Apoptosis Induction in Lymphoma Cells: Thiol Deprivation versus Thiol Excess

(apoptosis induction / thiol deprivation / thiol excess / lymphoma cells)

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Abstract. We studied the effects of thiol availability on apoptosis induction in B-cell lymphoma 38C13, T-cell lymphoma EL4, and also other cells. Compounds with a free SH group are required for survival and growth of 38C13 cells but not of EL4 cells. Thiol deprivation (2-mercaptoethanol concentrations about 0.3 μM and lower) induced apoptosis in 38C13 cells. On the other hand, thiol excess (2-mercaptoethanol concentrations higher than 300 μM) induced apoptosis in 38C13 cells and EL4 cells as well as in other cells (e.g. Raji, HeLa). L-cystine and non-thiol antioxidant ascorbic acid were unable to support survival of 38C13 cells. Ascorbic acid induced cell death at concentrations higher than 600 μM. Thiol cross-linking compound diamide (100 μM and higher) abrogated the survival-supporting effect of 2-mercaptoethanol (50 μM). Apoptosis induction by thiol deprivation and by thiol excess was not directly related to a specific significant change in the p53 level or p53 activation. Apoptosis induction by thiol excess was associated with a certain decrease in the Bcl-2 level while the Bax level did not change. We conclude that both thiol deprivation and thiol excess can induce apoptosis in lymphoma cells. Apoptosis induction by thiol deprivation is specifically related to the presence of a free SH group. However, apoptosis induction by thiol excess does not seem to be specifically related to the presence of a free SH group. It probably results from the excess of a reductant. Apoptotic control protein p53 does not seem to play a significant role in apoptosis induction either by thiol deprivation or by thiol excess.

Apoptosis is a genetically encoded programme of cell self-destruction. It plays a significant role in many physiological processes such as those involved in development or those related to the function of the immune system. Apoptosis also occurs in tumour cells responding to therapy (Thompson, 1995; Jacobson et al., 1997; Raff, 1998). Thus, the factors involved in control of apoptosis are of particular interest.

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Abbreviation: PBS – phosphate-buffered saline.

Proteins of the Bcl-2 family such as antiapoptotic Bcl-2 and proapoptotic Bax or protein p53 seem to play an important role in control of apoptosis induction. On the other hand, changes in mitochondrial function, particularly those related to the change of mitochondrial membrane permeability, seem to be decisive for switching on the cellular apoptotic programme (Adams and Cory, 1998; Green and Reed, 1998; Gross et al, 1999). The redox status of the cell very likely plays an important role in these decisive events (Sato et al., 1995; Marchetti et al., 1997; Falk et al., 1998; Hall, 1999). Therefore, thiol antioxidants may play a significant role in apoptosis regulation and thus also in strategies concerning cancer treatment.

It was shown earlier that 2-mercaptoethanol was required for growth of B cells (Ishii et al., 1981). More recently, it has been demonstrated that 2-mercaptoethanol and other thiol compounds promote survival and prevent apoptosis in various lymphoid cells (Sato et al., 1995; Delneste et al., 1996; Kinoshita, 1997; Falk et al., 1998; Neumann, 1998), and also in other types of cells (Castro-Obregon and Covarrubias, 1996; Aoshiba et al., 1999, Jayasurya et all., 2000, Yang et al., 2000). It has also been demonstrated that thiols can inhibit apoptosis induced by other apoptotic stimuli such as glucocorticoids and irradiation (McLaughlin et al., 1996; Marchetti et al., 1997; Mirkovic et al., 1997) or viruses (Lin et al., 1995). Antiapoptotic thiol effects are supposed to be based on the presence of a free SH group (Delneste et al., 1996; Deas et al., 1997; Falk et al., 1998).

It has been found that some thiol compounds can inhibit proliferation of human leukaemic cells (Jeitner et al., 1998). It has also been found that exposure to some thiols at relevant concentrations can even kill cells of non-haematopoietic origin (Takagi et al., 1974; Held and Melder, 1987; Held and Biaglow, 1994). The authors suppose that thiol cytotoxicity results from the generation of H_2O_2. However, the cell killing was not related to apoptosis in these papers.

Mouse B-cell lymphoma 38C13 was found previously to be highly sensitive to apoptosis induction by iron deprivation (Kovar et al., 1997). Later, the lymphoma was also found to be highly sensitive to 2-mercaptoethanol withdrawal from culture media while mouse T-cell lymphoma EL4 was found to be completely

resistant. Therefore, we decided to study the effects of thiol availability on apoptosis induction specifically in lymphoma cells, employing as a model lymphoma 38C13 versus lymphoma EL4.

Material and Methods

Chemicals and reagents

2-mercaptoethanol was from Fluka (Buchs, Switzerland), dithiothreitol was from Bio-Rad (Hercules, CA), L-cysteine, L-cystine, ascorbic acid and diamide were from Sigma (St. Louis, MO). Mouse monoclonal antibody PAb 240 against mouse p53 from Calbiochem (La Jolla, CA), mouse monoclonal antibody HZ 52 against mouse $p21^{CIP1/WAF1}$ from Neo Markers (Fremont, CA), Syrian hamster monoclonal antibody 3F11 against mouse Bcl-2 from Pharmingen (San Diego, CA) and rabbit polyclonal antibody (Sc-562) against mouse Bax from Santa Cruz (Santa Cruz, CA) were used.

Cells and culture conditions

The mouse B-cell lymphoma 38C13 and the mouse T-cell lymphoma EL4 were obtained from Prof. J. Kemp (University of Iowa, Iowa City, IA). For some confirmatory experiments, human Raji and HeLa cells were employed. The human Burkitt lymphoma Raji was obtained from Prof. G. Klein (Karolinska Institutet, Stockholm, Sweden), and human cervix carcinoma HeLa was obtained from Dr. A. Cvekl (Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic). The cell lines were routinely tested for mycoplasma contamination using the fluorescent Hoechst 33258 staining method (Chen, 1977).

