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$CD8^+$ Natural Killer Cells Have a Potential of a Sensitive and Reliable Biodosimetric Marker *in vitro*

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Summary

The aim of our work was to evaluate peripheral blood lymphocyte subsets as *in vitro* indicators of the received dose of ionizing radiation (biodosimetric markers) in the range of 3-20 Gy and to determine the appropriate time interval, during which a dose-dependent induction of apoptosis occurs upon γ irradiation. In lymphocyte subsets characterized by double color surface immunophenotyping, four-color flow cytometry was used for visualizing cell death-associated increase in superficial phosphatidylserine exposure and cytoplasmic membrane permeability by fluorinated Annexin V and propidium iodide, respectively. No differences between sham-treated and lethal dose (7 Gy)-irradiated samples were observed upon 6 h cultivation *in vitro*. Ten and 18 h later, about 50 % of lymphocytes were apoptotic, but only the minority of them was in the late apoptotic phase. The only difference in radioresistance of the $CD4^+CD8^-$ and $CD4^-CD8^+$ lymphocyte subsets was seen upon 2-day cultivation when huge depletion of intact cells and prevalence of the late apoptotic population became obvious. A dose-dependence study in 16 and 48 h cultures confirmed the effectiveness of major T cell subsets as biodosimetric indicators. On the other hand, the minor $CD8^+$ subset of natural killer (NK) cells has been identified as a radiosensitive lymphocyte population the disappearance of which correlated with the received dose. We demonstrated that the $CD3^-CD8^+$ NK subset can be used as a lethal/sublethal dose discriminator to 16 h cultivation. In addition, our data indicate that two-day cultivation followed by CD3/CD8 expression analysis in an intact lymphocyte population may provide a clue for low dosage biodosimetry.

Key words
CD8+ killer cells • Biodosimetric marker • In vitro • Apoptosis • γ irradiation

Introduction

For estimation of the doses received by victims of the Chernobyl radiation accident, detection of chromosomal aberrations was used in stimulated lymphocytes collected from peripheral blood. Estimated

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doses correlated well with a further clinical course of the disease and with a decrease in neuthrophilic granulocyte numbers later after irradiation (Gale *et al.* 1993). A generally acceptable dose detectable by this method is 0.25 Gy, but only when at least 1000 cells are evaluated (Blakely *et al.* 2002). However, this method has also its limitations. Most importantly, after higher doses of radiation it is difficult to find a necessary number of dividing cells because of delayed mitosis (cell cycle arrest in response to cell damage), interphase death of lymphocytes and their depletion. Another limitation of this method is its duration; incubation of lymphocytes with mitogens required for proper stimulation lasts at least 48 h. Faster biodosimetric techniques with comparable or higher reliability are thus still being looked for.

Upon *in vitro* γ-irradiation, peripheral blood lymphocytes die by programmed cell death (apoptosis) accompanied by numerous processes including superficial exposure of phosphatidylserine (PS) (Hertveldt *et al.* 1997, Louagie *et al.* 1998a). Annexin V is a phospholipid-binding protein, which binds selectively and with a high affinity to PS in the presence of calcium ions (Koopman *et al.* 1994) and has thus been used for distinguishing apoptotic cells from their intact counterparts. The combination of Annexin V binding assay and immunophenotyping has been chosen by several groups in studies focused on the sensitivity of lymphocytes and their subpopulations (T cells, B cells, NK cells and their subsets) to ionizing radiation. Philippé *et al.* (1997) irradiated peripheral blood mononuclear cells (PBMC) *in vitro* by the dose of 2 Gy of γ-radiation and they detected apoptosis by Annexin V binding 24 hours after irradiation. They proved that B lymphocytes are more sensitive to ionizing radiation than T cells. Louagie *et al.* (1999a) described changes in distribution of lymphocytes in various subpopulations in patients undergoing fractionated external beam radiotherapy to the pelvis. The total received dose was 50 Gy, delivered with daily doses 2 Gy of 25 MV X-rays using a linear accelerator. The dose was recalculated to an equivalent of whole-body irradiation dose. The fastest decrease was detected in the B cell fraction. After the equivalent of whole-body irradiation dose of 1.5 Gy, the numbers of B lymphocytes decreased to 10 % of the starting value. $CD4^+$ and $CD8^+$ T cell subsets were also investigated. T cells appeared less sensitive and, after the whole-body irradiation by the dose equivalent to 2.5 Gy, their numbers decreased to 20 % of the starting value. The number of NK cells characterized by the $CD3^-/CD56^+$ surface phenotype decreased slowly, but also reached 20 % of the initial values by the end of therapy. NK cells represent about 15 % of circulating lymphocytes and most of them can be characterized as $CD3^-/CD8^+$ cells. They can be classified into two subpopulations using cell-surface density of CD56 – $CD56^{hi}$ and $CD56^{lo}$. While the $CD56^{hi}$ NK cells (10 %) are less cytotoxic and have a higher capacity for cytokine production, the $CD56^{lo}$ NK cells (90 %) are more cytotoxic (Cooper *et al.* 2001). Ban and Cologne (1992) described that $CD56^+$ were less sensitive than $CD3^+$ cells. However, it is clear that the population of $CD56^+$ cells contains various subpopulations with different functions in the organism. After 48 hours of incubation of PBMC irradiated by doses of 2-10 Gy decrease in $CD16^+$ NK cells in comparison $CD3^+$ cells was observed by Rana *et al.* (1990).

