Potentiation of Apoptosis by Heat Stress Plus Pesticide Exposure in Stress Resistant Human B-Lymphoma Cells and Its Attenuation through Interaction with Follicular Dendritic Cells: Role for c-Jun N-terminal Kinase Signaling

Stephen E. Bloom,*†-‡ Ann T. Lemley,†‡ and Donna E. Muscarella*†

*Department of Microbiology and Immunology, †Institute for Comparative and Environmental Toxicology, and ‡Department of Textiles and Apparel, Cornell University, Ithaca, New York 14853

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B lymphocytes (B cells) become increasingly resistant to apoptosis induction during their differentiation in the microenvironment of the germinal center of lymphoid follicles. This is due to increases in the levels of Bcl-2 protein as well as survival signals generated through B-cell binding to follicular dendritic cells (FDC). However, it is not known whether this cellular resistance may be bypassed as a result of exposure to multiple environmental stress factors resulting in excessive apoptosis induction in B cells. We examined this question of whether apoptosis may be induced, and possibly potentiated, as a result of exposure of the human EW36 B-lineage cell line, having elevated Bcl-2 protein, to heat stress and pesticide combination exposures in a co-culture system with a human FDC cell line. This co-culture system recapitulates essential features of a human germinal center including adherence of B cells to FDC generating survival signals. We found that heat stress plus pesticide exposures resulted in substantial potentiation of apoptosis in EW36 cells, effectively bypassing their stress resistance. Similar results were obtained when paraquat was substituted for heat stress. Furthermore, the JNK pathway was activated by some combination exposures, such as heat stress plus antimycin A, but this pathway was found to play a cytoprotective role in EW36 cells. Importantly, EW36 cell binding to FDC reduced the extent of apoptosis induction by most combination exposures. These results reveal cell stress scenarios that can greatly augment apoptosis in stress-resistant human B-cells and a germinal center interaction that selectively attenuates pesticide-induced apoptosis.

Key Words: B lymphocytes; follicular dendritic cells; pesticides; apoptosis potentiation; JNK activation.

The appropriate regulation of B and T lymphocytes for engaging in apoptosis is essential during normal immune system development and lymphocyte differentiation, whereas improper regulation of apoptosis contributes to immune system dysfunction and the progression of lymphoid cancers (Kuppers, 2005; Lopez-Hoyos et al., 1998). Sensitivity to apoptosis induction in lymphocytes is regulated by developmentally expressed genes and by signaling pathways activated through lymphocyte interactions with supporting cells, such as follicular dendritic cells (FDC), in lymphoid compartments (Koopman et al., 1997; Leanderson et al., 1992). The germinal center is one such important immune compartment where particular B lymphocyte (B-cell) populations with high-affinity B-cell receptors (BCR) are selected and expanded while those B cells with low affinity BCR are deleted by apoptosis (Liu et al., 1996; MacLennan, 1994). Perturbations of germinal center cells and their interactions resulting from exposure to physical or chemical stress can potentially lead to longer-term alterations of the immune system. Therefore, it is of high interest, from a toxicological perspective, to evaluate the effects and mechanisms of drug- and toxicant-induced apoptosis in B cells within a germinal center microenvironment. Of particular interest is the question of whether survival signals that are typically generated through B-cell survival interaction with FDC also protect against drug- or toxicant-induced apoptosis in B cells.

Studies of mechanisms involved in toxicant-induced apoptosis in human B-cells in vivo, besides being difficult to conduct and controversial, are hampered by difficulty in obtaining adequate numbers of germinal centered derived B cells as well as FDC for studies (Kim et al., 1995). To address this problem human cell lines have become available for the development of in vitro model systems to study toxicant effects on human B cells alone or in the context of their interactions with germinal center-derived FDC (Choe et al., 2000; Kim et al., 1995; Kuppers et al., 2005).

Mechanistic toxicological studies have shown that the sensitivity of lymphoid and other cell types to chemically induced apoptosis is strongly influenced by Bcl-2 expression level and activation of mitogen-activated protein (MAP) kinase signaling pathways (Kroemer, 1997). This paradigm is
supported by many studies, including our own, with particular drug resistant Burkitt’s lymphoma (BL) cell lines that are useful models for germinal center B cells. For example, the EW36 BL cell line expresses high Bcl-2 and shows resistance to apoptosis induction by anti-cancer drugs, heavy metals, and mitochondrial-inhibiting pesticides. The related ST486 BL cell line expresses low Bcl-2 and is sensitive to apoptosis induction by these same chemicals (Muscarella and Bloom, 2002, 2003; O’Brien et al., 2001). Activation of MAP kinases can facilitate apoptosis or act in parallel with Bcl-2 to augment cellular stress resistance (Xia et al., 1995). For example, arsenite was found to activate the p38 pathway in the ST486 BL cell line, but its inhibition augmented arsenite-induced apoptosis (Muscarella and Bloom, 2002).

It is of particular interest that Bcl-2-mediated multi-drug resistance is partially reversible by certain drugs or combination of stresses. For example, isoquinoline carboxamide (PK11195), an antagonistic ligand for the mitochondrial benzodiazepine receptor, reverses the anti-apoptotic effects of Bcl-2 in B- and T-lymphoma and other cells (Hirsch et al., 1998; Larochette et al., 1999). Pretreatment with PK11195 sensitized EW36 cells to the induction of apoptosis by sodium arsenite and several pesticides (Muscarella et al., 2003). Furthermore, pretreatment with sub-lethal heat stress sensitized EW36 cells to substantial apoptosis induction by sodium arsenite (Muscarella and Bloom, 2002). However, it is unknown whether this case of potentiation of arsenite-induced apoptosis in EW36 cells extends to other environmental chemicals. Arsenite can inhibit mitochondrial functions as part of its molecular effects; thus we reasoned that other chemicals such as mitochondrial-inhibiting pesticides might act synergistically with heat stress to induced apoptosis in EW36 cells. Combinations of pesticides may also potentiate apoptosis in EW36 cells. This is of particular importance to study since few pesticides, and pesticide combinations, have been evaluated for their potential immuno-toxicity in human cell models or in vivo (Holsapple, 2002).