Defined serum-free culture media were used. The basic medium was RPMI 1640 containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), Hepes (15 mM), iron-saturated human transferrin (5 μg/ml), ethanolamine (20 μM), ascorbic acid (20 μM), hydrocortisone (5 nM) and 11 trace elements, as described previously (Kovář, 1988; Kovář and Franěk, 1989). Cells were routinely maintained in the basic medium supplemented with 2-mercaptoethanol (50 μM). In experiments, the effects of the basic medium supplemented with various concentrations of 2-mercaptoethanol, L-cysteine, L-cystine, dithiothreitol, ascorbic acid and diamide were tested. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Cell growth and survival analysis

Cells maintained in the basic medium supplemented with 50 μM 2-mercaptoethanol were harvested by low-speed centrifugation, washed with the basic medium and then seeded at 40 x 10^3 (20 x 10^3) cells/100 μl of medium into wells of a 96-well plastic plate. Cell growth and survival in the basic medium and in the basic medium with tested concentrations of 2-mercaptoethanol, L-cysteine, L-cystine, dithiothreitol, ascorbic acid and diamide were assessed after 24 h of incubation. The number of living cells was determined by haemacytometer counting after staining with trypan blue.

Propidium iodide staining analysis

Cells previously grown in the basic medium supplemented with 50 μM 2-mercaptoethanol were harvested by low-speed centrifugation, washed with the basic medium and seeded at 400 x 10^3 cells/ml of medium into plastic culture flasks. The effect of control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (basic medium without 2-mercaptoethanol) and thiol excess (basic medium with 1000 μM 2-mercaptoethanol) was tested. After 0, 8, 16 and 24 h of incubation, the cells were harvested by low-speed centrifugation and stained. Briefly, approximately 4 x 10^6 cells per sample were washed twice with 2 ml of phosphate-buffered saline (PBS) and then fixed in 2 ml of 70% ethanol for 1 h at 4°C. Fixed cells were centrifuged and washed with 2 ml of PBS. The cell pellet was treated with 1 ml of 0.1% Triton X-100 at 4°C for 3 min and centrifuged. Supernatant was removed and 1 ml of RNAse (100 U/ml of PBS) was added. After a 10-min incubation at room temperature, the samples were spun and supernatant was aspirated. Propidium iodide (1 ml, 50 μg/ml) was added and the samples were incubated in the dark at 4°C for 1 h. Stained cells were analysed in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

DNA fragmentation analysis

Cells grown in the basic medium supplemented with 50 μM 2-mercaptoethanol were harvested by low-speed centrifugation, washed with the basic medium and seeded at 400 x 10^3 (800 x 10^3) cells/ml of medium into plastic culture flasks. The effect of control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (basic medium without 2-mercaptoethanol) and thiol excess (basic medium with 1000 μM 2-mercaptoethanol), as well as the effect of basic medium with employed concentrations of L-cysteine, L-cystine and dithiothreitol, was tested. After 16 h (22 h) of incubation, the cells were harvested by low-speed centrifugation and analysed. Briefly, 10^6 cells per sample were lysed in 400 ml of lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100) for 5 min at 4°C. Lysates were centrifuged at 13000 x *g* for 15 min at 4°C. DNA in the supernatant was precipitated overnight at -20°C by addition of 700 μl of 96% ethanol and 120 μl of 5 M NaCl. Samples were centrifuged again at 13000 x *g* for 15 min at 4°C. The pellet was washed twice with 200 ml of 70% ethanol and dried at room temperature. The dried pellet was dissolved in 20 μl of TE buffer (10 mM Tris, 1 mM EDTA) at 65°C. After cooling to room temperature, 2 μl of RNAse (100 U/ml of PBS) and 2 μl of proteinase K (2 mg/ml) were added and samples were incubated for 10 min at room temperature. Finally, 6 μl of loading dye (40 mM Tris, 20 mM sodium acetate,

