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Flow Cytometry Analysis of DNA Degradation in Thymocytes of γ-Irradiated or Hydrocortisone Treated Rats

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Abstract. The pattern of DNA degradation in thymocytes of irradiated or hydrocortisone-treated rats has been studied by means of flow cytometry of the cells, treated with probes specifically bound to the AT or GC-pairs of DNA. It has been shown that the death of thymocytes is accompanied by a decrease in their DNA content. The main features of the occurrence and accumulation of cells with a DNA content less than the normal diploid level correspond with those of internucleosomal DNA fragmentation: such cells appear after a 1 hour lag-period, their accumulation is prevented by cycloheximide injection and is lower at 300 Gy than at doses of 10 to 30 Gy. At the same time, no increase in permeability of the cell membrane to ethidium bromide was observed up to the sixth hour after irradiation. Most of the thymocytes dying under the action of irradiation or hydrocortisone are in the G_0 or G_1 phases of the cell cycle. The method used allows detection of the cells with cleaved but not removed DNA.

Key words: Flow cytometry — Cell death — DNA degradation — Thymocytes — HOECHST-33258 — Mithramycin — Ethidium bromide

Introduction

It is well known that the death of lymphoid cells under the action of γ-irradiation, corticosteroids and alkylating agents is accompanied by degradation of their nuclear DNA (Cole and Ellis 1957; Ermolaeva and Vodolazskaya 1970; Ivannik et al. 1976; Matyášová et al. 1979; Skalka and Matyášová 1963; Umansky et al. 1981; Zhivotovsky et al. 1980). Further, the degradation of genetic material is characteristic of various forms of death of other cell types (Hanson 1979; Umansky 1982). In some cases the DNA degradation in the dying cell can be considered to

Abbreviations: EB — ethidium bromide; MI — mithramycin; HO — Hoechst dye N 33258

be one of the first recorded events. It is probable that this phenomenon is typical of apoptotic cell death. In this case one of the first cytological manifestations in the dying cell is pyknotization of the nucleus followed by the extrusion of some of the nuclear contents (apoptotic bodies) from the cell (Wyllie et al. 1980).

It has been suggested previously that the DNA degradation is simply a step in a chain of events leading to cell death rather than a result of hydrolytic enzymes activation in the dying cell (Hanson 1979; Umansky 1982; Wyllie et al. 1980). A number of experimental data have provided evidence for this suggestion. It is therefore of interest to elucidate the mechanisms involved in the process of degradation of genetic material in dying cells.

In this respect thymocytes are one of the most suitable models, since both γ-irradiation and hydrocortisone treatment of animals induce simultaneous death of a great number of these cells.

At present DNA degradation is assessed from the accumulation of cleavages in the DNA and decrease of its molecular weight (Ivannik et al. 1976). The isolation and analysis of chromatin degradation products are also used to determine DNA degradation (Ermolaeva and Vodolazskaya 1970; Matyášová et al. 1979; Skalka et al. 1976; Umansky et al. 1981; Zhivotovsky et al. 1980).

It has been shown that chromatin degradation is due to internucleosomal enzymatic fragmentation of nuclear DNA (Matyášová et al. 1979; Skalka et al. 1976; Umansky et al. 1981; Zhivotovsky et al. 1980). The breakage of DNA is not accompanied by proteolysis and accumulation of intranucleosomal cleavages (Korol et al. 1979; Skalka et al. 1981; Umansky et al. 1981). DNA degradation is prevented by cycloheximide injection (Ermolaeva and Vodolazskaya 1977; Ivannik et al. 1978; Niconova et al. 1982) and, therefore, is somehow associated with protein synthesis. After irradiation the proportion of DNA degraded to nucleosomes and their oligomeres coincides approximately with that of the cells with pyknotic nuclei (Umansky et al. 1981). It may well be that in dying cells almost all of the DNA is rapidly fragmented, while in the rest of the cells it remains practically intact. Some cytological observations (Wyllie et al. 1980) and indirect biochemical data (Valkovich and Hanson 1976) suggest that part of the fragmented chromatin can be extruded out of the dying cells, exactly occurs upon the terminal differentiation of mammalian lens cells or erythrocytes. Using flow cytometry we have attempted to obtain more complete and reliable information on the processes of DNA degradation in the thymocytes of irradiated animals. The results obtained provide evidence for the adequacy of this approach which gives new insight on the processes occurring in the lymphoid cells of irradiated animals.

