CD27^{+} peripheral blood B-cells are a useful biodosimetric marker *in vitro*

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Running title: CD27-positive B-cells as a biodosimeter

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Summary:

The $CD8^+$ natural killer (NK) subpopulation has recently been identified as a fast and reliable biodosimetric indicator within human peripheral blood mononuclear cells (PBMC) *in vitro.* In irradiated and subsequently cultivated PBMC, a decrease of the relative number of intact $CD3^-CD8^+$ lymphocytes 16 and 48 hr after treatment has allowed for estimating the received dose in the range of 0 – 10 Gy and lethal/sublethal dose discrimination, respectively. Here we show that suitable biodosimeters can also be found in the peripheral blood B-cell compartment. Multiparameter flow cytometric analysis of irradiated and subsequently cultivated human PBMC revealed that both the $CD27^+$ and $CD21^-$ B-cell subpopulations can be used as biodosimeters and the $CD19^+CD27^+$ lymphocytes have proved useful for retrospective determination of the received dose in the range of 0 – 6 Gy. In addition, several $CD19^+$ lymphocyte subsets characterized by co-expression of CD21, CD27 and CD38 have been shown to bear biodosimetric potential, too. However, when important parameters like the original size within the $CD19^+$ compartment, its radiation-induced changes and data variation had been taken into account, the $CD27^+$ subpopulation proved superior to the other B-cell subpopulations and subsets. It appears that, in the dose range of 0 - 6 Gy, the relative decrease of $CD27^+$ B lymphocytes provides more sensitive and reliable data than that of $CD8^+$ NK-cells due mainly to lower data variation. In contrast to $CD27^+$ B-cells, the proportions of $CD27^+$ subpopulations of T-cells were not affected by irradiation. We have also proposed a simple experimental protocol based on full blood cultivation and three-color CD27/CD3/CD19 immunophenotyping as a time-saving and inexpensive

approach for practical biodosimetric evaluations on simple, three-to-four color flow cytometers.

Key words
B-cells • CD27 • Biodosimetric marker • γ-irradiation

Introduction

One of the main tasks of biodosimetry is to identify naturally-occurring indicators (e.g. biopolymers, soluble factors or cell populations) generally called biodosimetric markers that can be used for determining the received dose in individuals exposed to ionizing radiation (Straume *et al* 1992, Pass *et al*. 1997, Mori *et al*. 2005, Marchetti *et al.* 2006). In most cases when accidentally exposed victims do not bear personal dosimeters body fluids and cells are the only available materials that can help with dose estimates and subsequent selection of post-irradiation treatment. It has been documented that allogeneic bone marrow transplantation at short notice can save the life at intermediate-to-high doses (4 - 10 Gy) (Weisdorf *et al*. 2006); at lower expositions, hematopoietic growth factor administration is often sufficient as supportive care while histoincompatible grafting can have adverse effects due to the graft versus host reaction (Baranov *et al.* 1989). In addition to their sensitivity in the range of 0 – 10 Gy, appropriate biodosimetric markers should be easily accessible and reliable and the dose estimate should be available within the first day after irradiation as time is a crucial factor in triage of irradiated patients in case of accidental irradiations and mass casualties (Young 1987). The simplicity and costs of the detection system may also play a role, especially when a mass number of suspected sufferers are to be examined (Walker and Cerveny 1989).

Peripheral blood represents an easily accessible source of cells and soluble substances. It can be repeatedly collected at well-defined time intervals after irradiation with essentially no impact on the examined individual. In addition, hematopoiesis belongs to the most radiosensitive systems within the body and peripheral blood leukocytes as well as their subpopulations and subsets have been well characterized in terms of their phenotype and function. Lymphocytes are well recognized as the most radiosensitive white blood cell population (Harrington *et al.* 1997, Chambers *et al.* 1998) and they have been thoroughly investigated as a possible source of biodosimetric information (Gridley *et al.* 2002). Several biodosimetric assays including micronuclei formation (Lee *et al*. 2000), dicentric chromosome enumeration (Gotoh *et al.* 2005, Belloni et al., 2005), detection of chromosomal translocations after chromosome painting by fluorescence in situ hybridization (Lucas *et al.* 1995, Szeles *et al.* 2006) or mutation frequency determination (Saenko *et al.* 1998) have been used in the attempt to retrospectively determine the dose received by an individual. The estimation of doses received by victims of the Chernobyl radiation accident by chromosomal aberrations in mitogen-stimulated peripheral blood lymphocytes correlated well with the decrease of both granulocyte numbers and further clinical course (Gale *et al.* 1993) and provided the lowest dose detection limit of 0.25 Gy. Such biodosimetric approaches have proved reliable and provided good dose estimates but they are labored and time consuming. It is thus obvious that faster and easier protocols with high reliability of results are required.

