2 pAb contains 0.1% sodium azide. In order to avoid distortion of results, we examined whether this molecule alone is able to induce apoptosis in our insect cell line. The cells were incubated with 0.1% sodium azide for 48 and 72 h at 26 °C, and the percentage of cell death was determined by flow cytometry.

Light microscopy

The cells were cytocentrifuged and stained with May-Grünwald and Giemsa (MGG) for morphological examination.

Cytofluorimetric analysis

Detection of apoptosis

As previously described [19, 20], cell death was detected by reduced fluorescence of the DNA-binding dye propidium iodide (PI) in the apoptotic nuclei, following Nicoletti et al. [22]. The cell pellet was centrifuged at 1800 rpm and resuspended in 1 ml of hypotonic fluorochrome solution containing 50 µg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100. After 30 min of incubation, the cells were analyzed by flow cytometry. Cytofluorimetric readings were taken using a FACScan (Becton-Dickinson, Mountain View, CA, USA) equipped with a single 488-nm argon laser. A total of 10,000 cells were

acquired in list mode and analyzed with the Lysys II software program.

Mitochondrial membrane potential

The mitochondrial membrane potential was detected using a lipophilic cationic 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) probe that enters mitochondria in a selective manner [23]. The procedure described by Cossarizza et al. [24] was used, as reported in a previous paper [20]. The JC-1 molecule shows a monomeric form when the mitochondrial membrane is depolarized (emission at 527 nm after excitation at 490 nm), whereas it has a J-aggregate form when the membrane is polarized (emission at 590 nm). The cell suspensions were incubated in Ex-cell 405 medium with 10 µg/ml of JC-1 for 10 min in the dark at RT. After incubation, the cells were washed twice in PBS, resuspended in a final volume of 1 ml and analyzed.

Statistical analysis

Each experiment was repeated in duplicate three times, and statistical analysis was performed using the Student *t* test.

Chemical reagents

dRib, NAC, valinomycin, sodium azide and Triton X-100 were purchased from Sigma Chemical (USA), anti-Bcl-2 pAb [(ΔC 21): sc-783] from Santa Cruz Biotech (USA), JC-1 from Molecular Probes (USA), DABCO anti-fading and FITC-labelled-anti-rabbit-IgGs from DAKO (Denmark) and Ex-cell 405 medium from JRH Biosciences (USA). All the reagents for western blot analysis were purchased from Bio-Rad (USA), except the blocking reagent and NBT/BCIP, which came from Boehringer-Mannheim (Germany).

Results

The confocal microscopy investigations show the presence of immunoreactive molecules to anti-Bcl-2 pAb in the insect cell line IPLB-LdFB. Immunofluorescence is present throughout the cell; however, as reported by Alnemri et al. [8], greater intensity is found around the nucleus (fig. 1). Both the experimental conditions (no fixation and no cellular permeabilization before adding the primary antibody anti-Bcl-2 pAb) and the method utilized allow us to assert that the antibody is able to diffuse across the plasma membrane. However, in order to clarify the translocation of anti-Bcl-2 pAb across the cellular plasma membrane, electron-microscope studies using peroxidase-labeled antibody will be performed.

The immunoperoxidase reaction also shows that the insect cell line contains molecules immunoreactive to anti-Bcl-2 pAb (fig. 2).