Materials and Methods

Male Wistar rats of 150—160 g were irradiated with ^{137}Cs γ-rays at a dose rate of 3.6 Gy/min. Hydrocortisone and cycloheximide were introduced at a dose of 10 mg and 300 μg per 100 g weight respectively. At different time after irradiation and hydrocortisone treatment the rats were decapitated, the thymus was removed and rubbed through a nylon mesh. The thymocytes were suspended in 0.14 mol.l^{-1} NaCl and fixed by 50% ethanol for 1.5 hours at −18°C. 90 μl of fixed cells (1.5×10^7 cells-/ml) were added to 3 ml of HO (1 μg/ml) in 0.1 mol.l^{-1} NaCl + 0.1 mol.l^{-1} Tris-HCl (pH = 7.4) or to 3 ml of a solution containing 12.5 μg/ml EB, 25 μg/ml MI, 3.75 mmol.l^{-1} $MgCl_2$, 0.1 mol.l^{-1} NaCl, 0.1 mol.l^{-1} Tris-HCl (pH 7.4) (Barlogie et al. 1976). The final concentration of the cell suspension was about 0.5×10^6 cells per 1 ml. The cells were stained for 30 min, filtered through a network palladium filter with pore dimensions of 22×22 μm and analysed on the flow cytofluorometer LAKS-1 with chamber 1 (Fig. 1). $(50—70) \times 10^3$ cells were measure at an analysis rate of 300 cells/s.

Estimation of cells with disturbed plasma membrane barrier function was performed as follows: freshly isolated cells were suspended in Hanks' medium, where phenol red was replaced by EB at a concentration of 10 μg/ml. 1—2 min later the suspension was filtered and supplied to a chamber 28 (Fig. 1) of the cytofluorometer. Cells with a high membrane permeability to EB were identified by fluorescence of the dye bound to nucleic acids (EB stains intact cells too slowly for them to be fully stained within the duration of the experiment). The mean number of fluorescent cells passing through the measuring chamber per unit time, was determined from $5 \div 7$ measurements of 10 s each at a constant flow rate. Permeability of the cells was increased by the addition of digitonin to a final concentration of 40 μg/ml into the medium, and then the measurements were repeated. The total duration of the experiment was $5 \div 7$ min. Since the flow rate of the cell suspension passing through the chamber is constant and the number of cells detected is high, the percentage of damaged cells could be calculated precisely by dividing the values obtained before and after digitonin addition.

The fluorescence of HO was excited at 334 and 365 nm, MI at 405 nm (mercury lines), EB at 488 nm (laser line). The fluorescence of HO was registered within the range of $\lambda \geq 450$ nm, EB − $\lambda \geq 600$ nm. It should be noted that in the experiments with EB + MI the excitation of MI led to EB fluorescence as a result of energy transfer (Barlogie et al. 1976).

The results obtained were analysed by using a nonparametric method of histogram preparation. Each histogram was normalized for 10^5 cells and smoothed according to a 9-point procedure. $(G_0 + G_1)$ and $(G_2 + M)$ peaks were approximated to a Gaussian function by a least squares method. The appropriate undisturbed parts of the peaks (left wing of G_1 peak and right wing of G_2 peak) were chosen as approximation intervals. The validity of the approximation was estimated by the χ^2-criterion. If approximation was difficult because of the complicated profile of a histogram, the latter was conventionally divided into regions, where the cells were referred to $(G_0 + G_1)$, S, $(G_2 + M)$ and $<2C$ types. For HO and EB + MI the ratio of the average values of peaks $(G_2 + M)$ and $(G_0 + G_1)$ was about 1.98 with equal coefficients of variation within the range of $1.6 \div 2.0\%$.