The present study concerns the detection, and mechanisms, of bypass of Bcl-2 mediated drug resistance induced by multiple stress treatments (particularly pesticides) in a germinal center model consisting of the EW36 B-lymphoid cell line and the HK FDC line. This model recapitulates essential basic features of a human germinal center, including adherence between germinal center B cells and FDC. The EW36 B cell line we used originated from a human germinal center and retains features of normal cells including expression of a functional B-cell receptor (BCR), binding to FDC via surface receptors, and expression of Bcl-2. The human HK FDC line was established from normal human lymph node tissue, and has been used previously to investigate germinal center reactions in immune studies (Kim et al., 1995).

The specific aim of the present study was to investigate whether, and to what extent, apoptosis induction is potentiated by heat stress and pesticide combination exposures in drug-resistant EW36 B-lineage cells. The specific stress exposure scenarios were (1) environmental heat stress plus a pesticide and (2) paraquat plus another pesticide. Furthermore, we evaluated whether JNK pathway activation by the multiple stress treatments is required for apoptosis induction. Finally, we investigated whether binding between EW36 B cells and human FDC, simulating a germinal center interaction, results in the attenuation of apoptosis induced by the combination stress treatments.

Importantly, we found that the heat stress plus pesticide combination exposure resulted in substantial potentiation of apoptosis in EW36 cells, effectively bypassing their stress resistance. The activation of the JNK pathway was found to play a cytoprotective role in EW36 cells. Importantly, EW36 cell binding to FDC reduced the extent of apoptosis induction by most, but not all, combination exposures. These results reveal cell stress scenarios that can greatly augment apoptosis in stress-resistant human B-lineage cells and a germinal center interaction with FDC that selectively attenuates pesticide-induced apoptosis.

**MATERIALS AND METHODS**

**Chemicals and antibodies.** The pesticides aclachlor, aldrin, dieldrin, paraquat, and pyridaben were purchased from Chem Service, Inc. (West Chester, PA), and antimycin A from Sigma-Aldrich (St. Louis, MO). These pesticides were of the highest purity available, 98–99%. The anticancer drugs vincristine and doxorubicin, the chemical inhibitors cycloheximide, thieno[3,4-c]pyridine-3-carboxamide (TTFA), and oligomycin as well as the fluorochromes Hoechst 33342 and propidium iodide were obtained from Sigma-Aldrich (St. Louis, MO). The JNK inhibitor SP600125 was purchased from BIOMOL (Plymouth Meeting, PA). Rabbit polyclonal antibodies specific for total JNK1/2, phosphorylated JNK1/2 (Thr183 and Tyr185), and phosphorylated c-Jun (Ser73) were purchased from Cell Signaling Technology (Beverly, MA). The antibodies for c-Jun and poly (ADP ribose) polymerase (PARP) were purchased from Stressgen (Victoria, BC). Dilutions of antibodies for immunoblotting were 1:2000 for PARP and c-Jun; 1:1000 for total-JNK and phosphorylated JNK.

**Cell culture system and treatments with heat stress and chemicals.** For this research we employed a novel co-culture system consisting of human Burkitt’s lymphoma-derived B-cell lines that adhere rapidly and strongly to monolayers of the human HK FDC line. This model recapitulates several important features of a human germinal center and mantle zone including protection of the B-cell lines from stress-induced apoptosis through stimulation of survival pathways. The human B-cell lines retain phenotypic features of germinal center (ST486) and mantle zone (EW36) B lymphocytes and show differences in drug sensitivity related to expression of Bcl-2 and Bax proteins. This system enabled us to evaluate the effects of heat stress and pesticides on apoptosis induction in B cells in suspension cultures compared to B cells adhered to HK cells.

The EW36 B-lineage lymphoma cell line (NCI, Bethesda, MD) expressing Bcl-2 protein at a high level and the related ST486 B-lineage lymphoma cell line (ATCC, Manassas, VA) expressing low Bcl-2 were cultured in RPMI 1640 medium with L-glutamine and 15% fetal bovine serum (O’Brien et al., 2001). The human HK cell line (Kim et al., 1994) was cultured in the same medium formulation as the B-cell lines, and HK cells were grown to 90% confluence before experiments. Standard incubation conditions for the cell lines were 37°C, 5% CO₂, and 95% humidity.
For all experiments, suspension cultures of EW36 or ST486 cells were set up at a density of 0.3 × 10^6 cells/ml and grown for 24 h. To study the potentiation of apoptosis by multiple stresses, EW3 cells were treated for 1 h at 42.5°C and then transferred to 6-well plates, with 4 ml cell suspension per well. The cells were then cultured for 2 h at 37°C (recovery) after which time the solvent control (methanol or DMSO) and graded concentrations of the particular test chemical were added to the wells, respectively. Control plates contained non-heated stressed cells, but were exposed to the solvent control and test chemical. Cells were harvested at 2 h post-treatment to evaluate activation of the JNK pathway and at 24 h post-treatment to measure the amount of apoptosis induced in the cell cultures. Furthermore, experiments were performed to determine whether paraquat could mimic the effects of heat stress for sensitizing EW36 cells to pesticide-induced apoptosis. EW36 cells were exposed to 400 μM paraquat for 1 h and then treated with antimycin A or with pyridaben for 24 h and 48 h.