Cornelissen *et al.* (2002a) compared the type of interphase death occurring upon administration of moderate and high doses of low LET γ-rays and high LET fast neutrons (5.5 MeV). Peripheral blood lymphocytes were irradiated *in vitro* with radiation doses of 5 and 20 Gy. According to morphological criteria, they classified cells into live, early apoptotic, late apoptotic and oncotic (often referred to as necrotic). They found that the dose of 5 Gy induced apoptosis within 24 hours both after gamma (22.6±3.3 %) and neutron (19.7±4.2 %) irradiation. The dose of 20 Gy delivered by neutron irradiation caused apoptosis (24.3±4.5 %) 24 hours after the irradiation, while after the same dose delivered by γ-irradiation the ratio of apoptotic (16 %) to oncotic (47 %) cells was 1:3. 48 hours after the neutron irradiation a significant increase in apoptotic cells was detected after both studied doses – 5 and 20 Gy.

We have previously documented dose-dependent apoptosis induction in MOLT-4 T-lymphocyte leukemia cells (p53 wild type) 16 hours after ionizing radiation treatment in the dose range of 0.2.-5 Gy (Vávrová and Filip 2002). Programmed cell death was detected by an APO2.7 antibody, which reacts with an antigen present in the mitochondrial membrane of cells committed to apoptosis. We also proved that a dose-dependent increase and phosphorylation of p53 (serine 15 and serine 392) occurred in MOLT-4 cells as early as 3 hours after the irradiation (Szkanderová *et al.* 2003). It appears that apoptosis induced by ionizing radiation in peripheral lymphocytes in the G_0 phase is independent of accumulation and phosphorylation of p53 in the cell

nucleus (Jones *et al.* 2004). Mechanisms of apoptosis induction after *in vitro* γ-irradiation of PBMC by the dose of 5 Gy were also studied by Louagie *et al.* (1999b). Twenty-four hours after irradiation they observed PS exposure on the surface and a decrease in mitochondrial membrane potential. On the other hand, they did not detect release of cytochrome c from the intermembrane mitochondrial space to cytosol and only a weak caspase 3 activation.

The aim of our work was to establish a convenient and reliable *in vitro* biodosimetric protocol based on different radiosensitivity of individual lymphocyte subsets defined by surface immuno-phenotyping.

Methods

Peripheral blood mononuclear cells (PBMC)

PMBC were isolated from volunteers' heparinized blood by centrifugation on the Histopaque-1077 (Sigma) cushion according to manufacturer's instructions. After double washing in cold PBS the cells were resuspended in Iscove´s modified Dulbecco´s medium (Sigma) supplemented with 20 % fetal bovine serum (PAA Laboratories GmbH, Austria), 2 mM glutamine (Sigma), 100 UI/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma) at the density of $5x10^5$ cells/ml. The cell suspension was maintained in an incubator (37 ^oC, humidified atmosphere with 5 % CO_2) till irradiation (2-3 hours).

Irradiation

Cells in 10 ml aliquots were transferred into 25 cm^2 flasks (Nunc) and irradiated at room temperature using ^{60}Co γ-ray source. The doses of 2-7 Gy were delivered with a dose-rate of 0.4 Gy/min at a distance of 1 m from the source, for the doses of 10 and 20 Gy 1.5 Gy/min dose rate and a distance of 0.5 m from the source were used. Immediately after irradiation the flasks were placed into a 37 ^oC incubator with 5 % CO_2 and aliquots of cells were collected at various time intervals after irradiation for analysis.