A diagram of LAKS-1 (Pechatnikov et al. 1982) is presented in Fig. 1. The working principles of the flow-chambers 1,28 are similar to those described previously (Göhde 1971; Göhde et al. 1980). A cell suspension stirred by a magnetic stirrer is delivered via channel 2. The chamber channel is illuminated using the Köhler principle by elements 3—12 or 3—10, 14—17. Fluorescent light is collected by objective 3 and reaches photomultipliers 26,27 through elements 4 and 21—25. Diaphragm 22 encloses the visible region over which measurements were taken. The signals from the photomultipliers pass through preamplifiers 31—32 to peak detectors 33—34 and to pulse width detectors 35—36. The detector outputs can be connected with multichannel pulse height analysers 38—39 in various combinations. Coincidence units of the amplitude analysers permit measuring of one parameter at a selected "window" of another. It is possible to selected only those pulses with durations corresponding to the time at which the cells pass through the measuring volume. This essentially prevents a false

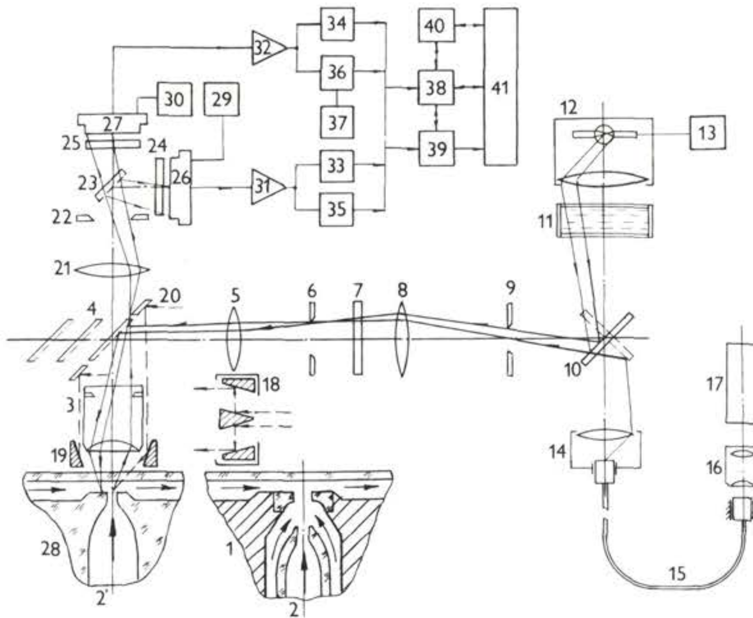

Fig. 1. Block-scheme of flow cytometer LAKS-1
1, 28 — Flow chambers, **2, 2'** — Cell transporting channels, **3** — Microscopic objective 100 × 1.3 or 50 × 0.8,**4, 23** — Dichronic mirrors, **5, 8, 21** — Lenses, **6** — Field stop diaphragm, **7, 24, 25** — Light filters**9**— Aperture diaphragm, **10** — Turning mirror, **11** — Warm-absorption filter, **12** — Illuminator with high-pressure mercury arc lamp DRSh-250-2, 250 W, **13** — Stabilized lamp power supply, **14** — Laser illuminator, **15**— Fiber-glass, **16** — Microscopic objective 10 × 0.4, **17** — Laser ILM-120 (Zeiss), **18, 19, 20** — Dark-field illumination elements, **22** — Measuring diaphragm, **26, 27** — Photomultipliers FEU-79, **29, 30** — High-voltage suppliers, **31, 32** — Preamplifiers with zero level reset, **33, 34** — Peak detectors, **35, 36** — Pulse width detectors, **37** — Cell concentration indicator, **38** — Multichannel analyser NTA-1024 (Hungary), **39** — Multichannel analyser NOKIA LP-4840 (Finland), **40** — Minicomputer EMG-666/B (Hungary), **41** — Peripheral equipment (magnetic tape recorder, X-Y plotter printer, paper tape punch, paper tape reader).

positive response of the circuits to electronic noise and to the signals from cell, which overlap partially in the measurement zone. In all experiments electronic signals were selected for duration.