For studies in the co-culture system, EW36 cells were added to 6-well plates containing monolayers of HK follicular dendritic cells at 90% confluency. The EW36 cells were allowed to bind to the HK cells for 3 h. The plates with the cocultured cells were then heat stressed at 42.5°C for 1 h, and recovered for 2 h at 37°C. Finally, the test chemical was added to the co-cultures, and EW36 cells subsequently harvested at 24 h to determine the extent of induction of apoptosis. Controls consisted of EW36 cells cultured without the HK dendritic cells and were either kept at 37°C or heat stressed prior to the addition of the test chemical.

**Pretreatments with pharmacologic inhibitors.** Protein synthesis was inhibited by pretreatment of EW36 cells with 0, 2.5, and 5.0 μg/ml cycloheximide. The EW36 cells were then subjected to the heat stress and test chemical treatments as described above. For inhibition of the JNK pathway, 50 μM SP600125 was added to EW36 cell cultures 1 h prior to the addition of the test chemical. The treatment with SP600125 was confirmed to inhibit the phosphorylation of c-Jun in EW36 cells by protein immunoblotting.

**Cytofluorometric detection and quantitation of apoptosis induction.** The induction of apoptosis and necrosis was carefully diagnosed, at an optimal time point of 24 h post-treatment, by a well-validated double fluorescence method (Lieberthal et al., 1998; Muscarella and Bloom, 1997). In the case of paraquat plus pesticide treatments, cells were sampled at 24 h and 48 h since apoptosis was induced with slow kinetics. This procedure allows for rapid, direct diagnosis of apoptotic cell phenotypes by observing condensed, segregated chromatin masses fluorescing blue. Necrotic cells are also easily detected in the same cell samples by observation of red fluorescence in cells due to propidium iodide uptake across damaged cell membranes. Briefly, 250 μl of cell suspension was transferred from the 6-well plates to culture tubes and stained in 20 μg/ml propidium iodide (emitting red fluorescence) and 100 μg/ml Hoechst 33342 (emitting blue fluorescence) for 15 min, at 37°C in the dark. The double fluorescence was detected with a Leitz Aristoplan microscope equipped with an epifluorescence system and a long-pass filter cube A. Digital images of fluorescently labeled cells were obtained using a Spectra-Imager 5000 cooled color microscopy image and analysis system with AlphaEase imaging software (Alpha Innotech, San Leandro, CA). Dead cells emit red and live cells blue fluorescence. Apoptotic cells have a characteristic phenotype of condensed, segregated chromatin bodies in intact but shrunken cells. The apoptotic phenotype was easy to detect and discriminate from necrotic cells, which were swollen, had irregular/damaged membranes and fluoresced red from propidium iodide that entered these damaged cells. Also, the chromatin in such cells was minimally condensed with some accumulation near the nuclear membrane. Typically, 500 cells were scored for each sample and classified as either necrotic, apoptotic, or normal/viable.

**Biochemical characterization and quantitation of apoptosis.** The induction of apoptosis in the B-cell lines was confirmed and further characterized by a biochemical assay. In the apoptotic pathway of the B-lymphoma and many other cell lines, activated caspase-3 cleaves the protein PARP in response to chemical treatments. In the PARP apoptosis assay, the ratio of the signals on immunoblots of the cleaved 85 kDa PARP fragment over the total PARP protein (uncleaved and cleaved) provides a measure of the extent of apoptosis in the cell population (Muscarella and Bloom, 2002).

**Protein immunoblotting detection of PARP cleavage and JNK pathway activation.** The extent of PARP cleavage and activation of the JNK pathway, by the heat stress and chemical treatments, was evaluated using protein immunoblotting as previously described (Muscarella and Bloom, 2002). Briefly, following heat stress and chemical exposure, cells were washed in phosphate buffered saline (PBS) and solubilized in 1× Laemml sample buffer (65.2 mM Tris-Cl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol). 20 μg of protein or 2 × 10^6 cells/sample was subjected to SDS-PAGE in a 4 to 15% gradient gel. Gels were electrophotographically transferred to nitrocellulose membrane (Bio-Rad) in 25 mM Tris, pH 8.3, 192 mM glycine, 20% MeOH. For detection of phosphorylated kinases, membranes were first probed with antibodies specific for the phosphorylated forms of JNK1/2 and c-Jun. Filters were subsequently re-probed using antibodies that recognize the proteins independent of phosphorylation status to insure that differences in signal were due to phosphorylation of the protein and not to differences in amounts of total protein. For PARP cleavage, an antibody that recognizes the 113-kDa, intact PARP and the 85-kDa cleavage product was used to simultaneously detect cleaved and uncleaved PARP (Stressgen, Victoria, BC). Membranes were washed in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) then blocked for 1 h in TBS containing 5% dried milk. Filters were then washed in TBS containing 0.1% Tween-20 then incubated overnight at 4°C with primary antibody diluted appropriately in TBS containing 5% bovine serum albumin. Filters were washed again, and incubated with the second antibody—horseradish peroxidase-conjugate. Detection was then performed using an enhanced chemiluminescent (ECL) system. Quantitation of the signals on films was performed using an Alpha Imager 3400 Documentation and Analysis System, equipped with AlphaEase version 3.2.1 software (Alpha Innotech, San Leandro, CA).