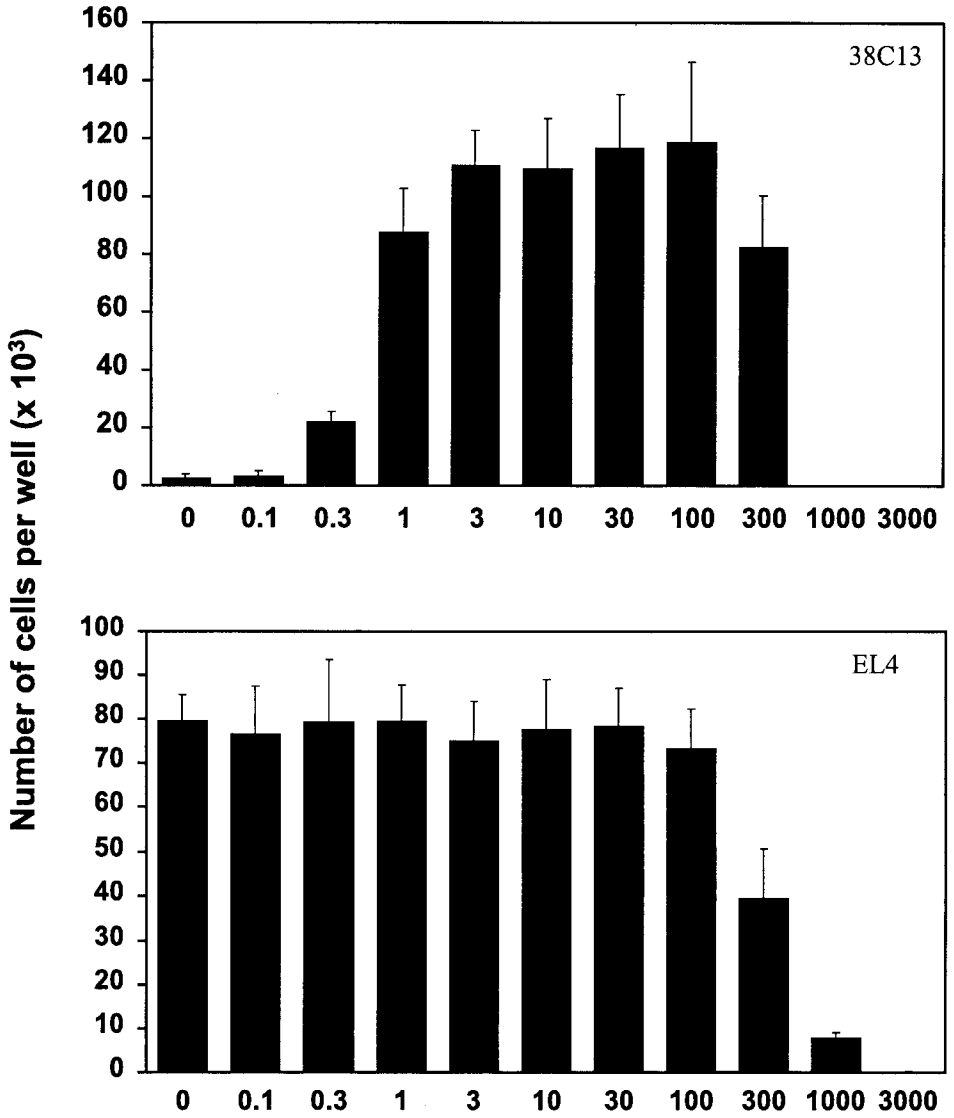

Fig. 1. Effect of 2-mercaptoethanol (0.1–3000 μM) on the growth and survival of 38C13 and EL4 cells. Control cells were incubated without 2-mercaptoethanol, i.e. in the basic medium. Cells were seeded at 40 x 10^3 cells/100 μl of medium in the well. The number of cells in the inoculum is shown as a dotted line. The number of living cells was determined after 24-h incubation. Each column represents the mean of at least four separate cultures ± SEM.

1 mM EDTA, 50% glycerol, 2% SDS, 20% saturated bromphenol blue solution) were added. Samples (20 μl) were run on a 1% agarose gel with ethidium bromide (10 μg/ml) in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA) at about 10 V/cm. DNA was visualized under UV light and photographed.

Indirect immunofluorescence analysis

Cells previously grown in the basic medium supplemented with 50 μM 2-mercaptoethanol were harvested by low-speed centrifugation, washed with the basic medium and seeded at 400 x 10^3 cells/ml of medium into plastic culture flasks. The effect of control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (basic medium without 2-mercaptoethanol) and thiol excess (basic medium with 2000 μM 2-mercaptoethanol) was tested. Indirect immunofluorescence according to the modified method of Pollice et al. (1992) was employed to assess the expression of p53, $p21^{CIP1/WAF1}$, Bcl-2 and Bax. After 0, 4, 8, 12 and 16 h of incubation, the cells were harvested by low-speed centrifugation and stained. Briefly, approximately 4 x 10^6 cells per sample were washed with 4 ml of PBS and then fixed in 2 ml of 0.25% paraformaldehyde in the dark for 15 min at room temperature. The cells were spun, washed with PBS and then fixed in 2 ml of 70% methanol for 1 h at 4°C. Fixed cells were centrifuged and washed with PBS. The cell pellet (approximately 1 x 10^6 cells per parallel) was resuspended and incubated in 50 μl of primary antibody (5 μg/ml of PBS) or in 50 μl of non-specific mouse, hamster or rabbit IgG (5 μg/ml of PBS) as a negative control. Mouse monoclonal antibody (IgG) PAb 240 against mouse p53, mouse monoclonal antibody (IgG) HZ 52 against mouse p21, hamster monoclonal antibody (IgG) 3F11 against mouse

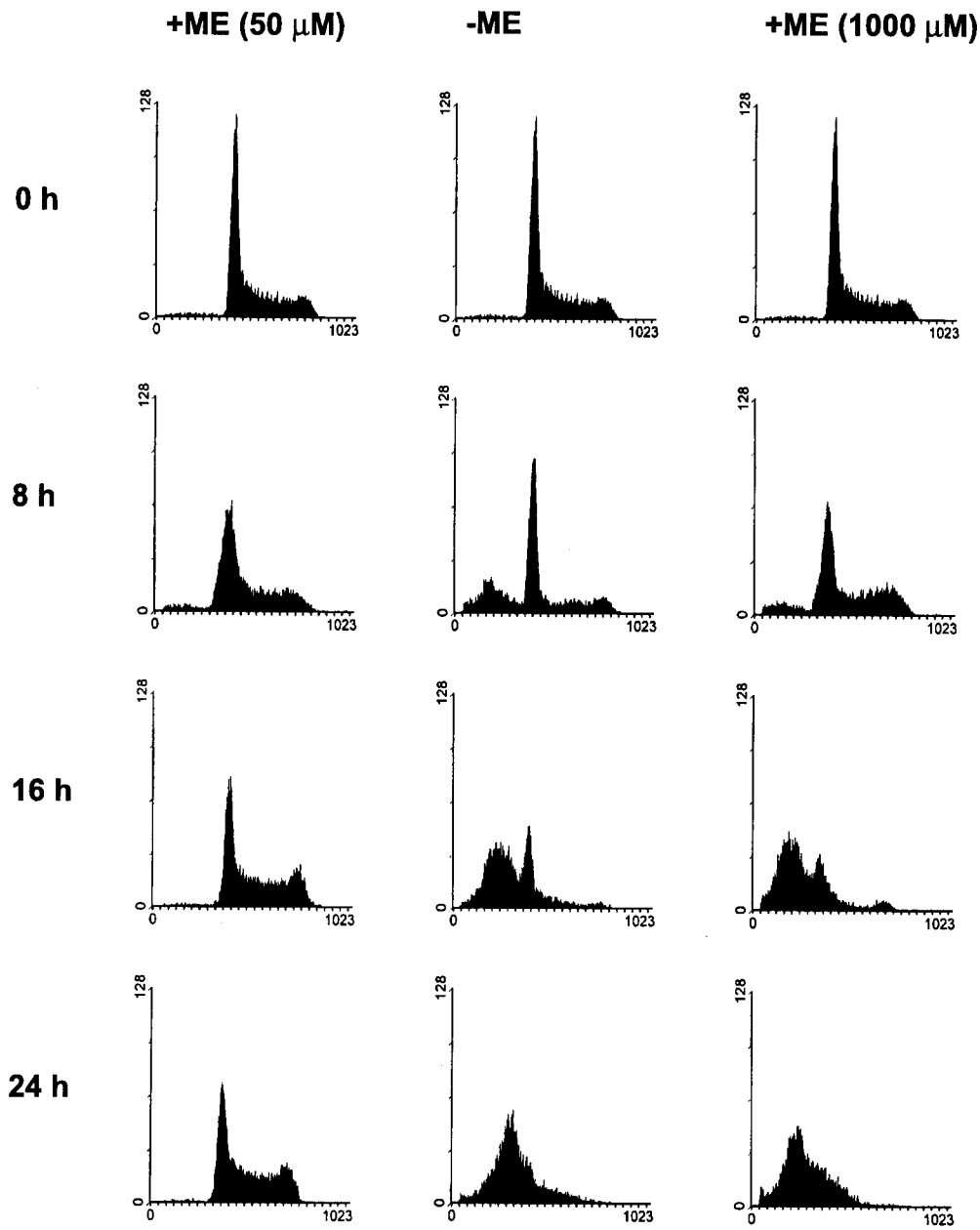

Fig. 2. Effect of availability of thiols, represented by 2-mercaptoethanol (ME), on DNA histograms of 38C13 cells. Control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (without 2-mercaptoethanol), and thiol excess (with 1000 μM 2-mercaptoethanol) were tested. After the incubation period (0, 8, 16 and 24 h), the cells were stained with propidium iodide and analysed by flow cytometry.

