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**A COMPARISON OF THE METHODS APPLIED TO DETECT
APOPTOSIS IN GENOTOXICALLY-DAMAGED LYMPHOCYTES
CULTURED IN THE PRESENCE OF FOUR ANTIMUTAGENS**

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Abstract: The sensitivity of the available methods of apoptosis detection in lymphocyte cultures was tested. Cells were preincubated with genotoxic agents: hydrogen peroxide (0.2 mM; 20min.) and benzo[a]pyrene (40 µM;90min.), and then cultured for 36h in the presence of a lectin (PHA-M;1%v/v) and one of the following potentially antimutagenic agents: alkylresorcinols, anthocyanins, todralazine and fluphenazine. It was established that staining with a mixture of fluorochromes (ethidium bromide and acridine orange) provided the highest amount of detected apoptotic cells, and the best repeatability of the results in subsequent experiments. Calculation of the Spearman's rank correlation coefficients proved that there was a high correlation between the results obtained by the ethidium bromide/acridine orange method and those obtained by identifying genomic DNA fragmentation by means of FIGE-electrophoresis. Therefore, these two methods were chosen for further studies of the tested antimutagens' impact on apoptosis in genotoxically-damaged lymphocytes.

Key Words: Apoptosis, Genotoxically-Damaged Lymphocytes, Antimutagenic Compounds

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INTRODUCTION

Apoptosis appears to be a precisely regulated process of cell suicide, by which dysfunctional and unwanted cells are removed from tissues in a silent, inconspicuous manner. This mechanism is crucial for normal physiological growth and development [e.g.1, 2]. It can be induced by a wide range of biological stimuli [2], as well as by chemical, physical and genetic factors [3]. The system of apoptosis is linked through several protooncogenes (c-myc) and tumor suppressor genes (p53) to other systems essential for cellular homeostasis, such as proliferation and the repair of DNA damage [4, 5]. Cells bearing DNA damage activate genes, the products of which function in DNA repair and/or cell cycle checkpoint activation. The best known process is the activation of the p53 gene in response to DNA damage, which triggers a transcription of genes involved in the cell cycle arrest (providing the cell with additional time for DNA repair), and/or apoptosis [6, 7]. If the level of DNA damage exceeds the efficiency of cellular repair systems, the last possibility of preventing fixation of the damage, i.e. mutation, is to induce suicide, an apoptotic program, which will eliminate the damaged cell(s) from the tissue [6, 7]. Thus apoptosis could be perceived as a repair process in genotoxically damaged tissues, able to reduce the level of mutations by selective elimination of heavily damaged cells [8].

The search for factors/compounds able to induce or enhance apoptosis in genotoxically-damaged cells constitutes an attractive branch of studies on antimutagenic mechanisms [e.g. 9, 10]. Antimutagenic compounds lower the level of mutations or prevent them from arising by diverse mechanisms of action, such as decreasing genotoxic agent uptake and transport, activating cellular systems which intercept and detoxicate mutagens, stimulating DNA damage repair, and/or eradicating heavily damaged cells in the apoptotic mode [10, 11]. Accordingly, each antimutagenic agent should be tested for its influence both on the DNA repair-system, and on apoptosis in genotoxically-damaged tissues or cell cultures.

In numerous papers, we have documented the strong antimutagenic effect of four compounds. Two of them are isolated from plants (anthocyanins from *Aronia melanocarpa* fruits and alkylresorcinols from cereal grains), and the other two are synthetic, currently-used drugs (an antihypertensive drug – todralazine, and a psychotropic drug – fluphenazine). Most of the antimutagenic effects were obtained in standard *in vitro* cytogenetic tests in human lymphocyte cultures [12-17]. The intriguing question was the influence of the tested antimutagens on apoptosis in genotoxically-damaged lymphocytes.

Extensive research has proved that the features of apoptotic cells may vary significantly, depending on the cell type, the nature of the apoptotic inducers, and the stage of apoptosis to be tested [e.g. 18-20]. In some test-systems, apoptosis is atypical, lacking one or more of its characteristic features.

Therefore, verification of the utility of the available methods in each experimental system is strongly recommended [18]. The mode of cell death should be confirmed by microscopic examination, for which a fluorochrome staining of cell smears is advisable [19]. The aim of the present study is to compare the sensitivity of the methods applied for the detection of apoptosis in genotoxically-damaged lymphocytes cultured *in vitro* in the presence of the tested antimutagens.