Four-color flow cytometry

The cells were washed in ice-cold washing and staining buffer (WSB, PBS containing 0.2 % gelatin and 0.1 % NaN_3) and kept in cold during subsequent processing to prevent further apoptosis development. For visualizing major lymphocyte subsets, we chose double surface immunostaining using the combination of mouse anti-human monoclonal antibodies (MoAb) conjugated to R-phycoerythrin (RPE) or allophycocyanin (APC) from the following panel: anti-human CD3/RPE (clone UCHT1, Immunotech), anti-CD4/APC (cloneMT310, Dako) and anti-CD8/APC (clone DK25, Dako). After 15 min incubation and a single wash cycle (2 ml WSB, centrifugation, vortexing) the cells were treated with fluorescein isothiocyanate-conjugated Annexin V (A-FITC) and propidium iodide (PI) from the Apoptest kit (Dako) according to manufacturer's instructions. Flow cytometry (FCM) was performed on a four-color FACSCalibur (Becton Dickinson, BD) analyzer equipped with the 15 mW argon laser (488 nm, 15 mW) and red diode laser (635 nm, 25 mW). Electronic compensation of emission spectra overlap was set using single stained samples with or without propidium iodide, which emits light both into the FL2 and FL3 fluorescence channels. The minimum of 50 000 events were analyzed in each sample and the results were evaluated using the CellQuest or PC-lysys (BD) analytical software. Too small objects including cell debris and the majority of non-adherent monocytes were excluded from analysis by a standard approach using the "lymphogate" region in the forward and side (right angle) scatter characteristics (FSC and SSC, respectively). Lymphogate was carefully set up to include smaller cells in the later stages of apoptosis into the analyzed population.

Statistical analysis

The results have been have statistically evaluated with the Student's t-test. The values represent the mean ± S.D. (standard deviation). Statistical significance of the difference of means in comparable sets is indicated.

Results

Multiparameter analysis of apoptosis in lymphocyte subsets

The staining protocol used in our study makes it possible to distinguish several stages of apoptosis and/or necrosis differing in Annexin V and/or propidium iodide binding. Together with double color immunostaining and excitation light scatter characteristics, numerous lymphocyte populations can be analyzed in each sample as demonstrated in Figure 1. Lymphogate setup in the FSC-SSC dot plot (R1 in Fig. 1A) excludes cell debris as well as the major part of red cell contamination and non-