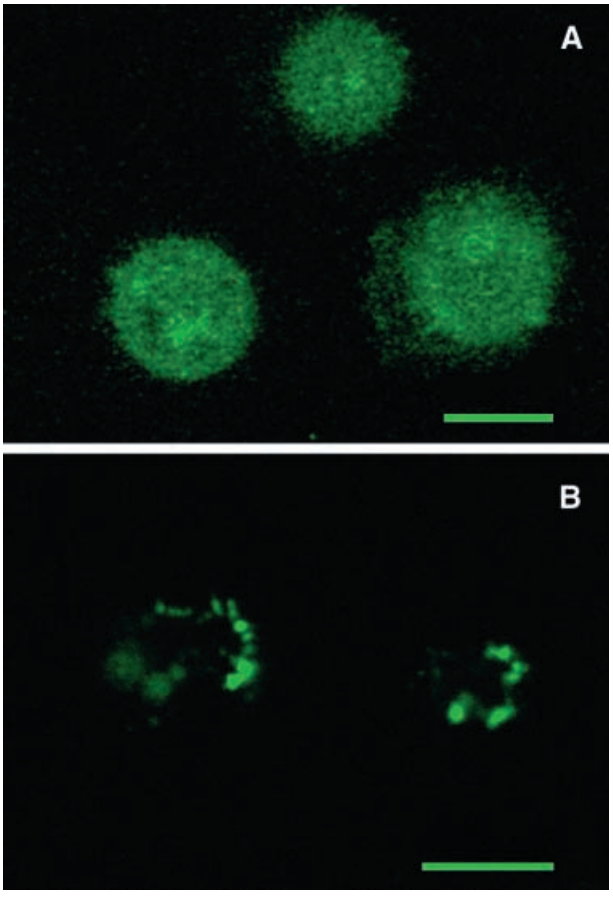

Figure 1. Confocal fluorescence micrographs of single optical section. (*A*) *Lymatria dispar* IPLB-LdFB cells showing immunoreactive molecules to anti-Bcl-2 pAb. (*B*) Negative control. Bar, 5 µm.

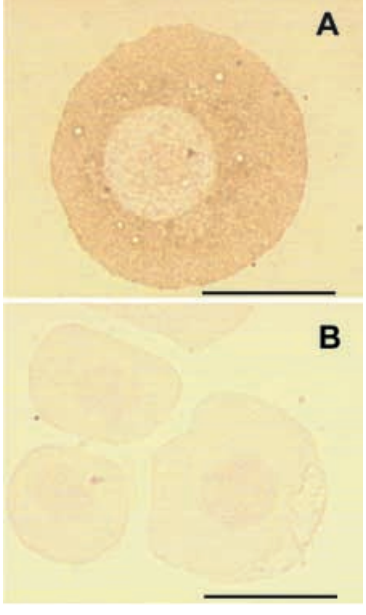

Figure 2. Immunoperoxidase staining of the *Lymatria dispar* IPLB-LdFB cells with anti-Bcl-2 pAb (*A*). Negative control, cell nuclei were counterstained with hematoxylin (*B*). Bar, 5 µm.

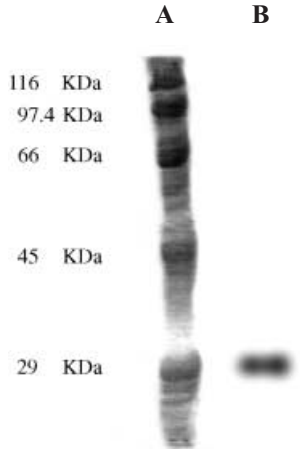

Figure 3. Western blot analysis of the Bcl-2 (corresponding to line *B*) performed using anti-Bcl-2 pAb. MW standard (line *A*).

The sodium azide present in the solution in which the antibody is dissolved shows no toxic effect on the cells. Indeed, evaluation by flow cytometry of the percentage of cell death gives values for control and treated cells in the same range at the two times considered: 4.60 (0.08, SD) and 4.65 (0.13, SD), respectively.

The western blot analysis of lysate insect cells shows that anti-Bcl-2 pAb recognizes a single immunoreactive band with a molecular weight (MW) of 29 KDa, corresponding to Bcl-2 expression in HL-60 and Jurkat whole-cell lysates demonstrated in the antibody-specificity test by Santa Cruz Biotech (fig. 3).

As expected [19, 20], incubation of the cells with dRib induces an increase in cell death, whereas anti-Bcl-2 pAb results in apoptotic values similar to those of controls (table 1; fig. 4). However, the antibody provokes a significant change in physical cell features, with an increase in cell density, as revealed by cytofluorimetric analysis (figs. 5 A, C). This is also confirmed by MGG staining in the presence of the antibody. The cells exhibit considerable morphological changes with respect to controls, i. e. a reduction in size, the disappearance of vacuola and a change in shape from a round (control) to an ameboid form (figs. 5 E, G).

Anti-Bcl-2 pAb also inhibits the dRib apoptotic effect, showing values of the same magnitude as those found in the presence of the antibody alone (table 1; fig. 4). Furthermore, morphological observations in the presence of anti-Bcl-2 pAb plus dRib show that the cells maintain their characteristics except for shape. The majority are round, whereas only a few are ameboid (figs 5 G, H).

In contrast, the cells incubated with dRib alone show a morphology similar to the control patterns, the only difference being an increase in cell size together, obviously, with a higher percentage of apoptotic bodies (table 1; figs. 5 E, F).

The mitochondrial membrane potential of the cells evaluated by flow cytometry after incubation with dRib and anti-Bcl-2 pAb used singularly or in combination shows the same range of values (table 2; fig. 6). A considerable

Table 1. Percentage of apoptotic *Lymantria dispar* IPLB-LdFB cell line.