MATERIALS AND METHODS

Chemicals

The blood cell separation solution – Histopaque-1077 – and the components of cell culture media – RPMI 1640, foetal calf serum (FCS) and L-glutamine – were purchased from Sigma (St. Louis, USA). Phytohemagglutinin (PHA-M) was obtained from Gibco (Gaithersburg, USA). Annexin V conjugated with biotin, and ExtrAvidin labelled with peroxidase were purchased from Sigma (St. Louis, USA). The other components of the peroxidase-anti-peroxidase (PAP) detection set, and a chromogen – diaminobenzidine (DAB) – were obtained from DAKO A\S (Glostrup, Denmark). The stains: acridine orange, ethidium bromide, propidium iodide, Hoechst 33258, azur II, eosine B and trypan blue were purchased from Sigma (St. Louis, USA). Benzo[a]pyrene, etoposide, dimethyl sulfoxide, 5-bromo-2'-deoxyuridine and demecolcine were also from Sigma. Hydrogen peroxide, 30% water solution, analytical grade, was obtained from POCH (Gliwice, Poland). The other reagents used for buffers and culture media preparation were from POCH (Gliwice, Poland).

The tested antimutagens

Alkylresorcinols (5-n-alk(en)ylresorcinols) were isolated and purified from a rye bran milling fraction at the Department of Lipids and Liposomes, University of Wrocław, following the procedure described previously [21]. The molecular weight of alkylresorcinols (AR) from such a preparation was established as being close to 376.33. The lyophilized powder was stored in a freezer. Anthocyanins (AN) were isolated from the fruits of *Aronia melanocarpa* at the Department of Fruit and Vegetable Technology, Wrocław Agricultural University, following the procedure included in Polish Patent No. PL 188707 and described in previous papers [22]. The molecular weight of the AN was taken as closely approximating 449.40. The lyophilized AN powder was stored in a freezer. Todralazine-HCl powder (TDR), research grade, molecular weight 268.7, was supplied by Polfa (Pabianice, Poland) and fluphenazine-HCl powder (FPh), research grade, molecular weight 510.4, was provided by Jelfa (Jelenia Góra, Poland). Both powders were stored at room temperature. On the day of the experiment, the tested antimutagens were dissolved in bidistilled water (AN, TDR, FPh) or in DMSO (AR), and filtered through a 0.2 µm Milipore filter

(Sartorius, Germany). Serial dilutions were prepared and the antimutagen solutions were added to the cell culture medium at a volume of 25 μ l at the dose needed to obtain the required final concentration.

Blood cell separation

Heparinized blood (20 ml) was obtained by venipuncture from four healthy male volunteers, between the ages of 40 and 50, each smoking 20-30 cigarettes per day. Lymphocytes were separated by the single-step continuous density-gradient centrifugation technique with Histopaque-1077 [23]. The separated lymphocytes were washed three times in the culture medium RPMI 1640 and counted under a microscope.

Lymphocyte pretreatment with genotoxic agents

The lymphocytes were suspended in the complete culture medium (RPMI 1640, 10% FCS, 2 mM L-glutamine) to a density of 3×10^6 cells/ml, and incubated with 0.2 mM hydrogen peroxide (HP) for 20 min. or with 40 μ M benzo[a]pyrene (B[a]P) for 90 min. at 37°C in a CO₂ incubator. The control cultures were incubated without the genotoxic agents. The culture media did not contain lectin, so the lymphocytes were treated with the genotoxic agents in a quiescent phase of the generation cycle (G₀-phase). Treatment with the genotoxic agents was terminated by dilution of the cell cultures with an excessive volume of a culture medium. Afterwards, the cultures were spun down and resuspended in a fresh medium without genotoxic agents.

Lymphocyte cultures with antimutagens

Cell samples previously incubated with HP or with B[a]P, as well as control samples (incubated without the genotoxic agents), were cultured for 36h in the complete culture medium and stimulated to mitogenesis with a lectin, PHA-M (1% v/v). The antimutagens: anthocyanins (AN), alkylresorcinols (AR), todralazine (TDR) and fluphenazine (FPh) were added, each to a separate culture, and were present in the medium for the whole culture time. After 36 h of culture, the cells were pelleted, suspended with phosphate buffered saline (PBS, pH 7.2), and aliquoted for staining procedures as well as for FIGE – pulsed-field electrophoresis.

For estimation of the dead cell fraction, cultures were stained with a 0.4% solution of trypan blue in PBS. After 10min. of incubation the glass slides were examined under a microscope, and the number of blue-stained cells (dead) within 2000 cells was counted [24]. Another procedure applied to revealing dead cells was the staining of the cell culture with propidium iodide (final concentration: 4.5 μ g/ml, incubation time: 10 min.) [25]. In this procedure dead cells, permeable to propidium iodide (red fluorescence), were counted with a Nikon G 2A filter block among 2000 cells randomly found under a microscope.

Staining procedures for detection of apoptotic cells

The staining of the cells with a mixture of fluorescent dyes – ethidium bromide/acridine orange (EtBr/AO) – followed the procedure described in the literature [26, 27]. The stains were dissolved with PBS, mixed and added to the cell suspension to make a final concentration of 40 nM each. After 10 min. of incubation, glass smear slides were made and examined under a fluorescence microscope equipped with a Nikon B 2A filter block. On the basis of the staining procedure four cell types can be identified [27]: 1) viable cells with green-stained, round-shaped nuclei, 2) early apoptotic cells – green-stained, irregularly-shaped nuclei with chromatin condensed on the edge, 3) dead cells with yellow to orange round-shaped nuclei, and 4) late apoptotic cells – yellow to orange cells with irregularly-shaped nuclei, holding condensed or fragmented chromatin. All four types of cell were distinguished within 300 cells randomly found under the microscope.