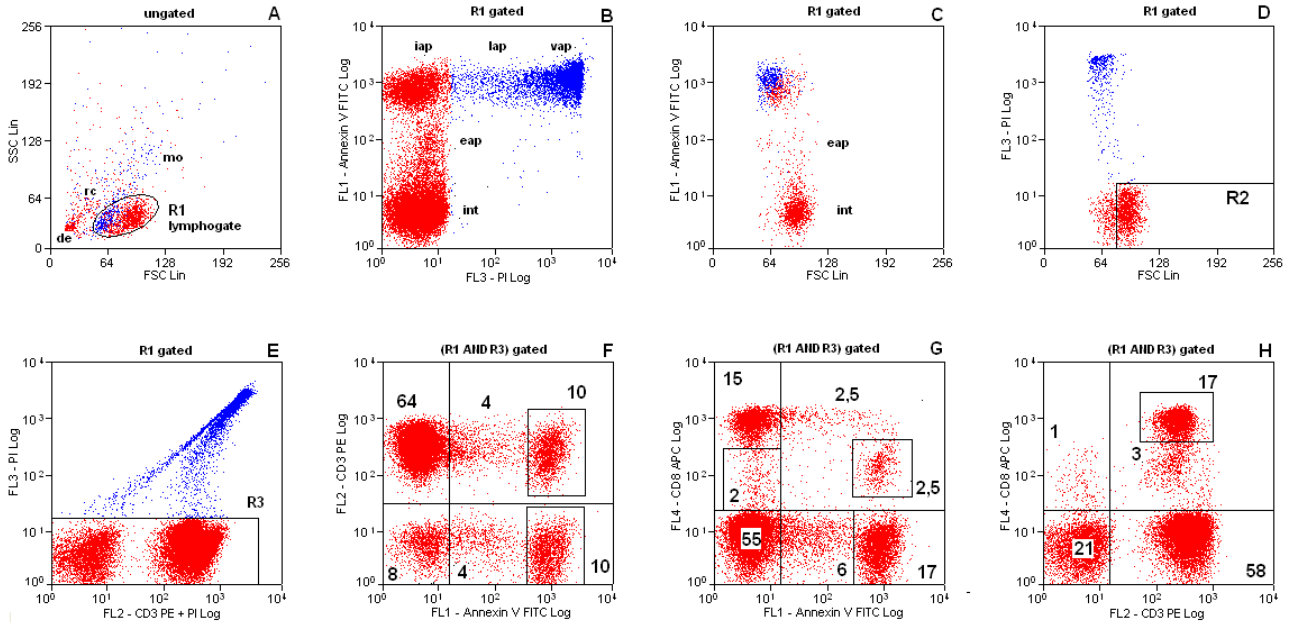

Fig. 1. Multiparameter flow cytometric analysis of in vitro irradiated (20 Gy) and subsequently cultivated (16 h) human PBMC stained with anti-CD3/PE and anti-CD8/APC MoAb and counterstained with Apoptest™. Lymphogate (R1 in A) was set to include all lymphocytes and exclude cell debris (de), the majority of erythrocytes (er) and non-adherent monocytes (mo) from analysis. Annexin V/FITC versus propidium iodide (PI) fluorescence dot plot (B) shows how intact (int) and early apoptotic (eap) subsets as well as cells in intermediate (iap), late (lap) and very late (vap) stages of apoptosis can be distinguished. Size (FSC) versus Annexin V/FITC and PI fluorescence analysis is shown in C a D, respectively. PI-negative cells (R3 in E) are shown as black dots while their PI^+ counterparts (cells outside the R3 region in E) are shown in grey. PI emits light both into the FL2 and FL3 channels (E) and double-color surface immunophenotyping can thus be evaluated on FACSCalibur in PI-negative population (R3) only. In PI-negative cells in lymphogate (the R1 AND R3 logical gate), the proportions of several lymphocyte subsets and the level of expression of surface markers can be determined in intact, early and intermediate phases of apoptosis as indicated in F, G and H, where relative numbers of individual subsets distinguishable by surface phenotype and/or Annexin V binding are shown. In A, C and D, only 2000 events are shown. B, E, F, G and H dot plots contain all cells in the logical gate defined on the top of dot plot windows.

adherent monocytes from the analysis. Annexin V versus propidium iodide staining profile of cells in the lymphogate (Fig. 1B) depicts apoptosis progression in the studied cell population and makes it possible to define successive stages of cell death. Intact cell population characterized by the lowest fluorescence signal both in the Annexin V-FITC (FL1) and propidium iodide (FL3) channels (A^-PI^-) is followed by cells in the earliest stages of apoptosis, which bind low-to-intermediate amounts of Annexin V while remaining cells are PI^- ($A^{lo}PI^-$). Intermediate apoptosis can be defined by high surface phosphatidylserine expression and null PI fluorescence ($A^{hi}PI^-$). Later stages of apoptosis become PI-positive with late apoptotic and very late apoptotic cells being $A^{hi}PI^{lo}$ and $A^{hi}PI^{hi}$, respectively. Two dimensional dot plots showing cell size (FSC) versus fluorescence of either Annexin V-FITC (FL1, Fig. 1C) or PI (in the FL3 channel, Fig. 1D) documents a cell volume decrease, which occurs in the stage of early apoptosis (Fig. 1C). This is not a trivial finding as, in consequence, gating for larger PI^- cells (R2 in Fig. 1D) makes it possible to analyze separately the frequency and phenotype of intact lymphocytes slightly contaminated with the cells in the earliest stages of apoptosis without using Annexin V-FITC, which saves time and money in large-scale experiments and opens an additional (FL1) channel for immunophenotyping. As expected, all cells in the terminal stages of apoptosis (PI^{hi}) are smaller than intact lymphocytes (Fig. 1D). PI emits light both into the FL2 and FL3 channels (Fig. 1E) and double-color surface immunophenotyping is thus impossible for PI^+ cells using our staining protocol and the FACSCalibur flow cytometer. However, when cells positive in the compensated FL3 channel are gated out (R3 in Fig. 1E), several lymphocyte subsets can be defined by surface expression of two selected surface markers and their proportions within the intact, early and mediate apoptotic populations can be determined (Fig. 1F-H). As demonstrated in Figure 1B, very few PI^+ cells binding null-to-low amounts of Annexin V were present in all samples studied, which is in agreement with formerly published data documenting that apoptosis is the hugely prevailing cell death mechanism after γ-irradiation.

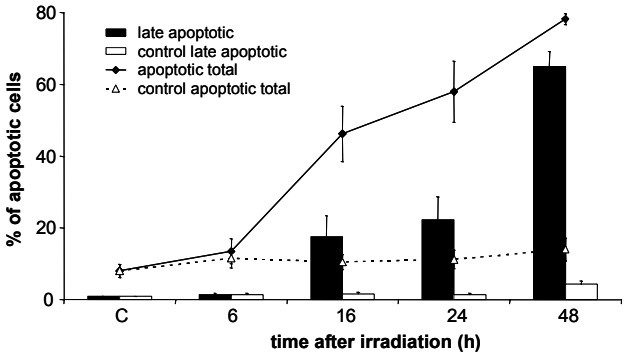

Fig. 2. Relative proportions of all apoptotic cells (A^+, lines) and cells in late or very late stages of apoptosis (A^+PI^+, bars) in 7 Gy irradiated (filled lines and bars) and control (dashed lines, open bars) lymphoid cells at different time points of *in vitro* cultivation. Mean values from 4 samples ± S.D. are given.