Bcl-2 and rabbit polyclonal antibody (IgG) Sc-562 against mouse Bax were used as primary antibodies. After 30 min of incubation on ice, 400 μl of PBS were added and cells were resuspended. The sample was underlain with 100 μl of foetal bovine serum (PAN Biotech, Aidenbach, Germany) and spun. The cell pellet was resuspended and incubated in 50 μl of secondary staining reagent (10 μg/ml of PBS). Corresponding (anti-mouse, anti-hamster and anti-rabbit) fluorescein-conjugated goat antibodies were used as the secondary staining reagents. After 30 min of incubation on ice, 400 μl of PBS were added and cells were resuspended. The sample was again underlain with 100 μl of foetal bovine serum and spun. Stained cells were resuspended in 300 μl of PBS and analysed in a FACScan flow cytometer (Becton Dickinson).

Results

Cell growth and survival under differing availability of thiols

We compared the effect of 2-mercaptoethanol in a wide range of concentrations (0.1–3000 μM) on the growth and survival of 38C13 and EL4 cells. Concentrations of 2-mercaptoethanol about 0.3 μM and lower

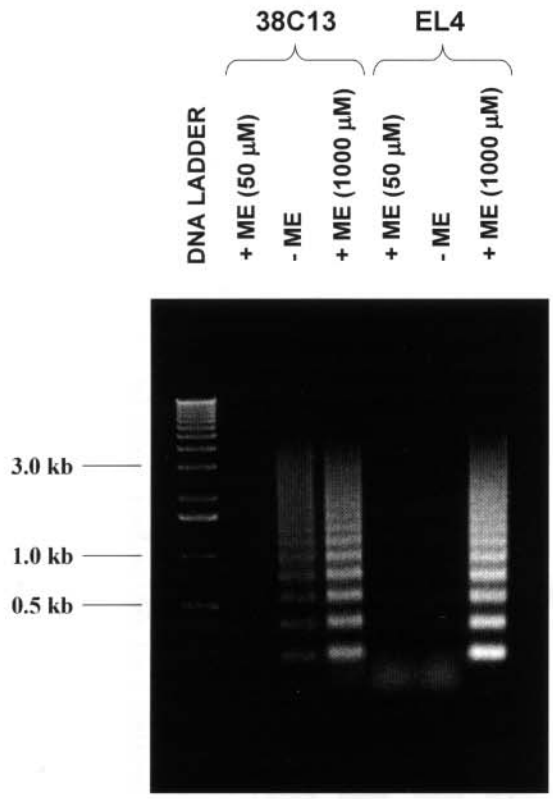

Fig. 3. Effect of availability of thiols, represented by 2-mercaptoethanol (ME), on DNA fragmentation in 38C13 and EL4 cells. Control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (without 2-mercaptoethanol), and thiol excess (with 1000 μM 2-mercaptoethanol) were tested. DNA fragmentation was determined after 16 h of incubation. Control DNA ladder is shown.

resulted in the death of 38C13 cells during 24-h incubation. On the other hand, 1000-μM and higher concentrations also resulted in cell death. The cells displayed growth in the presence of 1–300 μM 2-mercaptoethanol with the optimum at about 3–100 μM. In the case of EL4 cells, low 2-mercaptoethanol concentrations or even complete 2-mercaptoethanol withdrawal did not lead to cell death. Also, the growth of the cells under the deprivation of 2-mercaptoethanol was comparable with the growth under such 2-mercaptoethanol concentrations that were near optimal for 38C13 cells. Concentrations of 2-mercaptoethanol higher than 300 μM led to the death of EL4 cells similar to that seen in 38C13 cells (Fig. 1). The effect of another thiol compound, L-cysteine, was similar to the effect of 2-mercaptoethanol. However, effective concentrations were significantly higher (data not shown).

We also tested the effect of thiol deprivation (2-mercaptoethanol withdrawal) and thiol excess (1000 μM 2-mercaptoethanol) on the survival of several other cell lines. Both human Burkitt lymphomas Raji and Jiyoye were sensitive to thiol excess but resistant to thiol deprivation. Similarly, human T-cell leukaemia Jurkat and human cervix carcinoma HeLa were sensitive to thiol excess and resistant to thiol deprivation. All cell lines resistant to thiol deprivation, including EL4 cells, were able to proliferate without the presence of 2-mercaptoethanol permanently.

Apoptosis induction by thiol deprivation and thiol excess

Flow cytometric analysis, after propidium iodide staining, detected accumulation of cells with hypodiploid DNA content, typical for apoptosis, in the population of 38C13 cells after 8 h of incubation under thiol deprivation (2-mercaptoethanol withdrawal) as well as under thiol excess (1000 μM 2-mercaptoethanol). The apoptotic peak of hypodiploid cells increased and the number of cycling