Substances	Mean ± SD
Control	4.58 ± 0.39
dRib	21.20 ± 1.25*
Anti-Bcl-2 pAb	4.44 ± 0.37
Anti-Bcl-2 pAb + dRib	5.05 ± 0.60

The mean ± SD of six experiments is shown. dRib, 2-deoxy-D-ribose. Statistical analysis was performed by the Student *t* test. *$P < 0.05$ vs. control.

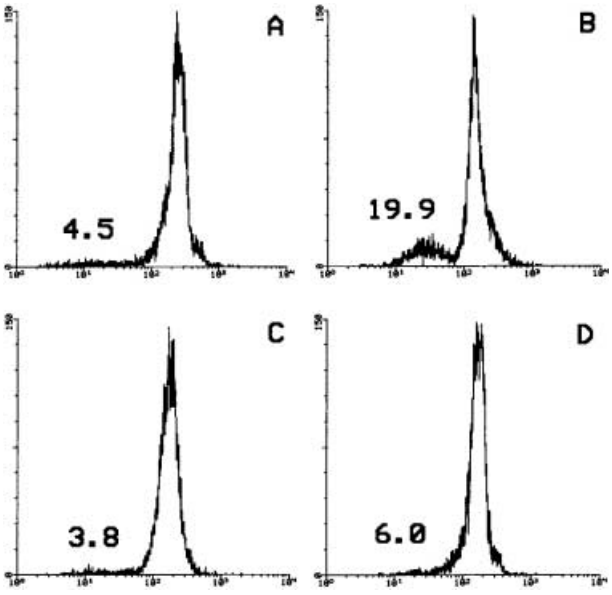

Figure 4. Cytofluorimetric analysis of apoptosis in *Lymatria dispar* IPLB-LdFB cells after 48 h incubation with different substances: medium (control) (*A*); 50 mM dRib (*B*); anti-Bcl-2 pAb (1 : 100) (*C*); anti-Bcl-2 pAb (1 : 100) + 50 mM dRib (*D*). The numbers in each panel refer to the percentage of apoptotic cells. One experiment representative of a set of six is shown.

Table 2. Percentage of cells with mitochondrial membrane depolarization (MMD) and of apoptotic cells (ACs) in *Lymantria dispar* IPLB-LdFB cell line.

Substances	% MMD mean ± SD	% ACs mean ± SD
Control	4.64 ± 0.24	5.09 ± 0.17
dRib	86.24 ± 1.10*	23.99 ± 0.93*
NAC	19.13 ± 0.52*	5.07 ± 0.05
Anti-Bcl-2 pAb	87.47 ± 1.84*	5.85 ± 0.35
Anti-Bcl2 pAb + dRib	88.45 ± 0.67*	5.37 ± 0.28
dRib + NAC	84.75 ± 0.72*	5.31 ± 0.29
Anti-Bcl-2 pAb + NAC	90.91 ± 0.81*	5.70 ± 0.42

The mean ± SD of six experiments is shown. dRib, 2-deoxy-D-ribose; NAC, *N*-acetyl-L-cysteine. Statistical analysis was performed by the Student *t* test. *$P < 0.05$ vs. control.