Another method used to detect apoptosis was the staining of cells with a mixture of fluorochromes: Hoechst 33258/propidium iodide (H258/PI). The general procedure followed this described in the literature, with minor modifications [28]. Cells were suspended in PBS and stained with H258 [10 μ M] for 10 min. at 37°C. Then PI was added [final concentration: 4.5 μ g/ml], and the cell suspension was incubated at 37°C for an additional 5 min. Glass smears were done, and the slides were examined under a fluorescence microscope to reveal cells permeated with H258 – blue fluorescence (Nikon UV 1A filter block) – and then the same microscopic image was checked for cells permeated with PI – red fluorescence (Nikon G 2A filter block). According to the data found in the literature [28], the staining procedure with H258/PI mixture can differentiate: 1. viable cells (unstained), 2. early apoptotic cells (blue fluorescence), 3. late apoptotic cells (blue fluorescence, and various intensities of red fluorescence), and 4. dead cells (prominent blue as well as red fluorescence). On each slide, a count was taken of the number of cells of each type within 300 cells.

Apoptotic cell detection was also carried out with annexin V, which binds to phosphatidylserine moieties exposed on the outer membrane leaflet from early stages of apoptosis [2, 29, 30], but also appears on the surface of dead cells [e.g. 2, 30]. Thus, it is necessary to estimate the dead cell number with propidium iodide (PI) and/or trypan blue-exclusion tests in cultures intended to be stained with the annexinV-PAP method. The number of apoptotic cells established by means of this method is a result of the subtraction of the number of propidium iodide-stained cells from the number of annexin V-positive cells. The main steps of the annexin V-staining procedure were fixation of cell smear slides in methanol:acetone:formaldehyde (19:19:2, v/v) for 90 sec., followed by 10 min. incubation with the annexin V-biotin solution in a 100 mM HEPES/NaOH buffer, pH 7.5, containing 140 mM NaCl and 2.5 mM CaCl₂. Afterwards, the slides were washed with PBS, immersed with Sigma peroxidase-labeled

ExtrAvidin in PBS, for 10min. under a hood, and subsequently stained with DAB in the presence of 0.025% H₂O₂ for 10 min. The number of positively-stained cells (yellowish-brown) was counted among 2000 cells randomly found under the microscope.

To check the repeatability of the cytochemical methods applied for detection of apoptosis, the apoptosis induction experiment and the staining procedures were repeated five times (at two week intervals) with lymphocytes separated from the same volunteer.

Field inversion gel electrophoresis (FIGE)

After preincubation with mutagens followed by a 36 hour-culture in the presence of a lectin, PHA-M (1% v/v) and the tested antimutagens, the cultures were spun down, the pellets suspended in PBS, and lymphocytes counted under a microscope. Aliquots of each culture containing 2×10^6 cells were suspended in PBS (100 μ l) and mixed with equal volumes of 2% low-melting point agarose at 37°C. The mixtures were then poured into a multiwell blockformer and left on ice for 30min. to solidify. Afterwards, the microslabs were transferred into eppendorf centrifuge tubes and incubated overnight at 56°C with 0.05% proteinase K in 50 mM Tris HCl, pH 7.0, containing 50 mM EDTA. The microslabs were washed twice with 50 mM Tris HCl, pH 7.0, 50 mM EDTA and polymerized into 1% agarose gel. The electrophoresis was run by means of a field inversion gel electrophoresis system (FIGE). The DNA size markers: MidRange I PFG Marker and Lambda Ladder PFG Marker (NEB Nucleic Acids, USA) were applied. The FIGE electrophoresis conditions were in general the same as previously described [31, 32] with the following modifications: the pulse field electrophoresis apparatus was from Renner GmbH, Germany, and the electrophoresis was run at 7.5 V/cm, $P_{t_1}=6s$, $P_{t_2}=2s$, $rt=0.02$, for 36 hours. Afterwards, the gels were stained with EtBr, inspected and photographed under a UV-lamp, and used for densitometric analysis. On the basis of the procedure described above, three electrophoretic domains can be detected in the electrophoretic slabs. They were identified with markers as the domain of megabase, highly-polymerized DNA, and the domains corresponding to 300–500 kb lengths and 30–50 kb lengths.