Lymphocyte populations, subpopulations and subsets differ in their sensitivity to ionizing radiation, which can be used for biodosimetric purposes (Crompton and Ozsahin 1997, Harrington *et al*. 1997, Mori *et al*. 2005). Higher radiosensitivity of

B-cells than T-cells was documented in mice (Anderson *et al.* 1977, Durum and Gengozian 1978), rats (Bazin and Platteau 1986, Bazin *et al.* 1986) and humans (Schwartz *et al*. 1983, Ashwell *et al.* 1986) a long time ago. Surface immunophenotyping combined with Annexin V binding to superficially exposed phosphatidylserine (PS) has proved an effective approach in studies focused on γ-radiation-induced apoptosis in lymphocyte subpopulations and subsets characterized by surface marker expression (Koopman *et al.* 1994, Hertveldt *et al*. 1997, Kern *et al.* 1999, Philippé *et al.* 1997). Hence, Schmitz *et al.* (2003) found B-cells the most radiosensitive lymphocyte population in a large set of human PBMC samples and higher radiosensitivity of $CD19^+$ lymphocytes (B-cells) than their $CD3^+$ counterparts (T-cells) was confirmed by Philippé *et al.* (1997). Higher radioresistance of $CD4^+$ T-cells than the $CD8^+$ ones has been documented by several groups (Seki *et al.* 1995, Wilkins *et al.* 2002, Schmitz *et al.* 2003, Vokurková *et al.* 2006). When T-lymphocyte subsets were analyzed in more detail, CD4-positive and CD45RA-negative cells were more prone to apoptosis than the CD8-positive and CD45RA-positive ones (Philippé *et al.* 1997). When compared to T-lymphocytes, B-cell subpopulations and subsets have received less attention. Riggs *et al.* (1988) showed that, in irradiated mice, splenic B-cells with the $sIgM^{++}/sIgD^{dim}$ surface phenotype are more radiosensitive than B-cells bearing much less sIgM than IgD on the surface. This finding was supported by histological analysis confirming enhanced radiosensitivity of the marginal zone B-cells. A similar conclusion was made from observations in rats irradiated with doses equal to or higher than 3.5 Gy where the non-circulating marginal zone B-cell population appeared more susceptible than circulating cells from the follicular compartment (Bazin and Platteau, 1984, Bazin *et al*. 1986). Interestingly, selective resistance of peritoneal B-1 cells to radiation-induced

apoptosis, which was not paralleled by either B-1 or B-2 splenocytes has recently been published (Otero *et al.* 2006).

We have recently identified a population of relatively radiosensitive small $CD3^-CD8^+$ PBMC with a biodosimetric potential in the range of 0 – 10 Gy (Vokurková *et al.* 2006). We have also shown that no significant increase in the proportion of Annexin V-binding cells occurs in irradiated PBMC during first 6 hr of *in vitro* cultivation, which may represent a useful time window for sample collection and immunophenotypic characterization of lymphocyte subsets before cells start dying. In this work we have focused on the B-cell compartment and studied relative radioresistance of $CD19^+$ peripheral blood B-cell subpopulations and subsets in the attempt to identify another reliable, easily accessible and highly sensitive biodosimetric markers.

Materials and Methods

Cell isolation

In three sequential experiments, seven peripheral blood mononuclear cell (PBMC) preparations (2, 3 and 2) and five full blood samples (0, 3 and 2) were analyzed. Heparin-treated blood obtained from seven different volunteer donors was diluted 1:1 with phosphate buffered saline (PBS) and either used directly (full blood samples) or PBMC were isolated by 400 g centrifugation through the Ficoll-Histopaque (density 1077, Sigma, St. Luis, MO) cushion (20 min, 20^{o}C). PBMC collected from the Ficoll / plasma interface were washed twice in sterile RPMI 1640 (GIBCO Life Technologies, Grand Island, NY) supplemented with 20% FCS, 2 mM glutamine, 100 UI/ml penicillin and 0.1 mg/ml streptomycin (all products from Sigma) and their density was finally set to 10^6 cells / ml.