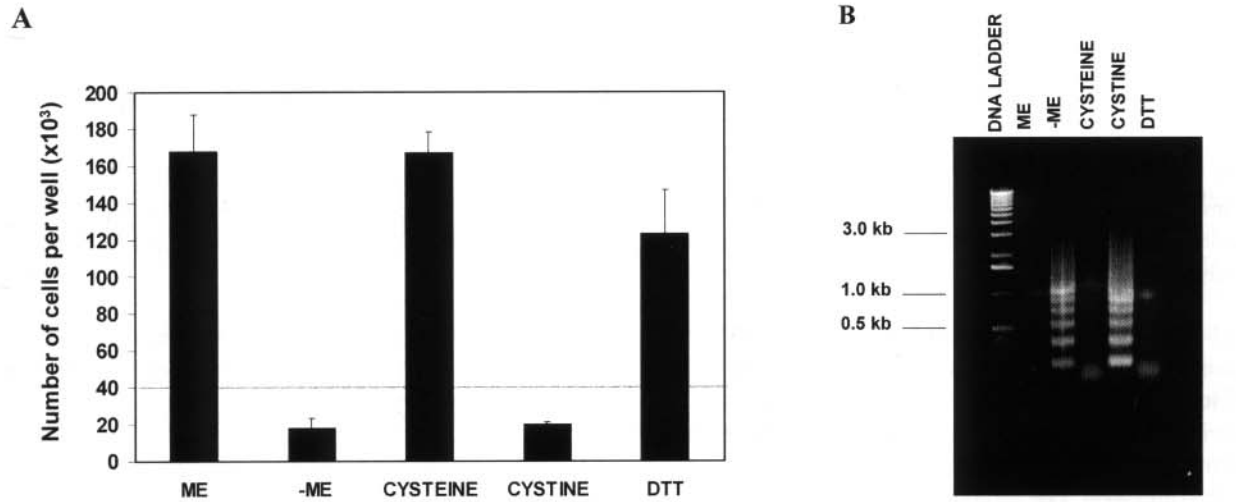

Fig. 4. Effect of 500 μM L-cysteine (CYSTEINE), extra 500 μM L-cystine (CYSTINE), and 500 μM dithiothreitol (DTT) on (A) growth and survival, and on (B) DNA fragmentation of 38C13 cells. Cells incubated with 50 μM 2-mercaptoethanol (ME) represent a positive control and cells incubated without 2-mercaptoethanol (-ME), i.e. in the basic medium, represent a negative control. The basic medium itself contains approximately 200 μM L-cystine. (A) Cells were seeded at 40 x 10^3 cells/100 μl of medium in the well. The number of cells in the inoculum is shown as a dotted line. The number of living cells was determined after 24- h incubation Each column represents the mean of at least four separate cultures ± SEM. (B) DNA fragmentation was determined after 22 h of incubation. Control DNA ladder is shown.

cells decreased with time. After 24 h, the G_1 peak completely disappeared (Fig. 2). In the case of EL4 cells, flow cytometric analysis detected accumulation of hypodiploid cells only under thiol excess (1000 μM 2-mercaptoethanol).

DNA fragmentation analysis by agarose gel electrophoresis showed that 38C13 cells after 16 h of incubation under thiol deprivation (2-mercaptoethanol withdrawal) and also under thiol excess (1000 μM 2-mercaptoethanol) produced a ladder typical for apoptosis. EL4 cells produced the ladder only under thiol excess (Fig. 3). Thus, flow cytometric analysis and DNA fragmentation analysis proved that thiol deprivation induced apoptosis only in 38C13 cells, while thiol excess induced apoptosis in both 38C13 and EL4 cells as well as in other cells (Raji, HeLa).

Correlation of thiol effects with the presence of an SH group

All tested compounds with a free SH group such as 2-mercaptoethanol (50 μM), L-cysteine (500 μM) or dithiothreitol (500 μM) supported survival and growth of 38C13 cells during 24 h of incubation. While L-cysteine supported survival and growth of the cells, L-cystine was without any supportive effect. The incubation with extra 500 μM L-cystine (approximately 200 μM L-cystine is a regular component of the culture medium) resulted in the death of 38C13 cells similar to the control incubation without 2-mercaptoethanol, i.e. without any compound with a free SH group (Fig. 4A). Compounds with a free SH group, i.e. 2-mercaptoethanol (50 μM), L-cysteine (500 μM) or dithiothreitol (500 μM), did not produce any ladder during 22 h of incubation either. On the other

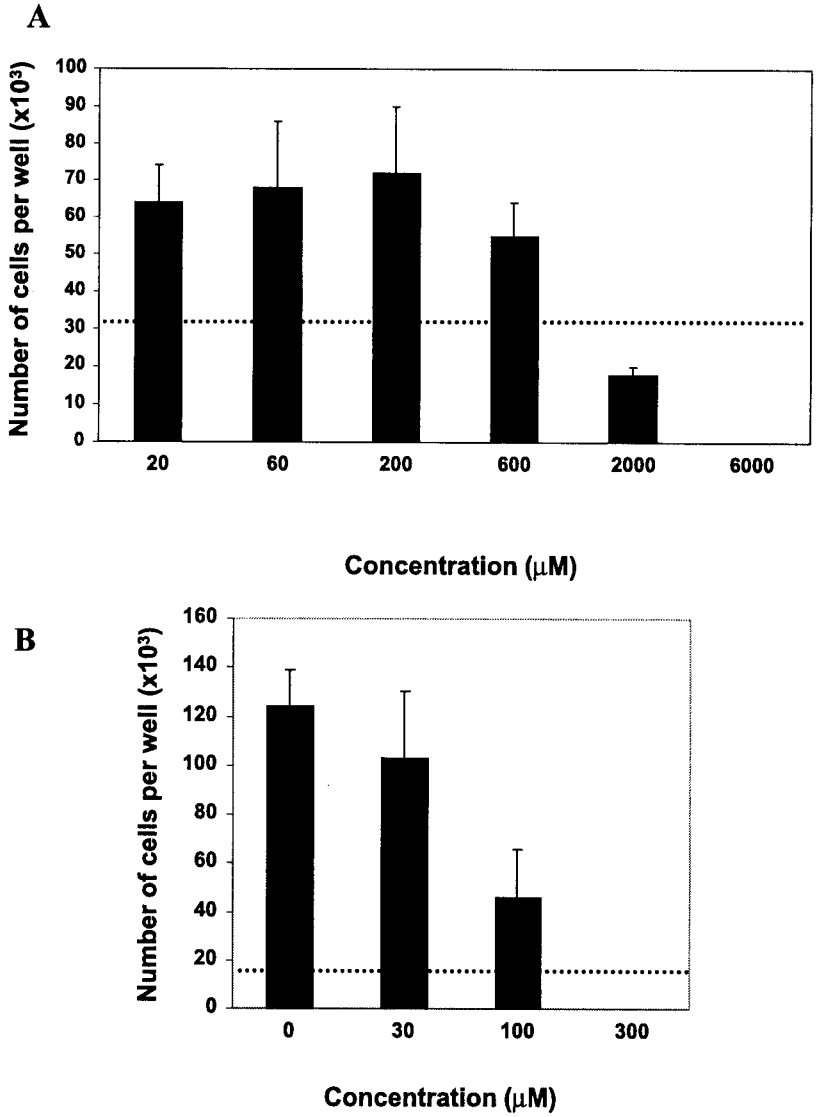

Fig. 5. Effect of (A) ascorbic acid (20–6000 μM) and (B) diamide (30–300 μM) on the growth and survival of 38C13 cells in the presence of 50 μM 2-mercaptoethanol. Control cells were incubated (A) with 20 μM ascorbic acid because the basic medium itself contains 20 μM ascorbic acid, or (B) without diamide. Cells were seeded at 40 x 10^3 cells/100 μl of medium in the well. The number of cells in the inoculum is shown as a dotted line. The number of living cells was determined after 24-h incubation. Each column represents the mean of at least four separate cultures ± SEM.