**Statistical analysis of the data.** Three experiments were performed in all cases, and replicate samples analyzed within experiments. Statistical evaluations of all data sets were performed using the statistical program NCSS 6.0 (Kaysville, UT). Percentage data were transformed by arc sine, prior to statistical analysis, to normalize the data. The data were analyzed by ANOVA. If the F-statistic was significant, post-hoc comparisons among control and treatment groups were made using Fisher’s least significant different test. All statistical evaluations were performed at a significance level of p < 0.05.

**RESULTS**

**The EW36 B-Lineage Lymphoma Cell Line, Expressing Bcl-2 Protein, Is Resistant to Chemically Induced Apoptosis.**

A range of concentrations was used to analyze robustly the induction of apoptosis in the human B-cell lines by the respective pesticides and drugs, some of which are mitochondrial inhibitors. This was important in the present study since higher concentrations of chemicals were needed to elicit some apoptosis induction in the drug resistant EW36 cell line. However, the lower concentrations of the mitochondrial inhibitors are generally considered to induce more specific inhibitory effects on complexes within the electron transport chain. Therefore, comparisons of potency for induction and potentiation of apoptosis were made at the low concentrations of these chemicals.

The EW36 B-cell line showed consistent resistance to the induction of apoptosis by the mitochondrial inhibitors...
antimycin A, pyridaben, and TTFA (Fig. 1) and the other chemicals alachlor, aldrin, dieldrin (data not shown), and oligomycin (Fig. 2D). Interestingly, EW36 cells were also fairly resistant to the potent anticancer drug vincristine, although some apoptosis was induced, about 35% at 24 h (Fig. 1A). By 48 h vincristine induced about 50% apoptosis, showing a delayed but robust response. In contrast, the related ST486 B-cell line, expressing low Bcl-2, was quite sensitive to the induction of apoptosis by these same chemicals (Fig. 1). The two pesticide-mitochondrial toxicants, pyridaben and antimycin A, known to inhibit complexes I and III, respectively, showed similar potency for inducing apoptosis in ST486 cells. In contrast, TTFA, a known mitochondrial complex II inhibitor, was a comparatively weak inducer of apoptosis. These results are consistent with our previous findings showing that differential sensitivity to drug-induced apoptosis in these

![Figure 1](image1)

**FIG. 1.** Comparative resistance of the EW36 compared to the ST486 B-lineage lymphoid cell lines to drug and pesticide-induced apoptosis. Cultures of EW36 (black bars) and ST486 (grey bars) were treated with the indicated concentrations of chemicals for 24 h. Cells were analyzed at the 24 h time point for the induction of apoptosis by cytofluorometric detection of apoptotic phenotypes of condensed, segregated chromatin masses fluorescing bright blue. Data points are from three experiments with replicate cultures. The results are presented as the mean ± SEM. The percentage of apoptotic cells was significantly lower (*p < 0.05*) for EW36 cells (asterisks) compared to ST486 cells at all concentrations assayed.

![Figure 2](image2)

**FIG. 2.** Potentiation of apoptosis in EW36 cells by the sequential treatments of 42.5°C heat stress and pesticide (antimycin A, pyridaben) or heat stress and drug (TTFA, oligomycin). EW36 cells were treated at 42.5°C for 1 h (controls at 37°C) followed by incubation for 2 h at 37°C, and then cultures treated with one of the chemicals for a total of 24 h. Cells were analyzed cytofluorometrically for the induction of apoptosis at the 24 time point. The complex III and I inhibitors, antimycin A and pyridaben, respectively showed the greatest degree of potentiation of apoptosis. Minimal necrosis was observed. Data points are from three experiments with replicate cultures. The results are presented as the mean ± SEM. The percentage of apoptotic cells was significantly higher (*p < 0.05*) in the 42.5°C groups for the four drugs at all concentrations compared to the 37°C controls (see asterisks).
cell lines is due to intrinsic factors regulating apoptotic pathways such as differing Bcl-2 and Bax protein expression levels and not due to differential drug accumulation (O’Brien et al., 2001). Furthermore, mitochondria within EW36 cells showed comparative resistance to mitochondrial membrane depolarization induced by antimycin A and other drugs, and this resistance was reversible upon exposure to agents that interfere with Bcl-2 protection (Muscarella et al., 2003; O’Brien et al., 2001).

**Apoptosis Is Potentiated by Sub-lethal Heat Stress Plus Pesticide Exposure in Drug-Resistant EW36 Cells**

We reported previously that drug-resistant EW 36 cells can be sensitized to sodium arsenite-induced apoptosis by prior exposure to sub-lethal heat stress (Muscarella and Bloom, 2002). However, it was unknown whether this effect was restricted to this particular combination of treatments. Also, we were interested in whether heat stress could sensitize EW36 cells to pesticides to which the EW36 cell line is resistant. Therefore, we examined possible potentiation of apoptosis by the combination treatments of heat stress and a pesticide. This first part of our study involved assessment of apoptosis potentiation involving the pesticides antimycin A, pyridaben, aldrin, dieldrin, and alachlor, which have different modes of action in cells. The main goal here was to compare potency of these chemicals for apoptosis induction and synergistic interaction with heat stress. The first four pesticides can inhibit the electron transport chain (Table 1). Comparisons with other known chemical inhibitors such as TTFA (complex II inhibitor) and oligomycin (a complex V or F1F0- ATP synthase inhibitor) were also made. Alachlor is a genotoxic herbicide, and vincristine is an anti-cancer drug that suppresses microtubule dynamics and blocks mitosis.