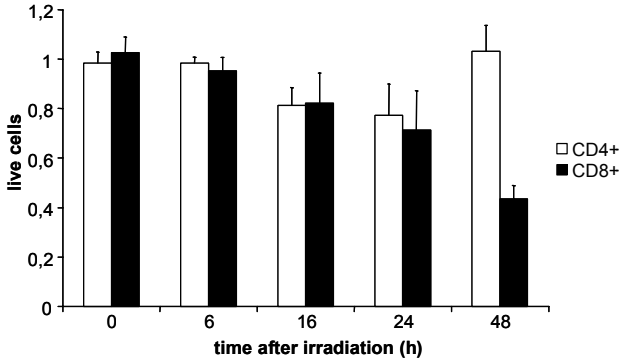

Fig. 3. Comparison of radioresistance of $CD4^+CD8^-$ (open bars) and $CD4^-CD8^+$ (filled bars) lymphoid cells. Cells were irradiated with 7 Gy and cultivated for the time intervals indicated. The ratio of intact (A^-PI^-) cells with the defined surface phenotype in the irradiated and sham-treated samples was calculated and plotted versus time of cultivation. Mean values from 4 samples ± S.D. are given.

Annexin and MoAb binding profiles confirm CD8 (and CD4, not shown) down-modulation during apoptosis progression described by Philippé *et al.* (1997). In contrast to their data, we recorded little if any decrease of antibody binding capacity to CD3 in the earliest stages of apoptosis (compare Fig. 1F and 1G, also apparent in Fig. 1H).

Taken together, the lymphoid compartment of PBMC cultured *in vitro* and stained with the cocktail of two MoAb, Annexin V and propidium iodide, is quite complex. Moreover, the momentary proportion of a selected apoptotic subset depends both on its lifetime and the size of all antecedent populations, which may change with time. Consequently, subset analysis of lymphocytes in different stages of apoptosis is not easy and experimental data must be carefully interpreted.

Dynamics of apoptosis progression upon lethal irradiation in vitro

As the first step, the lethal dose of 7 Gy was selected for studies on the time course of apoptosis progression in the total lymphocyte population. In parallel, the proportions of lymphoid subsets defined by mutually exclusive expression of CD4 and CD8 were compared upon 6, 16, 24 and 48 h cultivation. Sham-treated cells from all donors were used as control samples at the same time points.

Figure 2 shows that, upon 6 h cultivation, irradiated cells could not be distinguished from the controls in terms of apoptosis progression as both of the samples contained less then 10 % of A^+ events. Moreover, early apoptotic (A^+PI^-) cells strongly prevailed over the late apoptotic ones. On the other hand, 10 h later a clear difference between irradiated and sham-treated samples became apparent. While the percentage of apoptotic lymphocytes only slightly increased during cultivation reaching the maximum of about 10 % and the early apoptotic cells always prevailed in controls, huge apoptosis accompanied by superficial phosphatidylserine exposure and the loss of membrane integrity occurred in irradiated samples. The cultivation lasting 16 h and 24 h provided similar results: about half of the cells in the lymphogate bound Annexin V and the early and late apoptotic subsets were almost of equal size. On the other hand, almost all cells were apoptotic and the late apoptotic population strongly prevailed two days after irradiation.

Figure 3 compares radioresistance of $CD4^-CD8^+$ and $CD4^+CD8^-$ lymphocyte subsets in the same experiment. In the intact (Ann^-PI^-) cell compartment, the ratio of each of the two subsets in 7Gy irradiated and sham-treated samples was calculated and the resulting numbers were plotted versus the time of cultivation. This calculation has been chosen to normalize relative numbers of the two selected lymphocyte subsets in individual donors and to eliminate possible differences in the sensitivity of lymphocyte subsets to *in vitro* cultivation. In terms of radioresistance, no significant difference between $CD4^-CD8^+$ and $CD4^+CD8^-$ lymphocytes was observed during the first day of cultivation. In contrast, the existence of a radioresistant $CD4^+CD8^-$ subset in 48 h-lasting cultures, may be an explanation for the higher proportion of $CD4^+CD8^-$ cells in irradiated cultures. The analysis of the cell phenotype