Irradiation and cultivation:

500 µl and 1 ml aliquots of PBMC and PBS-diluted blood, respectively, were transferred into a 24-well polystyrene cultivation plate (Nunc). Individual plates were sham-treated or irradiated with the doses of 3, 6 or 10 Gy delivered with a rate of 1.29 Gy/min at a distance of 1.23 m from the Co^{60} source. Subsequently, the plates were transferred into a humidified incubator and cultivated for 16 hr at 37^{o} C. After cultivation, the cells were harvested by thorough pipetting and centrifuged (400 g for 10 min at 4^{o}C). Red cells were removed from full blood samples by 10 min exposure of the resuspended pellet to the EasyLyse solution (Dako, Glostrup, DK) and remaining peripheral blood leukocytes (PBL) were centrifuged. PBMC and PBL pellets were resuspended and washed twice in ice-cold washing and staining buffer (WSB, PBS containing 0.2 % gelatin from cold water fish skin and 0.1 % sodium azide, all reagents from Sigma) and their density was set to 5x 10^{6} / ml. Until data acquisition, the cells were kept in cold and at the presence of sodium azide to prevent apoptosis progression.

Flow cytometry:

Anti-CD19/Pacific blue (anti-CD19/PB, clone HD37, Dako), anti-CD5/Fluorescein isothiocyanate (anti-CD5/FITC, clone DK23, Dako), anti-CD27/FITC (clone M-T271, Dako), anti-CD3/R-Phycoerythrin (anti-CD3/RPE, clone UCHT1, Immunotech), anti-CD21/RPE (clone B-ly4, Becton Dickinson), anti-CD138/RPE (clone BB4, Immunotech), anti-CD19/RPE-cyanin 5 (anti-CD19/PC5, clone HD37, Dako) and anti-CD38/PC5 (clone LS198-4-3, Immunotech) conjugates of mouse anti-human monoclonal antibodies (moAbs) were used in the following cocktails for

multicolor immunophenotyping: CD19/PB + CD27/FITC + CD21/RPE + CD38/PC5, CD19/PB + CD5/FITC + CD138/RPE + CD38/PC5 and CD27/FITC + CD3/RPE + CD19/PC5. 100 µl of cell suspension (5 x 10^5 cells) was incubated with the moAbs cocktail for 30 min on ice. Then the cells were washed twice in ice-cold WSB and data were acquired on a nine-color flow cytometer CyAn ADP (Dako). Propidium iodide (PI) was added just before acquisition to the final concentration of 0.1 µg / ml. Acquisition and analysis were performed using the Summit software (Dako). The detector sensitivity and off-line compensation of FITC, RPE and PC5 emission spectra overlaps were set using unstained and single-color stained samples and the automatic compensation software utility. The emission of PB excited by the violet (405 nm) laser was not necessary to compensate with the set of fluorochromes used.

Statistical analysis

To eliminate differences between individual blood donors, we have defined "irradiated versus non-irradiated ratio" (IVNIR) by dividing the relative size of a selected cell population in the irradiated sample by its respective number in the non-irradiated control. IVNIR cohorts were statistically evaluated and results are given in the text and illustrated in figures as mean value +/- standard deviation and mean + standard deviation, respectively. In dose dependence experiments, differences between samples were assessed by the Student's t-test.

Results

Surface marker selection and gate logic

Since the main task of our work has been to identify readily accessible, easily detectable and reliable biodosimetric markers within the B lymphoid compartment we have chosen a panel of fluorochrome-labeled moAbs directed against differentiation

antigens present on the surface of peripheral blood B-cells. We have omitted anti-Ig heavy chain immunoreagents as extensive washing of blood leukocytes is required before staining to prevent blocking the anti-Ig binding sites by serum-born Ig, which would unnecessarily prolong sample processing. In addition, Fc-receptor bound Ig may represent a potential source of false positive signal in terms of B lineage identification. Last but not least, polyclonal antisera are traditionally used for Ig subclasses detection and we wanted to prevent batch-to-batch variation in long-term experiments and in practical ramifications in radiobiology. Therefore, anti-CD19 moAb was used to distinguish B-cells from other PBMC lineages and anti-CD5, anti-CD21, anti-CD27, anti-CD38 and anti-CD138 were selected for visualizing B-cell subpopulations and subsets.