Apoptosis was not induced in EW36 cells at low or moderate concentrations of antimycin A and pyridaben and only modestly at 100 μM at 24 h. However, the combination of sub-lethal heat stress followed by antimycin A or pyridaben resulted in dramatic potentiation of apoptosis, even at low concentrations (Fig. 2). The heat stress plus antimycin A combination proved to be somewhat more potent, as shown by higher apoptosis induction, particularly at 10 μM and 25 μM antimycin A. This finding was consistent with results from the PARP apoptosis assay, which detected caspase-3 mediated cleavage of the 113 kDa PARP [poly (ADP ribose) polymerase] protein, generating an 85 kDa fragment. For example, antimycin A induced more PARP cleavage compared to pyridaben at 24 h (Figs. 3B and 4B).

Apoptosis in EW36 cells was also potentiated by the combination of heat stress and aldrin or dieldrin; apoptosis was induced less potently by heat plus alachlor (Table 1). The combination of heat plus oligomycin effectively potentiated apoptosis at lower concentrations, but induced only necrosis above 20 μM, consistent with expected dramatic inhibition of ATP production (Fig. 2D). Interestingly, the heat stress plus

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**TABLE 1**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Mode of action</th>
<th>% Apoptosis at 10 μM chemical</th>
<th>With 42.5°C pretreatment</th>
<th>Fold increasea</th>
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<tbody>
<tr>
<td>Antimycin A</td>
<td>Inhibits C-III</td>
<td>5</td>
<td>50</td>
<td>4.4</td>
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<tr>
<td>Oligomycin</td>
<td>Inhibits C-V</td>
<td>8</td>
<td>30</td>
<td>2.6</td>
</tr>
<tr>
<td>Pyridaben</td>
<td>Inhibits C-I</td>
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<td>27</td>
<td>2.4</td>
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<tr>
<td>Aldrin</td>
<td>Inhibits C-I and C-V</td>
<td>9</td>
<td>25</td>
<td>2.2</td>
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<tr>
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<td>Inhibits CIII and C-V</td>
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<td>17</td>
<td>1.5</td>
</tr>
<tr>
<td>Alachlor</td>
<td>Genotoxin</td>
<td>2</td>
<td>15</td>
<td>1.3</td>
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<tr>
<td>TTFA</td>
<td>Inhibits C-II</td>
<td>3</td>
<td>NP</td>
<td></td>
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<tr>
<td>Vincristine</td>
<td>Microtubule inhibitor</td>
<td>33</td>
<td>NP</td>
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*aFold increase in the percent apoptosis at 10 μM chemical compared to solvent control with 42.5°C heat pretreatment. The percent apoptosis for the heat stress control was 11.5% on average. Thus, the fold increase = % apoptosis at 10 μM/11.5% control.

bInhibitors of complexes I, III, and V of the electron transport chain. C-V is the F1F0 ATPase.

NP = no potentiation of apoptosis with 42.5°C heat stress pretreatment.

The value of 33% apoptosis for vincristine is significantly different from control at p < 0.05. The values for percent apoptosis for the other chemicals at 10 μM at 37°C are not significantly different from control values.

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**FIG. 3.** Potentiation of apoptosis in EW36 cells by heat stress plus antimycin A exposures is associated with JNK activation (phosphorylation) and increased expression of c-Jun. (A) Immunoblot showing analysis of phosphorylated JNK and total JNK after 2 h of treatment with antimycin A (heat stress plus antimycin A). (B) c-Jun was analyzed at the 24 h time point. The cleavage of poly (ADP ribose) polymerase (PARP), reflecting apoptosis induction, was also analyzed. The percent cleaved PARP reflects the extent of apoptosis induction in the cell population and complements the cytofluorometric method. Twenty μM arsenic (AS) was included as a positive control for potent activation of JNK, c-Jun induction, and PARP cleavage.
TTFA combination failed to potentiate apoptosis (Fig. 2C), and a similar result was obtained with vincristine (Table 1). The hierarchy of potencies of the eight chemicals to synergize with heat stress to induce apoptosis is shown in Table 1. The mitochondrial inhibitors constitute the most potent of the chemicals evaluated, except for TTFA, the complex II inhibitor. Vincristine stands out by its potency to induce apoptosis in EW36 cells in the absence of heat stress.

The Potentiation of Apoptosis by Non-lethal Heat Stress and Antimycin A Is Associated with Activation of the JNK Pathway and c-Jun Expression

We examined the JNK pathway as a candidate for regulating stress-induced apoptosis in EW36 cells. Sub-lethal heat stress alone increased modestly the baseline level of phosphorylated JNK due to heat 42.5°C heat stress, but no further phosphorylation due to pyridaben exposure. (B) Immunoblot shows potentiation of PARP cleavage by heat stress plus pyridaben but no induction of c-Jun expression. AS is the arsenic positive control.

The Potentiation of Apoptosis by Sub-lethal Heat Stress Plus Pyridaben Occurs Independently of JNK Pathway Activation

The combination of heat stress and pyridaben resulted in the potentiation of apoptosis/PARP cleavage in EW36 cells (Fig. 4). However, apoptosis induction was not accompanied by JNK phosphorylation or by the induction of c-Jun expression (Figs. 4A, 4B). Thus, heat stress increases baseline phospho-JNK but the JNK pathway is not additionally activated by the pyridaben treatment. These results suggested that the potentiation of apoptosis by heat stress plus pesticide does not require the activation of the JNK pathway.