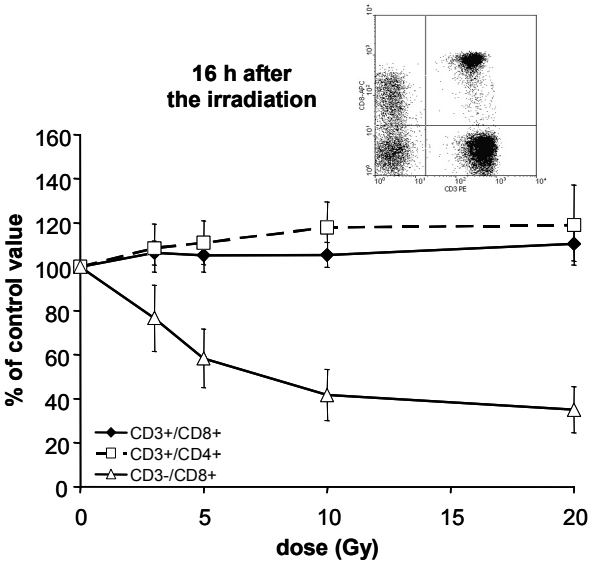

Fig. 4. Dose dependence of selected lymphocyte subset survival upon 16 h cultivation *in vitro*. The proportions of intact $CD3^+CD4^+$ T cells (open squares), $CD3^+CD8^+$ T cells (black diamonds) and $CD3^-CD8^+$ NK cells (open triangles) in irradiated samples were divided by the respective relative numbers in sham-treated controls, the resulting numbers were transformed to percentage and plotted versus the irradiation dose. Mean values from 6 samples ± S.D. are given. The insert in the upper right corner shows a representative CD3 versus CD8 expression dot plot of the A^-PI^- population in a 5 Gy irradiated sample.

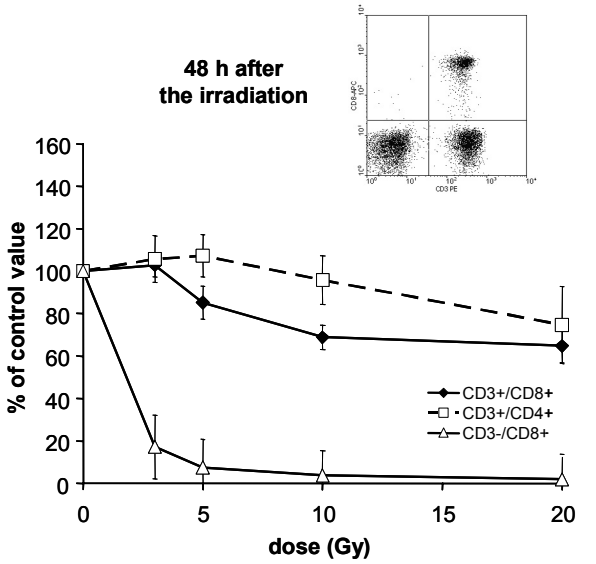

Fig. 5. Dose dependence of selected lymphocyte subset survival upon 48 h cultivation in vitro. The proportions of intact $CD3^+CD4^+$ T cells (open squares), $CD3^+CD8^+$ T cells (black diamonds) and $CD3^-CD8^+$ NK cells (open triangles) in irradiated samples were divided by the respective relative numbers in sham-treated controls, the resulting numbers were transformed to percentage and plotted versus the irradiation dose. Mean values from 6 samples ± SD are given. The insert in the upper right corner shows a representative CD3 versus CD8 expression dot plot of the A^-PI^- population in a 5 Gy irradiated sample.

in the A^+PI^- compartment has not provided any significant dose-dependent differences in the number of cells with the selected phenotype. Moreover, as such cells represent only transitional apoptotic population with as yet unknown lifetime and their frequency depends on the gradually decreasing number of intact cells, the analysis of the A^+PI^- cells cannot represent a reliable biodosimetric parameter under our experimental conditions. Based on these results describing the time dependence of apoptosis development in 7 Gy irradiated lymphocyte cultures and the survival of CD4/CD8 subsets, 16 h and 48 h cultivations were selected for subsequent dose-dependence studies.

Lymphocyte subsets as biodosimetric indicators.

As shown in Figure 1, multiparameter flow cytometry offers several possibilities how to study apoptosis development in irradiated lymphocyte preparations. In the biodosimetric part of our work we have continued with the analysis of intact (A^-PI^-) cells in an attempt to identify lymphocyte subsets and/or surface markers that would prove useful as biomarkers of the received dose. PBMC isolated from six donors were analyzed for the survival of lymphoid populations defined by CD3 versus CD8 and CD3 versus CD4 surface immunophenotyping.

The dose dependence of changes in proportions of intact lymphocytes with the $CD3^+CD4^+$ (helper and regulatory T lineages), $CD3^+CD8^+$ (cytotoxic T lineage) and $CD3^-CD8^+$ (a subset of NK cells) surface phenotype are shown in Figures 4 and 5 for 16 h and 48 h cultivations, respectively. In each irradiated PBMC preparation, the relative number of intact (A^-PI^-) cells with the selected surface phenotype was divided by the proportion of their counterparts in the sham-treated sample. The resulting numbers eliminate inter-donor differences in relative sizes of the lymphoid population studied and quantify the increase of apoptosis in irradiated samples versus spontaneous apoptosis in non-irradiated controls.