Results

In the first series of experiments, to choose the most favorable staining technique, two groups of dyes were used: HO which interact specifically with AT-pairs in DNA (Latt and Wohlleb 1975; Müller and Gautier 1975; Weisblum and Haensler 1974), and a mixture of EB + MI, the last of which binds specifically to the GC-pairs of DNA (Kersten et al. 1966; Ward et al. 1965).

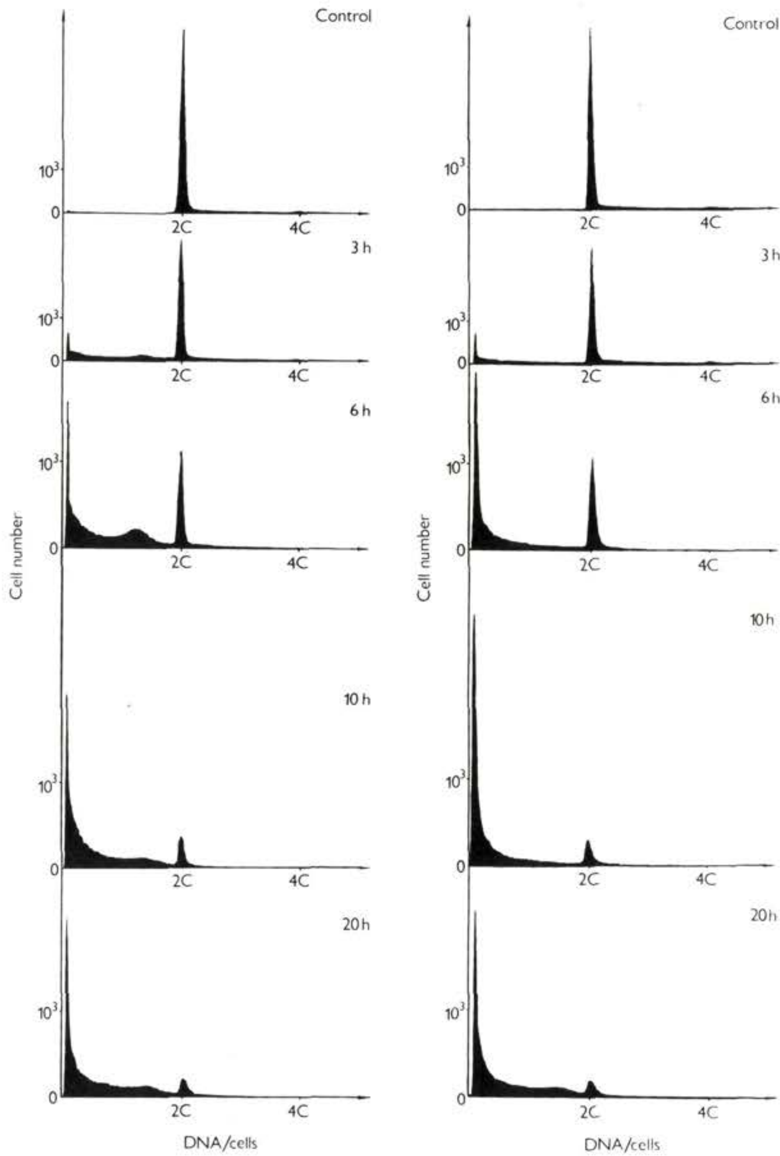

Fig. 2. DNA-histograms of thymocytes from γ-irradiated (10 Gy) rats, stained with HO (left column) or EB + MI (right column). Time after irradiation is indicated on the figures.