Densitometric analysis of FIGE slab gels

A quantitative analysis of the DNA bands separated by the FIGE method was carried out by means of computer densitometry. The analyzing set consisted of a CCD Panasonic WV 204 camera, a graphic card VFG-512 Frame Grabber, an analogue monitor and an IBM computer with a Pentium II processor, 500 MHz. The software used was an IM-AN programme with a “unidimensional densitometry” subprogramme, written by Wojciech Warcho³, Department of Biophysics, Medical Academy of Poznań. The measured parameter was an integral of optic density in selected electrophoretic bands. The optic density of

each band was finally expressed as a decimal fraction of the sum of the optic densities of all the measured bands. The integrals of optic density of the FIGE DNA domains, i.e. megabase DNA, the 300–500 kb domain and the 30–50 kb domain, were compared in each electrophoretic strip.

Statistical methods

The Kruskal-Wallis test and the paired t-test were performed and the Spearman's rank correlation coefficients calculated according to the routine statistical procedures [e.g.33].

RESULTS

By means of the staining procedure with the EtBr/AO-mixture, it was established that preincubation of lymphocytes with genotoxic agents, both HP (0.2 mM, 20 min.) and B[a]P (40 μ M, 90 min.), did not change the amounts of dead cells in the cultures, neither immediately after nor four hours after finishing the preincubation, as tested with the trypan blue- and propidium iodide-exclusion method and, separately, with the EtBr/AO-fluorochrome staining. Further, it was found that lymphocyte preincubation with the mutagens followed by a 36-hour culture of the cells with a lectin (PHA-M, 1% v/v) gave well-pronounced features of apoptosis in a number of lymphocytes both in the presence and in the absence of the tested antimutagens. A distinct diversification of cell subpopulations was also noticed – a significant number of cells could be classified as early apoptotic, late apoptotic, dead or viable. A shorter culture time (24h) provided only a small amount of early apoptotic cells and dead cells, whereas a longer culture period (48h) yielded a marked increase in dead cells and late apoptotic cell numbers. Therefore we chose the 36h-incubation time for further studies of apoptosis in this experimental system. The percentual representation of viable, early and late apoptotic and dead cells in the control lymphocyte cultures as estimated with the EtBr/AO-staining method are given in Tab.1.

As may be seen in Tab.1, the control cultures after 36h of culture in the presence of PHA-M consisted of about 10% dead cells and about 9% apoptotic cells (early and late apoptotic, together). A preincubation of cells with B[a]P followed by a 36h culture with PHA-M caused a mild increase in the early apoptotic and dead cell numbers, whereas preincubation with HP led to a marked increase in the number of dead cells, as well as early and late apoptotic cells. At the last culture, the dead cell fraction constituted about 30%, and the apoptotic cell fraction about 27% of the cultured lymphocytes.

Since it was not possible to assume the normal distribution of the compared features, the Kruskal-Wallis nonparametric test was chosen for evaluation of the statistical significance of the data presented in Tab 1. The test proved that the content of the estimated cell fractions was significantly different in three types

of culture, i.e. in those containing lymphocytes cultured with PHA-M and in those cultured with PHA-M after preincubation with genotoxic agents ($H = 125,37$; $df = 2$; $p < 10^{-4}$).

Tab.1. Fractions of viable cells, early and late apoptotic cells and dead cells evaluated in cultures preincubated without genotoxic agents then cultured for 36 h in the presence of PHA-M (1% v/v) (a), and lymphocyte cultures incubated with PHA-M after preincubation with genotoxic agents: B[a]P (40 μ M, 90 min at 37°C) (b), and HP (0.2 mM, 20 min at 37°C) (c).

cultures:	Percentual representation of cells, mean \pm SD, n = 4			
	viable	early apoptotic	late apoptotic	dead
a.	80.75 \pm 14.067	6.78 \pm 1.103	2.34 \pm 1.093	10.13 \pm 1.850
b.	71.05 \pm 10.657	7.12 \pm 1.207	4.73 \pm 0.906	12.10 \pm 1.953
c.	43.28 \pm 8.413	12.57 \pm 1.956	14.24 \pm 2.264	29.91 \pm 2.885

As a demonstration of the results obtained with the EtBr/AO-staining procedure, the microphotography taken from a microscopic image of the culture is provided, in which lymphocytes were exposed to HP (0.2 mM, 20 min., in G₀-phase), washed, and subsequently cultured for 36 hours in the presence of a lectin – PHA. This typical microscopic image is in Fig. 1.

Figure 1A shows normal, viable cells (green), dead cells (yellow to orange) and three viable apoptotic, irregularly-shaped cells (green). In the apoptotic cells the concentration of chromatin on the cell nucleus margin can be seen, as well as the apoptotic bodies (some of each are indicated by arrows). Various pictures of apoptotic cells, selected from other photographs of the same culture are given in Fig. 1B – 1I. Fig. 1B – 1F show viable apoptotic cells (green fluorescence), whereas Fig. 1G – 1I show dead apoptotic cells (yellow to orange fluorescence). Fig. 1B shows a cell with an irregularly-shaped nucleus, in which a condensation of chromatin on the margin is visible; Fig. 1C shows a cell with a fragmented nucleus and an intact cell membrane; Fig. 1D and 1E show the formation of apoptotic bodies; Fig. 1F shows advanced apoptosis with the remnants of a fragmented nucleus and a visible cell membrane blebbing. Fig. 1G shows three apoptotic bodies and, below, a viable lymphocyte with a normal, round cell nucleus. Fig. 1H shows a dead apoptotic lymphocyte which contains chromatin condensed on the edge of the nucleus and newly-forming apoptotic bodies. The cell presented in Fig. 1I contains a multifragmented nucleus in a dead apoptotic cell.