The Profile of JNK Pathway Activation following Apoptosis Potentiation by Paraquat Plus Antimycin versus Pyridaben Parallels That for Heat Stress

To further explore the versatility of the B-cell model system for detecting potentiation of apoptosis and the involvement of the JNK pathway, we examined whether heat stress could be replaced by pre-exposure to the pesticide paraquat. This chemical was also of interest in this study since paraquat has been shown to be a mitochondrial toxicant (Vicente et al., 2001). We found that combination treatments with paraquat and antimycin or paraquat and pyridaben potentiated apoptosis in EW36 cells detected at 48 h (Fig. 5). However, the extent of apoptosis potentiation was not as great, and it was slower compared to the heat stress plus pesticide exposure protocol. In the case of paraquat plus antimycin A exposure, apoptosis induction was accompanied by the induction of c-Jun expression (Fig. 5A). Paraquat plus pyridaben potentiated apoptosis in the absence of c-Jun induction (Fig. 5B). These findings show that another stress can substitute for heat shock as a sensitizing agent, but that the result for c-Jun expression is similar to the heat shock scenario. These collective results indicate that the second treatment with the pesticide, antimycin versus pyridaben, determines whether the JNK pathway is activated. The first treatment determines the degree of sensitization of cells and the rapidity of apoptosis induction, with the heat shock-mediated response faster (18–24 h) than the paraquat response (48–72 h).

Roles of Protein Synthesis and the JNK Pathway in Apoptosis Potentiation: Studies with Pharmacologic Inhibitors

EW36 cell cultures were pretreated with cycloheximide in order to investigate whether new protein synthesis is required for the potentiation of apoptosis by heat stress and pesticide treatments. Treatment of EW36 cells with 10, 25, and 50 µg/ml cycloheximide did not inhibit or attenuate apoptosis induction.

**FIG. 4.** Potentiation of apoptosis in EW36 cells by heat stress plus pyridaben exposures occurs in the absence of JNK pathway activation. EW36 cells were heat stressed and treated with pyridaben for 24 h. (A) Immunoblots show increase in baseline phosphorylated JNK due to heat 42.5°C heat stress, but no further phosphorylation due to pyridaben exposure. (B) Immunoblot shows potentiation of PARP cleavage by heat stress plus pyridaben but no induction of c-Jun expression. AS is the arsenic positive control.

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**The Potentiation of Apoptosis by Non-lethal Heat Stress and Antimycin A Is Associated with Activation of the JNK Pathway and c-Jun Expression**

We examined the JNK pathway as a candidate for regulating stress-induced apoptosis in EW36 cells. Sub-lethal heat stress alone increased modestly the baseline level of phosphorylated JNK (Fig. 3A). However, the combination of heat stress followed by antimycin A treatment resulted in greatly increased phosphorylation of JNK at all concentrations at 24 h (Fig. 3A). We also examined c-Jun protein expression representing a downstream consequence of activation of the JNK pathway. Levels of c-Jun protein increased in parallel with phospho-JNK protein (Figs. 3A and 3B). In addition, the data in Figure 3 show the parallel concentration—dependent increases in c-Jun expression and PARP cleavage, reflecting the extent of apoptosis. Other activator protein-1 (AP-1) transcription factors in the Jun family were examined. Jun D protein was found to be expressed at moderate levels but was not induced as a result of the heat stress and pesticide treatments (data not shown). No baseline or induced Jun B protein was detected. Therefore, c-Jun is the main Jun protein that is associated with the potentiation of apoptosis in EW36 cells.

**The Potentiation of Apoptosis by Sub-lethal Heat Stress Plus Pyridaben Occurs Independently of JNK Pathway Activation**

The combination of heat stress and pyridaben resulted in the potentiation of apoptosis/PARP cleavage in EW36 cells (Fig. 4). However, apoptosis induction was not accompanied by JNK phosphorylation or by the induction of c-Jun expression (Figs. 4A, 4B). Thus, heat stress increases baseline phospho-JNK but the JNK pathway is not additionally activated by the pyridaben treatment. These results suggested that the potentiation of apoptosis by heat stress plus pesticide does not require the activation of the JNK pathway.

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To further explore the versatility of the B-cell model system for detecting potentiation of apoptosis and the involvement of the JNK pathway, we examined whether heat stress could be replaced by pre-exposure to the pesticide paraquat. This chemical was also of interest in this study since paraquat has been shown to be a mitochondrial toxicant (Vicente et al., 2001). We found that combination treatments with paraquat and antimycin or paraquat and pyridaben potentiated apoptosis in EW36 cells detected at 48 h (Fig. 5). However, the extent of apoptosis potentiation was not as great, and it was slower compared to the heat stress plus pesticide exposure protocol. In the case of paraquat plus antimycin A exposure, apoptosis induction was accompanied by the induction of c-Jun expression (Fig. 5A). Paraquat plus pyridaben potentiated apoptosis in the absence of c-Jun induction (Fig. 5B). These findings show that another stress can substitute for heat shock as a sensitizing agent, but that the result for c-Jun expression is similar to the heat shock scenario. These collective results indicate that the second treatment with the pesticide, antimycin versus pyridaben, determines whether the JNK pathway is activated. The first treatment determines the degree of sensitization of cells and the rapidity of apoptosis induction, with the heat shock-mediated response faster (18–24 h) than the paraquat response (48–72 h).

**Roles of Protein Synthesis and the JNK Pathway in Apoptosis Potentiation: Studies with Pharmacologic Inhibitors**

EW36 cell cultures were pretreated with cycloheximide in order to investigate whether new protein synthesis is required for the potentiation of apoptosis by heat stress and pesticide treatments. Treatment of EW36 cells with 10, 25, and 50 µg/ml cycloheximide did not inhibit or attenuate apoptosis induction.
by heat stress plus antimycin or pyridaben (data not shown). This drug treatment did inhibit apoptosis induction by sodium arsenite alone or in combination with heat stress, consistent with our previous studies (Muscarella et al., 2003).