Sixteen as well as 48 h after irradiation, a trend of higher survival of $CD4^+$ cells with increasing dose could be observed within the $CD3^+$ T cell population. However, statistical analysis did not reveal significant differences between the two major T cell subsets. In contrast to that, the numbers showing radiation-induced decay of NK cells expressing CD8 on their surface (the $CD3^-CD8^+$ lymphoid population) show unambiguous

dependence of the relative decrease of $CD8^+$ NK on the irradiation dose. Dot plot inserts in Figures 4 and 5 represent typical CD3 versus CD8 staining profiles of the intact (A^-PI^-) lymphoid population in 5 Gy irradiated samples upon 16 h and 48 h cultivation, respectively. Higher irradiation doses and/or prolonged cultivation result in modified staining profiles with strongly reduced or almost completely missing $CD3^-CD8^+$ cells and the profiles of irradiated samples can thus be visually identified. Importantly, the comparison of the graphs in Figures 4 and 5 suggests that the size of the $CD3^-CD8^+$ NK cell population in cultivated samples is a promising candidate for a relevant biodosimetric marker within the dose range studied. In shorter (16 h) *in vitro* cultures it can be a sublethal versus lethal dose discriminator (the decrease below 50 % indicates doses above 5 Gy), while two-day *in vitro* cultures may provide relevant data reporting on low dosage (below 3 Gy) exposure.

Discussion

In contrast to immortalized cell lines (e.g. MOLT-4 T leukemia cells used in other our studies), where the cells undergo intensive proliferation, peripheral blood mononuclear cells are almost exclusively in the quiescent, G_0 phase of the cell cycle. This provides two possible approaches how to study radiation-induced cell death *in vitro*: apoptosis progression directly from G_0 phase can be evaluated in cultured cells in selected time intervals or the cells can be activated with a mitogen (e.g. phytohemagglutinin) and apoptosis can be determined in proliferating cells. With our final goal in mind – to identify lymphocyte populations that would be suitable as early indicators of the received dose during radiation accidents – we decided to study apoptosis on quiescent irradiated mononuclear cells.

In the first approach we irradiated PBMC by the high lethal dose of 7 Gy and studied the dynamics of apoptosis induction in an attempt to select appropriate time intervals for further studies. We found that apoptosis in such primary cultures occurs more slowly than in the MOLT-4 cell line used as a model in our previous studies. MOLT-4 cells proliferate vigorously and, in the logarithmic phase of the culture, 40 % of cells are in the S phase of the cell cycle. Upon irradiation with the dose of 7.5 Gy, nearly all MOLT-4 cells are late apoptotic (APO2.7 positive without permeabilization) within 24 h (Vávrová and Filip 2002). In one-day PBMC cultures, on the other hand, a similar dose induces programmed cell death in 60 % lymphocytes only, about half of them being in the early (PI^-) apoptotic phases. Prevailing apoptosis with the majority cells being strongly stained with PI was found 24 h later. At this time point, a minor population of peripheral blood lymphocytes still had a character of intact cells and this was the only case when a statistically significant difference between surviving $CD4^+$ and $CD8^+$ lymphoid subpopulations was found. We explain this by existence of radioresistant $CD4^+$ subset(s) that become visible after a great majority of lymphocytes had disappeared from the intact cell compartment. Similar results were obtained by Wilkins *et al.* (2002), who studied apoptosis induction 48 h after *in vitro* irradiation of PBMC by the doses of 0.1-2 Gy and described an increasing CD4/CD8 ratio in the surviving population. They discussed the importance of cultivation conditions as the presence of $CD4^+$ cells may have a protective effect on $CD8^+$ lymphocyte survival.

Based on a quantification of apoptosis progression in lethally (7 Gy) irradiated cells we have chosen two time intervals for subsequent work with γ-radiation-treated and control PBMC samples. From the practical point of view, shorter (16 h) cultivation may represent a relatively fast biodosimetric assay while 48 h lasting cultivation may shed some light on the existence of radioresistant lymphoid subsets that could prove useful as biodosimetric indicator in individuals exposed to low doses of ionizing radiation (Wilkins *et al.* 2002). In the time intervals and dose ranges studied, apoptosis was the main if not the sole mechanism of γ-radiation-induced cell death. Both in irradiated and control samples, the apoptotic pathway characterized by the successive A^-P^-, $A^{lo}PI^-$, $A^{hi}PI^-$, $A^{hi}PI^{lo}$, $A^{hi}PI^{hi}$ stages strongly prevailed and putative necrotic cells with the A^-PI^+ or $A^{lo}PI^+$ characteristics were essentially missing. We have concluded that apoptosis is the only cell death mechanism that should be taken into account for further analysis. This conclusion is in good agreement with our previously published data showing that human promyelocytic leukemia cells HL-60 follow exclusively the apoptotic pathway in a broad range of γ-irradiation doses 20-100 Gy (Vávrová *et al.* 2001) and observations by Cornelissen *et al.* (2002b) comparing results of flow cytometry and electron microscopy in 5 Gy irradiated lymphocytes. We thus disagree with the conclusions made by the same group that high doses (20 Gy) induce lymphocyte necrosis as ascertained from electron microscopic preparations (Cornelissen *et al.* 2002a). In any case, as we decided to compare the radioresistance of