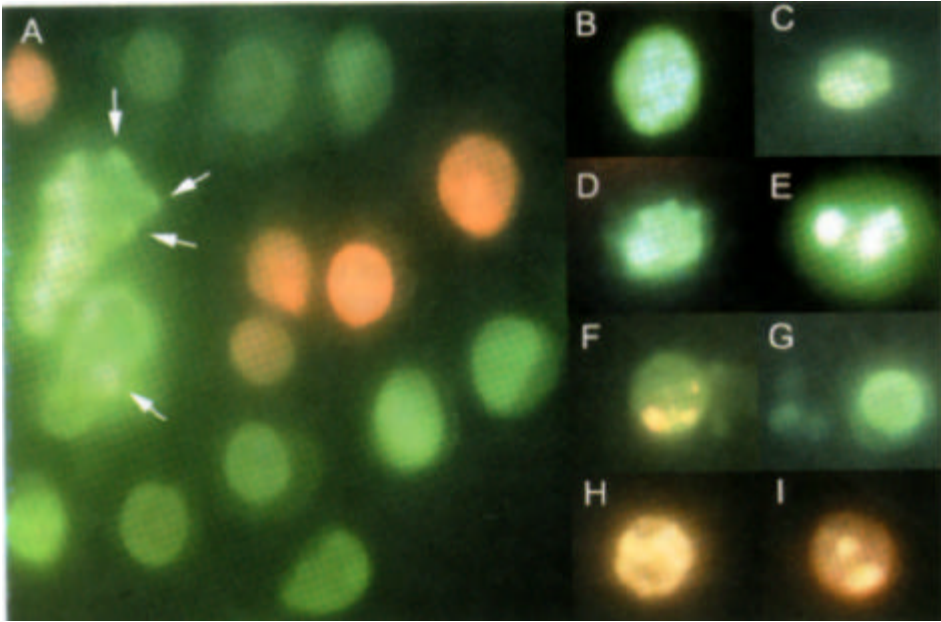


Fig. 1. Apoptosis detection wby the double fluorescence-staining method (EtBr/AO) in cultures of lymphocytes preincubated with HP (0.2 mM, 20 min., at 37°C), and then cultured for 36h in the presence of PHA-M (1% v/v).

Another staining procedure used for the detection of apoptosis, was the staining of cells with the mixture of H258/PI fluorochromes. Four types of cell can be distinguished with this method: viable cells, early apoptotic cells, late apoptotic cells, and dead cells, as described in the Materials and Methods section. The results obtained with the H258/PI staining method were similar to those obtained with the EtBr/AO staining procedure.

The method of the annexinV–PAP staining was also used to identify apoptotic cells, combined with a determination of the dead cell fraction with propidium iodide (PI). However the results obtained with this method varied markedly in subsequent experiments.

In a separate series of lymphocyte cultures, the utility of those staining methods for detection of apoptosis in this experimental system was assessed. Lymphocyte cultures were repeated five times (at two-week intervals) with cells isolated from the venous blood obtained from one smoking volunteer. Each experiment included cultures of lymphocytes preincubated with genotoxic agents (HP and B[a]P) and cultures preincubated without the genotoxic agents and with the solvents only (25 μ l of bidistilled water as a solvent of HP and 25 μ l of DMSO as a solvent of B[a]P). Cell smears were routinely stained by the

EtBr/AO, H258/PI or annexin V binding methods. The fraction of apoptotic cells was established under a microscope. The number of cells positively stained with annexin V binding method and the overall number of apoptotic cells (sum of early and late apoptotic) stained with EtBr/AO and H258/PI obtained in a five-culture series were compared by means of the Kruskal-Wallis nonparametric test. This statistical analysis was used to obtain the following information: 1. whether the differences in apoptotic cell numbers noted in five experiments are statistically significant in each staining method and 2. whether the differences between apoptotic cell numbers in the control cultures, HP-pretreated and B[a]P-pretreated cultures estimated in five experiments are significant.

As may be seen in Tab.2, the differences between experiments were significant for the annexin V binding method – the results obtained with this method were poorly repetitive in this experimental system.