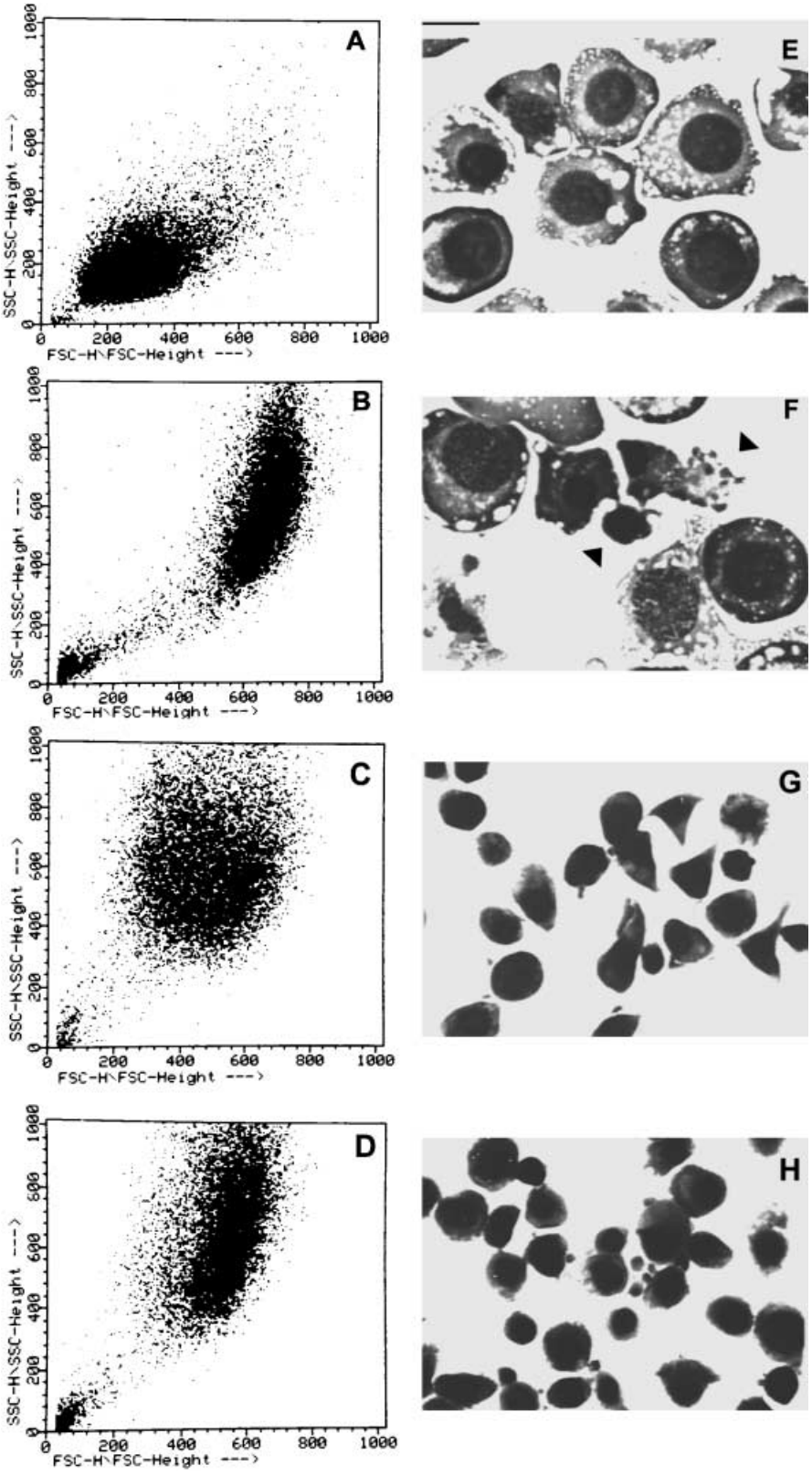

Figure 5. *Lymatria dispar* IPLB-LdFB cells distinguished by cytofluorimetric analysis on their different forward (FSC) and side (SSC) light scatters in the presence of medium (control) (*A*); 50 mM dRib (*B*); anti-Bcl-2 pAb (1:100) (*C*) and anti-Bcl-2 pAb (1 : 100) + 50 mM dRib (*D*). Morphological staining of the cells with MGG in the presence of medium (control) (*E*), 50 mM dRib (apoptotic bodies = arrowheads) (*F*); anti-Bcl-2 pAb (1 : 100) (*G*) anti-Bcl-2 pAb (1 : 100) + 50 mM dRib (*H*). Bar, 5 µm. One experiment representative of a set of six is shown.