Typical histograms of thymocytes stained with HO and EB + MI are presented in Fig. 2. It should be noted that 6C and 8C peaks are not observed in the histogram patterns (no data are presented), which provides evidence for a very slight degree of cell clumping in the suspension studied.

In control experiments both staining techniques showed a fairly equal distribution of cells between the phases of the cycle. 3—20 hours after irradiation a cohort of cells with a DNA content lower than the 2C DNA appeared. When staining with HO, the additional peak especially pronounced 6 h after irradiation, is also apparent in <2C region, whereas in the case of EB + MI it is not observed. One can suppose that in the part of cells chromatin is already cleaved but has still not been eliminated. During the staining with HO, low molecular weight chromatin fragments disappear from the cells. This, probably, does not take place when EB + MI is used because of the presence of Mg^{2+}, which decreases the solubility of chromatin fragments. To verify this assumption, fixed cells were preincubated with or without Mg^{2+} and then stained with HO or EB + MI for 10 min. Both experiments yielded similar results (Fig. 3). In the absence of Mg^{2+} the additional peak is clearly seen and drifts to the region of the lower DNA content. Mg^{2+} inhibits the removal of chromatin fragments. Electrophoretic analysis has shown that chromatin fragments washed from the fixed thymocytes in the absence of Mg^{2+} are nucleosomes and their oligomers (no data are presented). Thus, staining without Mg^{2+} reveals not only the cells with lower DNA content but also the cells which contain DNA which has been fragmented but not eliminated. That is why HO was generally used in the later experiments.

The proportion of cells with $<2C$ DNA increases with time and levels off by 8 h after irradiation (Fig. 4). The staining with EB + MI reveals the less amount of $<2C$ cells only in the period between the second and sixth hours. By 8 hours the results obtained with the two stains coincide. Thus, by this time the DNA cleavage has ceased and the chromatin fragments have been removed.

Luminescent microscopy of the cells stained with HO and EB + MI, performed in parallel, showed that intact cells, but not the nuclear fragments extruded out of the cells, are the sources of weak signals. It should be noted that a marked increase in the number of such cells is observed after a 1 h lag-period, which is consistent with the pattern of DNA degradation in the thymocytes of irradiated animals (Cole and Ellis 1957; Ermolaeva and Vodolazskaya 1970; Ivannik et al. 1976; Skalka et al. 1965; Umansky et al. 1981). The number of $<2C$ cells revealed with EB + MI corresponds with that of the cells with pyknotic nuclei (Umansky et al. 1981). Therefore, one can assume that cells containing fragmented but not extruded DNA do not appear pyknotic and can not be detected by other methods. With the increase of $<2C$ cells the number of $2C$ cells falls. The number of cells in S and $G_2 + M$ phases decreases 3 h after irradiation.