Tab.2. Differences in the results obtained in five tests and differences between the control culture and HP- and B[a]P-pretreated cultures as evaluated with the Kruskal-Wallis test.

staining method	differences between experiments; df = 4	differences between HP-, B[a]P- pretreated and control cultures; df = 2
EtBr/AO	0.89; NS	11.58; p<0.01
H258/PI	4.73; NS	9.64; p<0.05
annexin V	10.12; p<0.05	6.02; p<0.05

The results obtained with EtBr/AO and H258/PI were highly repetitive – the differences among the results obtained from five experiments were not significant. The compared staining methods differentiated between HP-, B[a]P-pretreated and control cultures, and the differences in the results were statistically significant, especially in the EtBr/AO staining .

Field inversion gel electrophoreses (FIGE) were carried out with lymphocytes cultured for 36 h in the presence of the tested antimutagens as well as with lymphocytes preincubated in the G₀-phase with mutagens and subsequently cultured for 36h with the antimutagens. All the cultures were carried out in the presence of a lectin, PHA-M (1% v/v).

The FIGE gave separation of genomic DNA into three domains: highly-polymerized, megabase DNA and two other domains which were identified with markers as corresponding to 300–500 kb length and 30–50 kb length. By

means of this system the impact of AN, AR, TDR and FPh on apoptotic events in the control lymphocyte cultures was compared with their influence on apoptosis in genotoxically-damaged lymphocytes. This paper provides an example of the results obtained in the testing system with FIGE electrophoresis. It shows the AR impact on apoptosis in control lymphocyte cultures and on lymphocytes damaged with HP (0.2 mM, 20 min.). The microphotography of the slab gel is shown in Fig. 2.

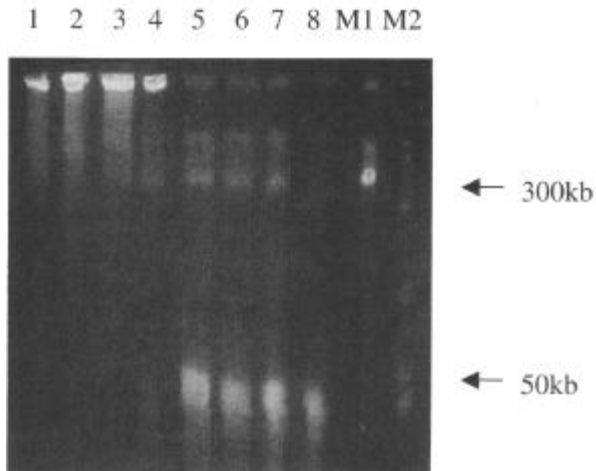


Fig. 2. Field inversion gel electrophoresis (FIGE)-separation of DNA from PHA-M stimulated lymphocytes: not preincubated with the genotoxic agent (lines 1 – 4), and preincubated with the genotoxic agent – HP 0.2 mM, 20 min., (lines 5 – 8). The influence of alkylresorcinols – AR (10 μ M, 20 μ M and 100 μ M, respectively) on the DNA separation profiles in genotoxically-damaged cells was compared to that for undamaged lymphocytes (lines 6 – 8 versus lines 2 – 4).

Lane 1 (from left) shows the separation of DNA from the control lymphocytes cultured in the presence of the solvent only (DMSO, 25 μ l), and lanes 2–4 show DNA separated from cultures performed in the presence of AR at concentrations of 10, 50 and 100 μ M, respectively. As can be seen, the 30–50 kb DNA domain was absent in lanes 1–4, and in line 4 the 300 kb length DNA domain appeared. Lane 5 shows the electrophoretic domains of DNA from lymphocytes preincubated with HP (0.2 mM, 20min.) and subsequently cultured for 36 h in the presence of the solvent only (DMSO, 25 μ l). Lanes 6–8 have the electrophoretic separation results for cells taken from cultures preincubated with HP and then cultured for 36 h in the presence of AR at concentrations of 10, 50 and 100 μ M, respectively. It should be noted that the presence of AR in the culture medium of the lymphocytes preincubated with HP caused a dose-

dependent decrease of DNA content in the 300–500 kb domain as well as in the domain of highly-polymerized, megabase DNA, whereas the content of the 30–50 kb domain slightly increased.

The densitometric data presented below were selected from the experiments and concern lymphocytes preincubated with the genotoxic agents and then cultured with the highest concentration of each antimutagen. The reference values were those obtained in the cultures pretreated with the genotoxic agents and subsequently cultured with the solvent (25 μ l of water or DMSO) instead of antimutagens. The results obtained in four independent experiments are presented in Tab. 3 as the mean \pm SD, and the effect of each antimutagen is compared to the reference lymphocyte culture (without antimutagen) by means of the paired t-test.