Exposure of EW36 cells to heat stress plus antimycin or pyridaben results in preferential induction of the JNK pathway by the former chemical. These results may indicate a lack of requirement of the JNK pathway for apoptosis potentiation or that different mechanisms are operative for each chemical, i.e., JNK is required for antimycin, but not for pyridaben potentiation of apoptosis. We therefore performed experiments to test whether the JNK pathway is required for apoptosis potentiation by heat stress and antimycin A. EW36 cultures were treated with 12, 25, 50, or 100 μM SP600125, a widely employed specific inhibitor of JNK (Bennett et al., 2001). The potentiation of apoptosis by heat stress and antimycin was not inhibited at any of the SP600125 concentrations (data not shown). The SP600125 treatment was determined to inhibit JNK-mediated phosphorylation of c-Jun as well as the induction of c-Jun expression in treated EW36 cells. These results suggest that the JNK pathway is not required for potentiation of apoptosis by heat stress plus antimycin, but may be a stress response that serves a survival function.

Partial Rescue of EW36 from Pesticide-Induced Apoptosis by Co-culture with FDC

Previous studies have shown the importance, and relevance for immunotoxicity evaluation, of assessing B-cell responses in the context of interactions encountered at the germinal center and mantle zone. In particular it is known that B-lymphocyte interactions with FDC can promote B-cell survival. We used a co-culture system consisting of EW36 B-lineage cells and the HK FDC line to examine whether direct interactions of these cell types inhibit apoptotic responses. In this co-culture system EW36 cells bind to HK cells rapidly and strongly. This part of our study was focused on the most important pesticides, namely pyridaben and antimycin A. Pyridaben is a pesticide widely used in agriculture to control insects on fruits and other crops. It is well documented to inhibit complex I in mitochondria, is considered moderately toxic but little is known about its tissue-specific cellular and molecular effects. Antimycin A is a well-documented inhibitor of complex III and is used to control fish populations (piscicide). In light of the known but different modes of action, wide uses, but lack of information on cellular toxicity, the mechanistic work was focused on these two pesticides.

Importantly, we found that the potentiation of apoptosis by the sequential treatment of heat stress and pyridaben was attenuated by about 35% when EW36 cells were adhered to monolayers of HK cells (Fig. 6A). This effect was dependent on EW36 cell adhesion to HK cells and was not due to soluble factors. Contrastingly, the potentiation of apoptosis by heat stress and antimycin A was not altered through adhesion to HK cells (Fig. 6B). Sodium arsenite-induced apoptosis is potently synergized by heat stress exposure in EW36 cells (Muscarella and Bloom, 2002), but this response was inhibited in co-cultures with HK cells (Fig. 6B, “As” data). The potentiation of apoptosis by heat stress plus other chemicals (oligomycin, aldrin, dieldrin, or alachlor) attenuated apoptosis by about 25% (for each chemical) compared to controls (data not shown). Experiments where EW36 cells were cultured in conditioned medium from HK cell cultures showed that soluble factors were not responsible for the attenuation of apoptosis in this model system. These combined results show that the interaction of EW36 B cells with HK cells provides protection.
against apoptosis induced by heat stress and pesticides, except for antimycin A.

DISCUSSION

Can Bcl-2-based Drug Resistance Be Reversed through Exposures to Multiple Environmental Stresses?

During differentiation of germinal center and mantle zone B cells, levels of Bcl-2 protein increase upon exposure to a variety of survival stimuli, and cells become more resistant to various stresses. This latter effect extends to B-cell lymphomas where increased expression of Bcl-2 plays a major role in oncogenesis and resistance to anticancer drug-induced apoptosis (Cory and Adams, 2002). Thus, Bcl-2 expression level plays a major role in lymphocyte survival, differentiation, lymphomagenesis, and in multi-drug cellular resistance.

Of great interest is the question of whether, and under what circumstances, Bcl-2-based cellular resistance can be bypassed, resulting in extensive cell death in lymphocyte populations.

The present study was directed toward addressing this question using the EW36 cell line as a model of human B-lineage lymphoid cells expressing high Bcl-2 protein and resistant to the induction of apoptosis by diverse chemical agents. We previously found that exposure of EW36 cells to sequential sub-lethal heat stress and sodium arsenite potentiated the induction of apoptosis (Muscarella and Bloom, 2002). However, it was not established whether this effect extended to other classes of chemicals such as pesticides, which have not been thoroughly studied for effects on lymphocytes (Holsapple, 2002). Furthermore, studies of B-cell sensitivity to individual and multiple environmental stresses should take into account possible reduction in lymphocyte apoptosis resulting from cell-cell interactions that occur in germinal centers of lymphoid follicles. Therefore, the model used in our study involved the evaluation of multiple stress effects on human EW36 B cells with and without binding to human FDC.

Reversal of Stress Resistance in EW36 Cells through Heat Stress Plus Pesticide Exposure

We found that the EW36 cell line was resistant, over a wide range of concentrations, to apoptosis induction by several pesticides as well as the anticancer drug vincristine. These results are consistent with our previous findings of resistance of EW36 cells to the uncoupler carbonyl cyanide m-chlorophenylhydrazone and sodium arsenite, associated with Bcl-2 expression (Muscarella and Bloom, 2002; O’Brien et al., 2001).