lymphocyte subsets by surface immunophenotyping within the intact A^-PI^- lymphocyte population representing the temporary survivors, apoptosis versus necrosis discrimination accuracy did not influence results presented in our paper. It is worth mentioning that this is not the only advantage of our approach that differs from experimental design used by other researchers who focused on apoptotic populations (Seki *et al.* 1995, Loaugie *et al.* 1998b). Apoptosis is a dynamic process and individual apoptotic populations are transitional. The momentary size of each of them influences the number of cells in subsequent stages of programmed cell death. It is thus extremely difficult to compare apoptotic populations-based results at different time points, especially when the lifetimes of individual apoptotic populations have not yet been determined. Qualitatively, our results indicate that the relative size of the populations in early and intermediate phases of apoptosis (eap and iap in Fig. 1B, respectively) do not significantly change until the intact population becomes very small 48 h after irradiation (Fig. 2). This finding indicates that these two dying populations are short living and apoptotic cells accumulate in later stages of apoptosis. In contrast to this, comparing the survival rate of individual lymphocyte subsets provides unambiguous data on their radioresistance. Moreover, the combination of scatter parameters with propidium iodide exclusion assay allows for omitting Annexin V from the staining protocol in large scale experiments, which opens up the possibility of involving more colors in the immunophenotyping protocol and makes lymphocyte-based biodosimetry time and cost effective.

The decrease of radiation-induced antibody binding capacity on apoptotic cells described by Philippé *et al.* (1997) was observed in our samples during early-to-intermediate apoptosis transition and it was accompanied by cell shrinking as reflected by excitation beam scattering (a decrease of the FSC parameter). Interestingly, while CD4 and CD8 behaved in a similar manner, intact and $A^{hi}PI^-$ cells appeared to bind similar amounts of anti-CD3 MoAb, which suggests that a cell volume decrease during the transition to the intermediate apoptotic stage is not accompanied by reduced density of CD3 on the surface. In spontaneously dying cells in the control samples such an effect was much less obvious and the existence of a pronounced $A^{hi}PI^-$ population is a hallmark of irradiated samples.

The radiation dose-oriented part of our study has confirmed that studies focused on the $CD4^+$ or $CD8^+$ lymphoid compartment do not provide any reliable biodosimetric data with the exception of long-term cultures that are inconvenient for rapid determination of the received dose. This finding has a reasonable explanation as both phenotypes include numerous differentiation stages of the two major T cell lineages, i.e. naive, activated, effector, regulatory and memory cells that may possess various radioresistance. Moreover, a subset of NK cells bear CD8 on their surface (Cooper *et al.*, 2001) and analyzing CD8 lymphoid cells thus mixes up two distantly related lymphoid lineages. We have thus tried to distinguish T and NK cells expressing CD8 on their surface. This approach resulted in the finding that $CD4^+$ T cells tend to appear more radioresistant in the dose range studied (3-20 Gy) than their $CD8^+$ counterparts, but the biodosimetric value of this phenomenon is low due to its low or non statistical significance.

Most importantly, CD3 versus CD8 immuno-phenotyping has revealed a $CD8^+$ NK subset with an outstanding biodosimetric potential. Upon 16 h cultivation, the irradiation dose above 5 Gy induces approximately 50 % $CD8^+$ NK cell reduction when compared to the controls and this subset can thus serve as a low versus high dose discriminator within a reasonable time interval. In addition, long-term cultivation provides a possibility to study low dosage irradiation using the same NK cell subset. The usefulness of the suggested approach for *ex vivo* determination in irradiated organisms must be determined. It must be emphasized that as the dose determination is based on NK subset depletion, immediate (or within 6 h) blood collection from irradiated individuals is essential to ensure reliable results. The exceptional biodosimetric potential of $CD8^+$ NK cells can be elucidated by its relative homogeneity – in contrast to other lymphoid populations studied so far these cells may represent a uniform population with well-defined radiosensitivity.

Radioresistance of NK cells remains a matter of discussion. Philippé *et al.* (1997) described that after the irradiation by the dose of 2 Gy the NK cells ($CD56^+$) are the least sensitive population of lymphocytes. In our hands, the $CD8^+$ NK cells disappear much faster than T cells upon irradiation *in vitro*. Pilot *in vivo* experiments running in our laboratory indicate that $CD8^+$ NK cell depletion from the circulation is followed by a re-appearance of such a subset later on. Both the differences between the situation *in vivo* and *in vitro* and different radioresistance of mature cells can be connected to the

observed phenomena. Although more detailed analysis within the NK population and careful comparison of *in vitro* experiments with the *in vivo* situation are required for better understanding of NK cell radiosensitivity, our data unambiguously show where the population with great biodosimetric potential can be found.

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