Tab. 3. A densitometric analysis of the DNA domains separated by the FIGE method. The share of each DNA domain was presented as a decimal fraction of the sum of the DNA contents in the three domains in this electrophoretic strip (mean \pm SD, n = 4). The results obtained with the tested antimutagens were compared to the reference values estimated in cultures preincubated with the mutagen and then cultured for 36h with the solvent instead of the antimutagen (paired t-test, * p < 0.05, ** p < 0.01).

preincubation in G ₀ -phase	culture with antimutagens	D N A d o m a i n s (F I G E) :		
		30–50 kb	300–500 kb	megabase
HP (0.2 mM, 20 min.)	AR (100 μ M)	0.60 \pm 0.045*	0.30 \pm 0.031**	0.10 \pm 0.014*
	AN (200 μ M)	0.37 \pm 0.052*	0.36 \pm 0.043	0.27 \pm 0.035**
	TDR (300 μ M)	0.34 \pm 0.048*	0.44 \pm 0.061	0.22 \pm 0.031*
	FPh (10 μ M)	0.57 \pm 0.027*	0.31 \pm 0.037	0.12 \pm 0.011
	--- (solvent)	0.46 \pm 0.061	0.39 \pm 0.056	0.15 \pm 0.030
B[a]P (40 μ M, 90 min.)	AR (100 μ M)	0.14 \pm 0.023**	0.59 \pm 0.057**	0.27 \pm 0.030**
	AN (200 μ M)	0.0 ---	0.0 ---	1.0 ---
	TDR (300 μ M)	0.0 ---	0.0 ---	1.0 ---
	FPh (10 μ M)	0.17 \pm 0.016**	0.52 \pm 0.045*	0.31 \pm 0.027**
	--- (solvent)	0.01 \pm 0.008	0.31 \pm 0.048	0.68 \pm 0.140

As can be seen in Tab.3, the impact of the tested antimutagens on the DNA content in the 30–50 kb domain in the case of the lymphocytes preincubated with HP was different for different antimutagens: AR and FPh increased, whereas AN and TDR decreased the DNA content in this domain. This corresponds to the inverse effect on the DNA content in the megabase, highly polymerised DNA domain, which was reduced in the case of AR and FPh and significantly elevated in the case of AN and TDR.

In the case of lymphocytes preincubated with B[a]P and cultured without the antimutagens, the DNA content in the 30–50 kb domain was very small, almost negligible (about 1–2% of the total DNA content for the three domains), and almost 70% of the DNA remained in the domain of the highly polymerised, megabase DNA. The addition of AN and TDR to the culture medium resulted in an increase of the megabase DNA content, and the domains of 30–50 kb and 300–500 kb were virtually absent in these electrophoretic strips. On the other hand, the presence of AR and FPh in the lymphocyte culture led to a marked increase in the DNA content in the 30–50 kb domain. It should be noted from the data in Tab. 3 that only the changes in the 30–50 kb domain were significant in the paired t-test in all the cultures carried out in the presence of antimutagens. Calculating the Spearman's rank correlation coefficients (r_s), the data obtained in the EtBr/AO staining method were compared with those obtained with FIGE electrophoresis. The Spearman's non-parametric correlation test was used since it was not possible to presume a normal distribution of the compared results, and the data compared were expressed in different units. The results of this calculation are given in Tab. 4.

Tab. 4. Spearman's rank correlation coefficients (r_s) calculated for the data obtained by microscopic examination of the EtBr/AO stained cells and the FIGE separation patterns of DNA large- scale domains. Lymphocyte cultures were preincubated with genotoxic agents (HP or B[a]P) and subsequently cultured in the presence of the tested antimutagens. The statistical significance of r_s was estimated for $n=10$ pairs of correlated data, at two-sided significance level (* $p < 0.02$, ** $p < 0.01$).

Cell fractions exposed with EtBr / AO staining	DNA electrophoretic domains		
	megabase	300–500kb	30–50 kb
	correlation coefficients (r_s) :		
viable cells	0.77*	-0.56	0.08
early apoptotic cells	-0.52	0.21	0.16
late apoptotic cells	-0.83**	0.49	0.81**
dead cells	0.01	0.10	0.02

The highest r_s in Tab. 4 was calculated for the late apoptotic cell number and the DNA content in the 30-50 kb domain ($r_s = 0.81$), whereas the greatest negative r_s was established between the late apoptotic cell numbers and the DNA content in the megabase domain ($r_s = -0.83$). In those three comparisons the r_s was estimated to be statistically significant, whereas the other correlation coefficients given in Tab.4 were assessed as not significant.

The results presented in Tab.4 show that the correlation between the DNA content in the megabase and 30–50 kb domains was especially strong in the case of late apoptotic cells.

DISCUSSION

We previously described the activity of four compounds able to prevent mutations in lymphocytes obtained from smoking blood donors, induced *in vitro* by standard mutagens [12-17]. A study of apoptosis in lymphocytes obtained from smoking blood donors is a natural complement to the research cited above. The intention and objective of this project was a comparison of the impact of four antimutagens' on apoptosis in lymphocytes isolated from the blood of male smokers; it could help to develop a mutation-preventive design addressed to heavy smokers.