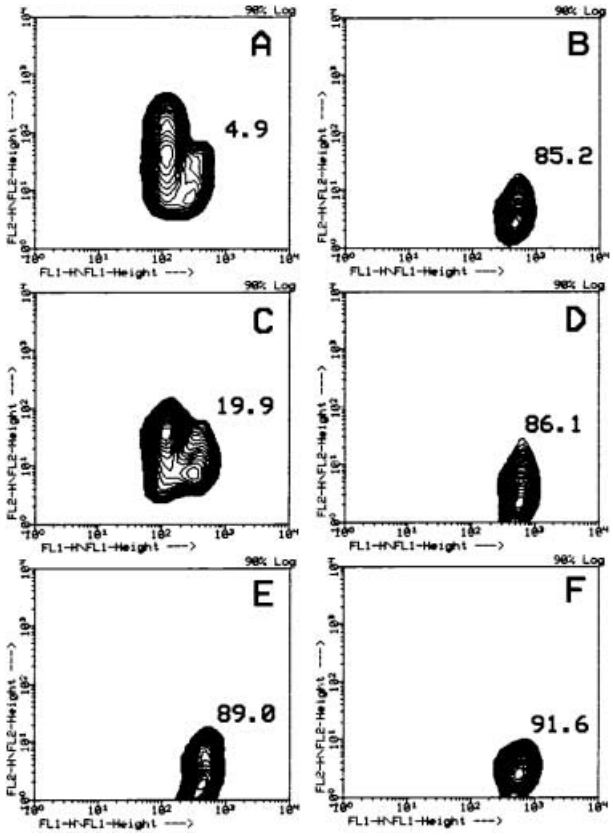

Figure 6. Cytofluorimetric analysis of the mitochondrial membrane potential of *Lymatria dispar* IPLB-LdFB cells stained with the JC-1 probe after 48 h incubation with different substances: medium (control) (*A*); 50 mM dRib (*B*); 10 mM NAC (*C*); anti-Bcl-2 pAb (1:100) (*D*); anti-Bcl-2 pAb (1:100) + 50 mM dRib (*E*) and anti-Bcl-2 pAb (1:100) + 10 mM NAC (*F*). One experiment representative of a set of six is shown.

increase in the percentage of cells with mitochondrial membrane depolarization (MMD) is observed. NAC is unable to reverse MMD caused by dRib or the antibody, provoking rather a marginal increase in MMD (table 2; fig. 6). As previously reported [20], NAC inhibits the apoptotic effect of dRib, whereas anti-Bcl-2 pAb either with or without the antioxidant gives the same apoptotic values (table 2).

The results obtained with the K+ ionophore valinomycin are reported in Table 3. Valinomycin provokes a significant increase in MMD without apoptotic phenomena at all the concentrations used and also inhibits the dRib apoptotic effect.

Table 3. Percentage of cells with mitochondrial MMD and of ACs in *Lymantria dispar* IPLB-LdFB cell line.

Substances	% MMD mean ± SD	% AC mean ± SD
Control	5.40 ± 0.10	4.37 ± 0.07
dRib	94.36 ± 1.20*	20.07 ± 0.12*
VM (50 nM)	90.09 ± 1.09*	4.51 ± 0.10
VM (30 nM)	83.69 ± 2.40*	4.71 ± 0.03
VM (10 nM)	74.84 ± 4.63*	4.43 ± 0.16
VM (50 nM) + dRib	95.71 ± 0.37*	5.73 ± 0.20
VM (30 nM) + dRib	96.81 ± 0.38*	5.70 ± 0.17
VM (10 nM) + dRib	96.48 ± 0.36*	5.30 ± 0.20

The mean ± SD of six experiments is shown. dRib, 2-deoxy-D-ribose; VM, valinomycin. The cells were incubated for 24 h. Statistical analysis was performed by the Student *t* test. * $P < 0.05$ vs. control.

Discussion

The anti-Bcl-2 pAb results can be summarized as follows: (i) the antibody is able to cross the plasma membrane of the fat body cell line IPLB-LdFB from the insect *L. dispar*; (ii) it reveals the presence of Bcl-2-like molecules in the insect cells, (iii) in lysate cells, it recognizes a single band with a MW of 29 KDa, corresponding to mammalian Bcl-2; (iv) it blocks dRib apoptotic action and provokes morphological changes in the insect cell line, i.e. a reduction in cell size, the disappearance of the vacuola and changes in shape; (v) it provokes MMD which NAC is unable to prevent; (vi) it shows behaviour similar to the K^+ ionophore valinomycin.

The results obtained with the anti-Bcl-2 pAb are surprising, in that the antibody does not induce apoptosis. In contrast, it inhibits the dRib-induced apoptotic effect. This was unforeseen, as the role of Bcl-2 protein is important for cellular survival [9, 25].

Consequently, we have studied the effect of the K^+ ionophore valinomycin on our insect model to verify how the anti-Bcl-2 pAb acts. Valinomycin provokes marked MMD [24] and at high doses induces apoptosis [26, 27], whereas at low concentrations it is able to induce only a decrease in mitochondrial membrane potential, as seen in the present experiments.

These results reveal that anti-Bcl-2 pAb and the K^+ ionophore have the same effects in the insect cell line, i.e. they are unable to induce apoptosis, provoke MMD and counteract the dRib effect, suggesting that Bcl-2 is not essential in regulating cell death.

In general, the findings with regards to morphology, apoptosis, MMD and valinomycin support the hypothesis that Bcl-2 is mainly involved in the maintenance of the integrity of the cellular components of IPLB-LdFB cells, e.g. mitochondria, rather than constituting a survival pathway.

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