The analysis of cell viability, by the criterion of EB permeability, showed that

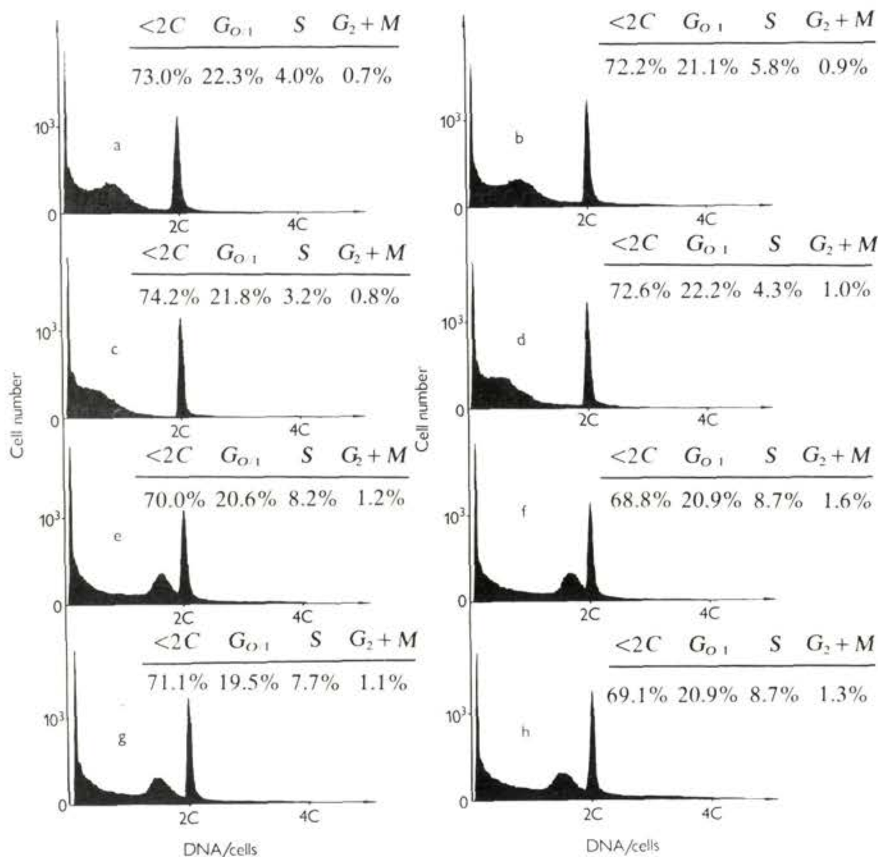

Fig. 3. Dependence of histograms on preincubation of cells with or without Mg^{2+}. Thymocytes from rats irradiated with 10 Gy (6 h after treatment) were fixed, preincubated 20 (*a, b, e, f*) or 50 (*c, d, g, h*) minutes in 0.1 mol.l^{-1} NaCl + 0.1 mol.l^{-1} Tris-HCl (pH = 7.4) without (*a—d*) or with (*e—h*) 4 mmol.l^{-1} $MgCl_2$ and stained for 10 min with HO (*a, c, e, g*) or EB + MI (*b, d, f, h*).

throught the 6 h period after irradiation the number of cells stained with EB is almost unchanged. Quite similar data were obtained earlier with erythrosin B (Scaife and Vittorio 1964).

In the next series of experiments the dose dependence of the yield of <2*C* cells 6 hours after irradiation (Fig. 5) was been analysed. In this case, too, the results obtained are consistent with those obtained with other methods (estimation of cells with pyknotic nuclei, measurement of chromatin fragments formed). Cells with a low DNA content are observed at doses as low as 0.25 Gy. High doses of irradiation inhibit DNA degradation.

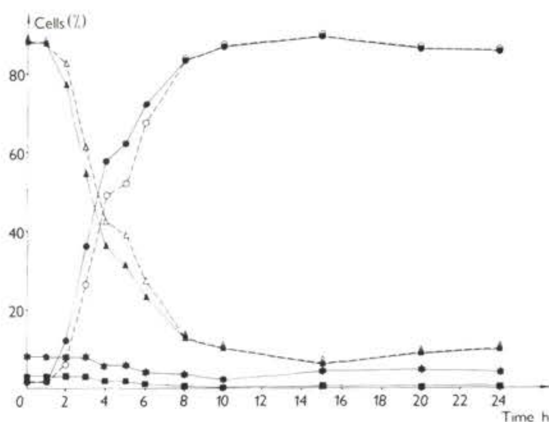

Fig. 4. Distribution of thymocytes with respect to DNA content after irradiation of rats with 10 Gy. Open symbols- staining with EB + MI, filled symbols — staining with HO. ▲ — $(G_0 + G_1)$; ∗ — S; ■ — $(G_2 + M)$; ◑ — $<2C$.