The antimutagenic effect of the four tested compounds was established in the standard cytogenetic lymphocyte tests (the sister chromatid exchange test, the cytokinesis-blocked micronucleus assay and the thioguanine resistance test) in cultures stimulated to mitogenesis with a lectin – PHA-M [12-17]. Thus, examination of the antimutagen influence on apoptosis in the same experimental system was important for direct comparison of the results.

That is why it was decided to examine apoptosis in lymphocyte cultures activated to mitogenesis with PHA-M (1% v/v). It was documented in the literature [34, 36] that cultures of lymphocytes activated by mitogens were a more sensitive system for the detection of apoptosis than the assays with resting lymphocytes. As was suggested, lymphocytes often receive signals that prime them for apoptosis, but they do not inevitably undergo a cell death programme until the final signal is received [37, 38]. Being cultured in the presence of mitogens, the primed cells can be driven fully into the death cascade [38-40], probably because an important additional signal for apoptosis is provided, for instance, by mitogen-activated protein kinases [39, 40] and cyclin-dependent kinases [41].

On the other hand, the cytokinesis-blocked micronucleus assay documented that PHA-P was able to induce apoptosis in normal lymphocytes, whereas preincubation of the cells with a genotoxic agent (HP) followed by a 72-96h culture in a PHA-containing medium caused a significant inhibition of apoptosis accompanied with a marked increase in the number of dead cells as well as of micronucleated cells [42]. We did not treat those results as contradictory to our own data. Having cultured the lymphocytes for twice as long as we did, the authors probably observed a more advanced stage of apoptotic cell self-destruction, i.e. secondary necrosis. Under our experimental conditions, the control cultures consisted of about 10% dead cells and about 9% apoptotic cells after 36h of culture in the presence of PHA-M. A preincubation of lymphocytes with HP followed by a 36-hour culture with

PHA-M yielded up to 30% dead cells and 27% apoptotic cell fractions. We noticed that a prolonged culture period (48h) yielded a marked increase in dead cell and late apoptotic cell numbers. It was documented in the literature that a lymphocyte culture time longer than 48 hours provided a decrease in the apoptotic cell number proportional to the rise in the secondary necrotic cell number [43]. Under *in vivo* conditions, apoptotic cells were quickly removed by phagocytosis, which is obviously not possible *in vitro*, in cultures of purified lymphocytes [43].

In our experience, of the three cytochemical methods used to detect apoptosis, the microscopic examination of EtBr/AO-stained cell smears can be recommended as the most reliable method, since makes it possible to perform high-quality studies of cell morphology, and nuclear and chromatin disintegration, and to distinguish viable cells, dead cells, and early- and late-apoptotic cells. As shown in Tab.2, this staining method provides results which are highly repetitive in subsequent experiments, and it also differentiates well between control and genotoxically-damaged cell cultures.

In the literature, the opinion prevails that the microscopic examination of cytological changes in apoptosis is a necessary component of each set of methods used to detect and study apoptosis [e.g.18-20]. For instance, several characteristic morphological traits of apoptosis occur in the absence of detectable DNA fragmentation [44-48]. More recent studies show that DNA degradation is a relatively late event in the apoptotic cascade of events [49] and chromatin fragmentation to large-scale 300–500 kb and 30–50 kb fragments usually precedes the degradation of DNA to small internucleosomal sections [45-47]. Sometimes apoptosis-associated DNA degradation stops after generating several 30–50 kb fragments and does not proceed to internucleosomal sections [44, 47]. For the reasons given above, the opinion prevails that the pulsed-field gel electrophoresis technique and its modifications (such as field-inversion gel electrophoresis, FIGE) should be chosen for the electrophoretic detection of apoptosis, especially for identifying the early stages of apoptosis [e.g. 19]. In contrast to these discrete apoptotic DNA bands, the electrophoretic separation of DNA from necrotic cells provides smeared and distorted bands on agarose gels as a result of intensive, random DNA cleavage in those cells [e.g. 50, 51]. Thus, the electrophoretic separation of DNA on agarose gels is recommended as a method of qualitative discrimination between apoptosis and necrosis [51]. Accordingly, our results presented in Tab.3, documenting the differences between the FIGE-separation profiles of genotoxically-damaged lymphocytes cultured for 36h in the presence of PHA and one of the four tested antimutagens, should be perceived as a mirror of the diverse influence of the antimutagens on apoptosis in cultures.

In conclusion: we confirmed the utility of the FIGE method of revealing DNA disintegration patterns, as well as the utility of the microscopic examination of EtBr/AO-stained cell smears for counting the number of apoptotic cells. Each

method provided reliable, repeatable results and distinguished well between control cultures and cultures exposed to the genotoxic agents, as well as between cultures performed in the presence and in the absence of the antimutagens. Also, the results obtained with these two methods were correlated well. Thus, we have used both of these methods in further studies of the four antimutagenic compounds' effects on apoptosis in lymphocyte cultures.

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