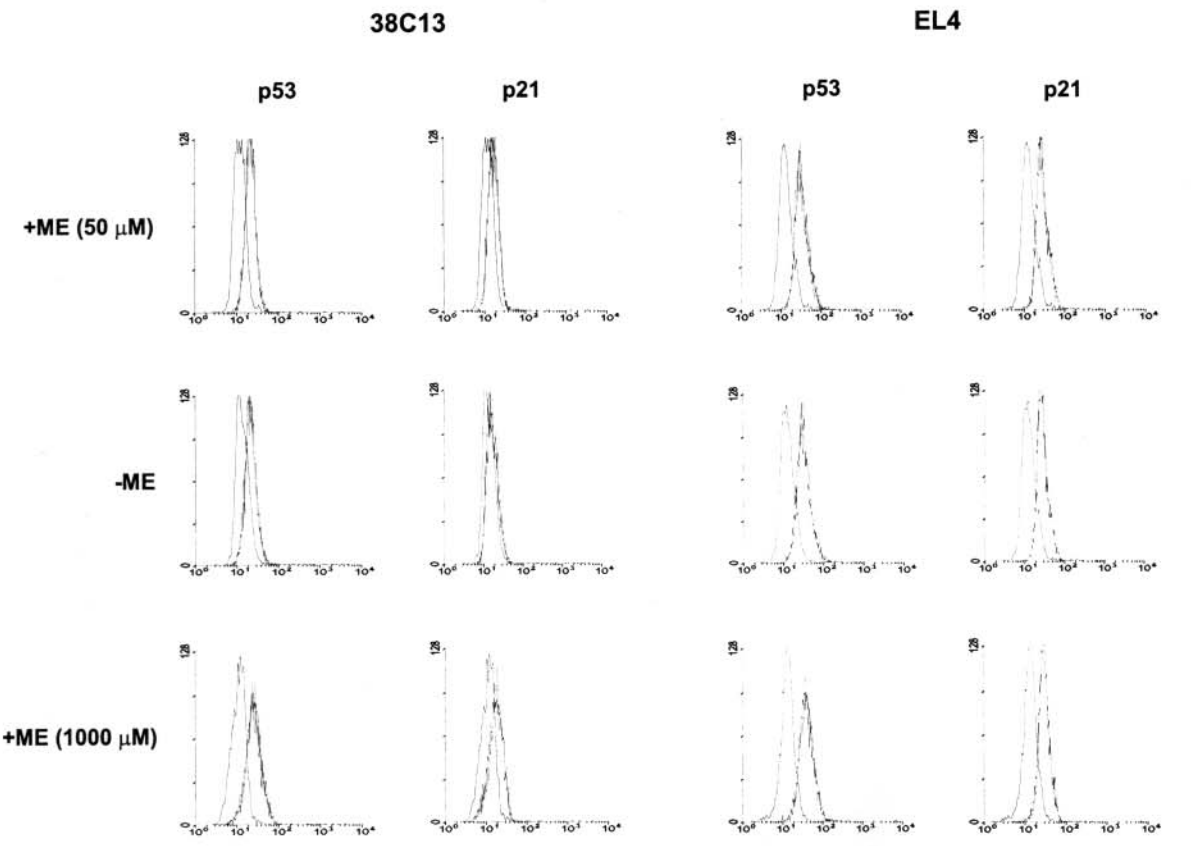

Fig. 6. Effect of availability of thiols, represented by 2-mercaptoethanol (ME), on the expression of p53 and $p21^{CIP1/WAF1}$ by 38C13 and EL4 cells. Control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (without 2-mercaptoethanol), and thiol excess (with 1000 μM 2-mercaptoethanol) were tested. After 12 h of incubation the cells were stained with specific antibody (mouse monoclonal IgG antibody PAb 240 specific for mouse p53, mouse monoclonal IgG antibody HZ 52 specific for mouse p21), as well as with relevant control non-specific immunoglobulin (non-specific mouse IgG), and analysed by flow cytometry. The bold line represents staining with specific antibody and the fine line represents staining with control non-specific immunoglobulin. The data shown were obtained in one representative experiment of three independent experiments.

hand, the incubation with extra L-cystine (500 μM), like in the control incubation without 2-mercaptoethanol, produced a typical ladder (Fig. 4B).

We also tested the effect of non-thiol antioxidant ascorbic acid in the range of concentrations 20–6000 μM. Ascorbic acid alone was unable to support survival of 38C13 cells at any concentration used during 24 h of incubation (data not shown). When ascorbic acid was applied together with 50 μM 2-mercaptoethanol, concentrations higher than 600 μM resulted in cell death. The cells grew in the presence of 600 μM and lower concentrations of ascorbic acid (Fig. 5A). Thiol cross-linking compound diamide at concentrations higher than 100 μM completely abrogated the survival-supporting effect of 50 μM 2-mercaptoethanol (Fig. 5B).

The findings showed that the presence of compounds with a free SH group was required for the survival-supporting and growth-supporting effects. However, the death-inducing effect of high thiol concentrations did not seem to be specifically related to the presence of a free SH group.

p53 and $p21^{CIP1/WAF1}$ expression under thiol deprivation and thiol excess

In order to acquire a better understanding of mechanisms involved in apoptosis induction by thiol deprivation and thiol excess in the studied cells, we assessed the expression of p53 and p21 under thiol deprivation and thiol excess. Repeated experiments employing indirect immunofluorescence showed that thiol deprivation (2-mercaptoethanol withdrawal) and also thiol excess (1000 μM 2-mercaptoethanol) did not significantly change the p53 level in 38C13 as well as in EL4 cells during 12-h incubation. Concerning the p21 level, there also was no change for both 38C13 and EL4 cells under thiol deprivation as well as under thiol excess. Data for a 12-h incubation period are shown in Fig. 6. Similar data were obtained with Raji and HeLa cells. In the case of Raji cells, even a certain decrease in the p21 level was seen for thiol excess after 24-h incubation (data not shown).