Importantly, the exposure of EW36 cells to sub-lethal heat stress plus a pesticide resulted in the rapid and substantial potentiation of apoptosis. The five pesticides were grouped based on their potency for synergizing with heat stress to induce apoptosis. The most potent of the pesticides in this multiple stress exposure scenario was antimycin A. Pyridaben and aldrin were grouped as moderately potent; dieldrin and alachlor were the least potent for inducing apoptosis. The pesticides in this study have in common known direct effects on mitochondrial functions. For example, antimycin A inhibits the electron transport chain at complex III, as is believed to be the case for dieldrin that can also inhibit oligomycin-sensitive Mg$^{2+}$ ATPases (Mehrotra et al., 1982). Both antimycin A and dieldrin have been shown to rapidly decrease mitochondrial membrane potential, associated with apoptosis induction (Kitazawa et al., 2001; O’Brien et al., 2001). Aldrin, a structural analog of dieldrin, can inhibit electron flow at complex I as well as oligomycin-sensitive Mg$^{2+}$ ATPases (Mehrotra et al., 1982; Pardini et al., 1971). Pyridaben, a widely used miticide, binds to the PSST subunit of complex I and inhibits electron flow (Schuler et al., 1999). Pyridaben has not been fully evaluated for possible immunotoxic effects, but our results show that it is a potent inducer of apoptosis in ST486 cells and in EW36 cells in combination with heat stress.
Contrastingly, the complex II inhibitor TTFA did not induce apoptosis.

These combined results suggest that the potentiation of apoptosis by heat stress plus pesticide may result from successive, multiple insults to particular mitochondrial components resulting in great enhancement of apoptosis and bypass of Bcl-2 protection. The combined treatments could impair homeostatic regulation of mitochondrial function by the permeability transition (PT) pore complex leading to release of apoptogenic proteins. In addition, inhibition of electron flow at complexes III and I can generate excess reactive oxygen species, and an oxidative stress condition, contributing to mitochondrial dysfunction (Chen et al., 2003; Garcia-Ruiz et al., 1997; St-Pierre et al., 2002).

**JNK Activation and Protein Synthesis Are Not Required for Apoptosis Potentiation**

The role of the JNK pathway in regulating the engagement in apoptosis can vary, as it can promote, suppress or have no apparent role in cell death. Several studies suggest that JNK may not necessarily be an intrinsic component regulating apoptosis. Rather, JNK is proposed to be a modulator of apoptosis, and particular outcomes are cell-type and stimulus-dependent (Lin, 2003). We used a well-validated JNK inhibitor, SP600125 (Bennett et al., 2001), to further explore a role (s) of the JNK pathway in the potentiation of apoptosis by antimycin A. However, the potentiation of apoptosis and JNK pathway activation by heat stress plus antimycin A was not inhibited by SP600125. Rather, JNK activation was revealed to be a stress response that is partially cytoprotective, i.e., suppresses stress-induced apoptosis. Similar results suggesting a cytoprotective role of JNK activation have been observed in other studies. Dougherty et al. (2002) found that activation of the JNK pathway promotes survival of cardiac myocytes exposed to an oxidative stress condition. CD40 receptor engagement in WEHI-231 B lymphoma cells induced the JNK and p38 pathways correlating with cell survival (Sutherland et al., 1996). A role for JNK in IL-3 stimulation of proliferation, but not apoptosis, was observed in the BAF3 pre-B cell line (Smith et al., 1997). We also observed in studies with cycloheximide that the potentiation of apoptosis by heat stress plus pesticide does not require protein synthesis. Thus, apoptosis potentiation may be propelled by more direct effects of these stresses on mitochondria and/or by the activation of other signaling pathways that have yet to be identified.

**Contact between EW36 and HK-FDC Attenuates Apoptosis Induced by Most Chemicals**

Previous studies highlight the importance of direct interactions between B cells and FDC in the germinal center for promoting B-cell survival (Nossal, 1994). However, few studies to determine the effects of such interactions, and mechanisms involved, on modulating the extent of drug- and toxicant-induced apoptosis have been conducted. We used the HK FDC line in a co-culture system with the EW36 B-lineage cell line to determine whether the extent of apoptosis induced by heat stress plus chemical (pesticides and arsenite) is attenuated through direct binding of EW36 cells to HK cells. We found that apoptosis was partially attenuated through this cell-cell interaction in the combinations of heat stress plus pyridaben or with arsenite as well in with other chemicals, aldrin, dieldrin, alachlor, or oligomycin. This attenuation of apoptosis was shown to require contact between EW36 and HK cells and was not significantly influenced by soluble factors. Apoptosis induction was not reduced in the cases of heat stress plus antimycin A. Thus, the EW36 cell-HK cell interaction enhanced cellular protection against most but not all chemical stresses. The mechanism of this differential protection was not determined in our study, and it is presently unclear what pro-apoptotic pathway (s) that is induced by pyridaben and arsenite is readily antagonized by the EW36-HK cell interaction. Also, it is unknown how heat stress plus antimycin combination induces apoptosis in an HK-independent manner.

**Working Model for Potentiation of Apoptosis in Stress Resistant B Cells**

In summary, our study demonstrated, for the first time, dramatic potentiation of apoptosis resulting from exposures of EW36 B cells to the combination of sub-lethal heat stress plus a pesticide. This potentiation was found for several different pesticides. Of additional significance is that apoptosis can be potentiated by the combination of paraquat plus a pesticide. This suggests that additional combination of pesticides or other chemicals may act synergistically to induce apoptosis in stress resistant B cells. Along these lines it has recently been reported that mixtures of pesticides, such as lindane and malathion, can act synergistically to induce apoptosis in murine thymocytes (Olgun et al., 2004). These studies highlight the importance of determining the mechanisms involved in apoptosis induction by individual and mixtures of pesticides in B- and T-lineage lymphocytes.

We also extended our study to include possible modulation of apoptosis resulting from adherence of EW36 B cells to HK FDC, modeling an important germinal center interaction. Importantly, multiple stress induced-apoptosis was attenuated through this interaction, extending knowledge of the effects of such cell-cell interactions to include pesticides and heavy metals.

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