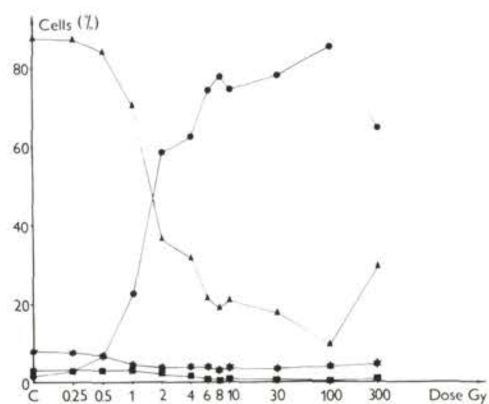

Fig. 5. Dose dependence of thymocyte distribution with respect to DNA content (6 hour after irradiation). Staining with HO. ▲ — $(G_0 + G_1)$; ∗ — S; ■ — $(G_2 + M)$; ● — $<2C$.

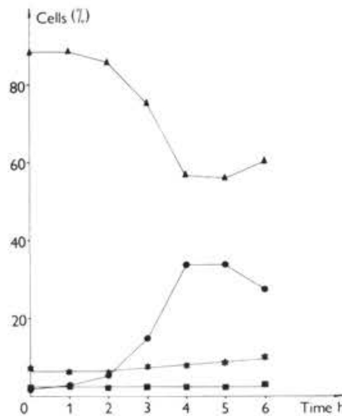

Fig. 6. Distribution of thymocytes with respect to DNA content after hydrocortisone injection. Staining with HO. ▲ — (G_0+G_1); $*$ — S; ■ — (G_2+M); ● — $<2C$.

The $<2C$ cells were also observed after hydrocortisone injection at the dose lethal to hormone-dependent thymocytes (Fig. 6).

It has already been shown that chromatin degradation in the thymocytes of irradiated or hydrocortisone-treated animals is prevented by introduction of protein synthesis inhibitors (Ermolaeva and Vodolazskaya 1977; Niconova et al. 1982). It was therefore of interest to study the influence of cycloheximide on the degradation of genetic material by using a flow cytofluorometric method. It was shown that application of cycloheximide decreases the proportion of $<2C$ cells from $72 \div 75\%$ to $12 \div 14\%$ and from $27 \div 28\%$ to $12 \div 13\%$ 6 hours after irradiation (10 Gy) and hydrocortisone treatment respectively.

Discussion

The results presented show that the method of flow cytofluorometry provides new information on the processes in dying cells. For the first time it has been shown that the death of lymphoid cells due to γ-irradiation or hydrocortisone is accompanied not only by internucleosomal fragmentation of chromatin, but also by a significant decrease in the DNA content of the cells. The patterns of formation and accumulation of cells with a low DNA content are similar to those of internucleosomal DNA fragmentation (Umansky et al. 1981). Such a cell type appears after a 1 h lag period. Introduction of cycloheximide decrease the accumulation of $<2C$ cells substantially and at high doses of radiation their numbers are reduced.

The lowering of DNA content in the cells can be accounted for only partially by degradation of DNA to acid-soluble products, because the necessary number of

intranucleosomal cleavages in the DNA has not been observed (Korol et al. 1979; Skalka et al. 1981; Umansky et al. 1981). It is more likely that part of the fragmented chromatin is extruded from the cells and manifests itself cytologically in the formation of apoptotic bodies (Wyllie et al. 1980).

It is noticeable that up to the sixth hours after irradiation no increase in the EB permeability of the cell membrane is observed. This provides further evidence that degradation of chromatin, and even its extrusion from the dying cell, precede the significant perturbations in the other cell structures.

It should be noted that flow cytometry detects not only the cells with reduced DNA content but also those with cleaved but not removed DNA. This is possible due to the removal of low molecular weight chromatin fragments during the incubation of fixed cells.

The death of irradiated cells is usually classified into two types, interphase and reproductive. Interphase death is typical of lymphoid cells. Indeed, the data presented indicate that the cells with $<2C$ DNA content are accumulated mainly at the expense of the cells in G_0 and G_1 phases. It is difficult to analyse the death of reproductive thymocytes because of the low content of diving cells in the thymus. DNA degradation upon the death of proliferating cells needs to be studied on cell culture. These experiments are now in progress.

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