No change in the p53 level could be seen for apoptosis induction by thiol deprivation and thiol excess in 38C13 and EL4 cells. Apoptosis induction in both 38C13 and EL4 cells was not associated with any change in the p21

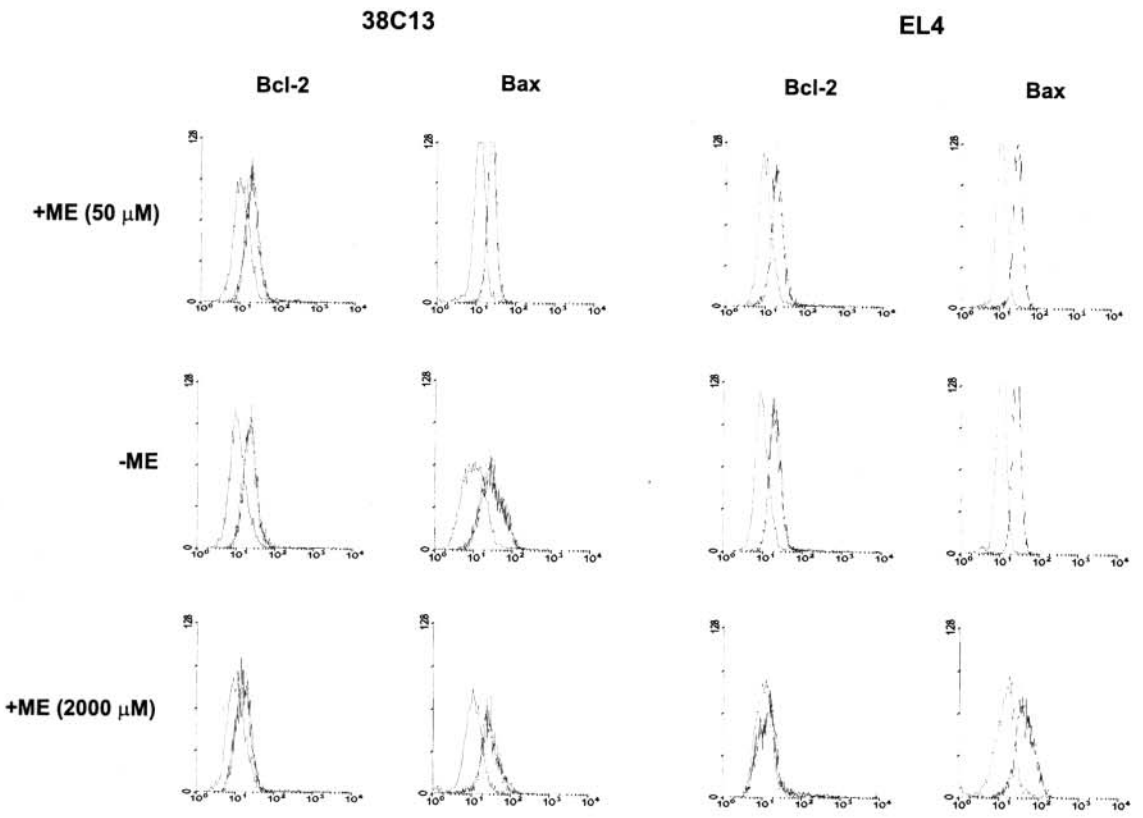

Fig. 7. Effect of availability of thiols, represented by 2-mercaptoethanol (ME), on the expression of Bcl-2 and Bax by 38C13 and EL4 cells. Control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (without 2-mercaptoethanol), and thiol excess (with 2000 μM 2-mercaptoethanol) were tested. After 12 h of incubation the cells were stained with specific antibody (hamster monoclonal IgG antibody 3F11 specific for mouse Bcl-2, rabbit polyclonal IgG antibody Sc-562 specific for mouse Bax), as well as with relevant control non-specific immunoglobulin (non-specific hamster IgG, non-specific rabbit IgG), and analysed by flow cytometry. The bold line represents staining with specific antibody and the fine line represents staining with control non-specific immunoglobulin. The data shown were obtained in one representative experiment of three independent experiments.

level, either. Thus, it seems that apoptosis induction by thiol excess and also apoptosis induction by thiol deprivation are not directly related to a specific significant change in the p53 level and particularly in p53 activation (detected by an increase in the p21 level).

Bcl-2 and Bax expression under thiol deprivation and thiol excess

We also assessed the expression of antiapoptotic Bcl-2 and proapototic Bax. Repeated experiments employing indirect immunofluorescence showed that apoptosis induction by thiol excess in 38C13 cells and also in EL4 cells correlated with a decrease in the Bcl-2 level during 16 h of incubation under thiol excess. On the other hand, thiol deprivation in both 38C13 and EL4 cells did not significantly change the Bcl-2 level. Data for a 12-h incubation period are shown in Fig. 7 and Table 1. Apoptosis induction by thiol excess and by thiol deprivation in 38C13 cells was not associated with a significant change in the Bax level during 16 h of incubation. Simi-

Table 1. Effect of availability of thiols, represented by 2-mercaptoethanol (ME), on the expression of Bcl-2 by 38C13 and EL4 cells[a]

Cells	Bcl-2 expression[b]		
	+ ME (50 μM)	- ME	+ ME (2000 μM)
38C13	22.2 (11.8)	23.7 (11.5)	17.0 (11.1)
EL4	21.1 (10.5)	21.8 (10.1)	15.0 (12.2)

[a]Control conditions (basic medium with 50 mM 2-mercaptoethanol), thiol deprivation (without 2-mercaptoethanol), and thiol excess (with 2000 μM 2-mercaptoethanol) were tested. After 12 h of incubation the cells were stained with specific antibody (hamster monoclonal IgG antibody 3F11 specific for mouse Bcl-2) as well as with relevant control non-specific immunoglobulin (non-specific hamster IgG) and analysed by flow cytometry.

[b]Mean fluorescence intensities were obtained in one representative experiment of three independent experiments. Values in brackets represent control staining with non-specific immunoglobulin.

larly, apoptosis induction by thiol excess in EL4 cells was not associated with a significant change in the level of the Bax protein. Data for a 12-h incubation period are shown in Fig. 7.

Apoptosis induction by thiol excess in both 38C13 and EL4 cells seemed to correspond with the decreased level of antiapoptotic Bcl-2. The level of proapoptotic Bax was not changed. However, apoptosis induction by thiol deprivation in 38C13 cells was not associated with any change in the level of antiapoptotic Bcl-2 or in the level of proapoptotic Bax.

Discussion

We found that thiol deprivation induced apoptosis in mouse B-cell lymphoma 38C13 but not in mouse T-cell lymphoma EL4 and that thiol excess induced apoptosis in 38C13 as well as in EL4 and also in other lymphoid cells. Thus, we demonstrate that not only thiol deprivation can induce apoptosis in sensitive lymphoma cells, as it was supposed for several cell types previously (Sato et al., 1995; Castro-Obregon and Covarrubias, 1996; Neumann et al., 1998; Aoshiba et al., 1999), but also that thiol excess induces apoptosis in lymphomas. Actually, apoptosis induction by thiol excess is not a surprising finding (Takagi et al., 1974; Held and Melder, 1987; Held and Biaglow, 1994). However, it has not been demonstrated explicitly until now. Interestingly, 38C13 cells, which are highly sensitive to thiol deprivation, were found previously to also be highly sensitive to iron deprivation (Kovar et al., 1997). On the other hand, EL4 cells, which are resistant to thiol deprivation, are also resistant to iron deprivation. However, the sensitivity to thiol deprivation does not correlate with the sensitivity to iron deprivation for all cell types tested (our unpublished data).

It has been demonstrated that only compounds with a free SH group are able to prevent apoptosis in sensitive cells and thus only deprivation of such compounds, i.e. thiols, results in apoptosis. There are several lines of supportive evidence: (i) we and others (Sato et al., 1995; Marchetti t al., 1997) show that the thiol cross-linking agent diamide, which mimics disulphide bridge formation, abrogates the thiol prevention of apoptosis. (ii) We show that while L-cysteine, in a manner similar to other thiols, prevents apoptosis in sensitive cells, L-cystine, with a disulphide bridge, is without any effect. (iii) We also show that ascorbic acid, a non-thiol antioxidant, is unable to substitute for thiols in preventing apoptosis. Similarly, other non-thiol antioxidants have been shown to be unable to substitute for thiols (Delneste et al., 1996; Deas et al., 1997). However, there are some contradictory data showing that the antioxidants ascorbic acid, catalase and vitamin E can substitute for thiols and prevent apoptosis (Aoshiba et al., 1999; Ikeda et al., 1999). 2-mercaptoethanol seems to be the most potent thiol tested. Differences in efficient concentrations probably reflect differing chemical reactivity of the thiols involved.

As discussed above, the mechanism of apoptosis induction by thiol deprivation is very likely related to a specific function of thiols, i.e. compounds containing a free SH group, in preventing apoptosis. This thiol function probably concerns the redox status of the cell (Sato et al., 1995; Castro-Obregon and Covarrubias, 1996; Marchetti et al., 1997; Falk et al., 1998; Aoshiba et al., 1999; Hall, 1999; Cai et al., 2000; Yang et al., 2000). It was speculated that the redox state of mitochondrial thiols could regulate opening of mitochondrial permeability transition (PT) pores and thus switch on the apoptotic programme of the cell (Marchetti et al., 1997). Recently it has been shown that oxidation of a thiol residue of the adenine nucleotide translocator (ANT), one of the proteins of the permeability transition pore complex (PTPC), leads to mitochondrial membrane permeabilization (Constantini et al., 2000). Another considered mechanism of apoptosis induction by thiol deprivation is upregulation of Fas receptor expression resulting from the change in the redox state of the cell due to thiol depletion. However, the relation between the control of Fas receptor expression and redox changes remains unclear. Autocrine apoptosis induction by the Fas ligand can be involved here (Delneste et al., 1996; Deas et al., 1997; Neumann et al., 1998). On the other hand, Furuke and coworkers (Furuke et all., 1999) showed that thiol deprivation suppressed the expression of the Fas ligand in activated NK cells. Several other mechanisms of apoptosis induction by thiol deprivation, such as thiol inhibition of NF-kappa B activation (Lin et al., 1995) or thiol regulation of the uptake of indispensable cystine by its reduction to cysteine (Falk et al., 1998), are also considered.

The mechanism of apoptosis induction by thiol excess, demonstrated in this study, can be related to the generation of H_2O_2. Thiol cytotoxicity resulting from the generation of H_2O_2 has been described previously (Takagi et al., 1974; Held and Melder, 1987; Held and Biaglow, 1994). However, our data concerning particularly the cell death induced by relevant high concentrations of non-thiol reductant ascorbic acid show that the mechanism is not necessarily related to the function of compounds with a free SH group and point at the possibility that it could be related to the effect of excess of any reductant.

Our data concerning the expression of p53 and $p21^{CIP1/WAF1}$ support the suggestion that thiol availability controls apoptosis independently of the p53 control system. Similarly, our data concerning the expression of Bcl-2 and Bax correspond with the suggestion that apoptosis induction by thiol deprivation

is not associated with the function of proteins of the Bcl-2 family (Falk et al., 1998; Constantini et al., 2000). On the other hand, apoptosis induction in both 38C13 and EL4 cells by thiol excess was found to be associated with a certain decrease in the level of antiapoptotic Bcl-2. However, there is no direct evidence that this change is related to the control of apoptosis induction.

Taken together, we can conclude that both thiol deprivation and thiol excess are able to induce apoptosis in lymphoma cells, but the sensitivity to thiol deprivation does not correlate with the sensitivity to thiol excess. We also conclude that p53 does not play a decisive role in apoptosis induction by thiol deprivation or by thiol excess. On the other hand, the control system of proteins of the Bcl-2 family could be somehow involved in the control of apoptosis induction by thiol excess but not in the control of apoptosis induction by thiol deprivation. The mechanism of apoptosis induction by thiol deprivation is related to the function of a free SH group, probably to its function in maintaining the required intracellular redox state of thiols. The mechanism of apoptosis induction by thiol excess is likely related to the effect of an excess of reductants. However, further studies are required to elucidate both mechanisms of apoptosis induction completely.

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