We and others have previously demonstrated that superficial phosphatidyl serine exposure at the early stage of apoptosis is rapidly followed by cell shrinking and decreased surface marker expression (Hertveldt *et al.* 1997, Vokurková *et al.* 2006). The great majority (> 95%) of Annexin V-binding cells in the intermediate and late stages of apoptosis can thus be excluded from analysis by gating them out using a region closely fitting the predominant population of small mononuclear cells clearly visible in irradiated as well as sham-treated PBMC cultures (region R1 in Fig. 1A). The left margin of R1 (the vertical bar in Fig. 1A, B and C) was set to exclude the majority of PI^+ events and cells with diminished CD19 expression (Fig. 1B and 1C, respectively) from analysis. Immunophenotyping within the “R1 AND R2 AND R3” logical gate has made it possible to determine IVNIR for subpopulations and subsets of small (R1) and propidium iodide-negative (R3) lymphocytes with unimpaired CD19 expression (R4). This approach has provided us a platform for identification of suitable biodosimetric markers within the peripheral blood B-cell compartment.

B-cell subpopulation and subset definition and their biodosimetric potential

To reveal putative biodosimetric candidates with short response time among B-cell subpopulations and subsets, a high lethal dose irradiation (10 Gy) followed by mid term (16 hr) cultivation and four-color immunostaining was chosen as the first step. Hereafter, all $CD19^{+}PI^{-}$ events in R1 (Fig. 1A) with comparable expression of one of the differentiation markers studied will be referred to as a subpopulation of small intact B-cells. We also define a subset of small intact B-cells as all $CD19^{+}PI^{-}$ events in R1 characterized by surface expression of two differentiation markers. Fig. 2 shows an example of CD21, CD27 and CD38 co-expression profiles on intact small B lymphocytes in sham-treated (A, B, C) and 10 Gy irradiated (E, F, G) PBMC cultivated for 16 hr after treatment. We disregarded fine resolution of surface marker density and defined the positive and negative B-cell subpopulations for every single marker as shown by quadrant statistics in Fig. 2. For example we refer to $CD21^{+}$ and $CD21^{-}$ B subpopulations as the sum of the subsets mapping to the upper and lower quadrants, respectively, in Fig. 2A, D. In the same dot plots, $CD27^{+}$ B subpopulation is represented by the two subsets in both of the right quadrants while its $CD27^{-}$ counterpart can be found in the left quadrants only. Analogously, the $CD21^{+}CD38^{-}$ single positive B-cell subset is defined as all events in the upper left quadrants in Fig. 2B and 2E. Double positive and double negative B-cell subsets for all marker combinations analyzed in Fig. 2 can thus be found in upper right and lower left quadrants, respectively. Inter-donor differences in proportions of B-cell subpopulations and subsets in peripheral blood have been eliminated by introducing IVNIR, the number reflecting relative radiosensitivity of individual B-cell subpopulations and subsets.

Summary of γ-radiation induced changes in the relative numbers of B-cell subpopulations and subsets are given as IVNIR statistics in Fig. 3A and 3B, respectively. In contrast to $CD27^+$ B-cells, the relative number of which obviously decreases in irradiated cultures *in vitro*, the $CD21^+$ population of B-cells relatively increases its size at the expense of their negative counterparts (Fig. 3A). The overall changes in the CD38-defined compartments are less pronounced and the $CD38^+$ and $CD38^-$ subpopulations appear similarly radioresistant, although the $CD19^+CD38^-$ subpopulation can be slightly more radiosensitive. Results shown in Fig. 3A suggest that it is the $CD21^-$ and $CD27^+$ subpopulations of B-cells that could represent promising radiation biomarkers since prominent changes in their relative size with reasonably low data variation can be observed in a relatively short time after high dose γ–irradiation. When the two subsets are compared, the $CD19^+CD27^+$ B-cells appear to represent the superior biomarker for the following reasons. First, the relative numbers of $CD27^+$ B-cells exceeded the size of the $CD21^-$ subpopulation in all non-irradiated samples studied: 18 – 35 % small $CD19^+$ lymphocytes were $CD27^+$ while the relative numbers of $CD19^+$ $CD21^-$ B-cells were in the range of 15 - 23 %. Second, IVNIR variation of the $CD27^+$ subpopulation was lower than that of $CD21^-$ B-cells. Third, the positive and negative populations are easier to distinguish for CD27 than for CD21 (Fig. 2). Experiments with anti-CD5 and anti-CD138 moAbs did not reveal any significant differences between irradiated and non-irradiated samples and these markers appear useless for biodosimetric purposes in peripheral blood (data not shown).

Two parameter immunophenotyping within the B-cell compartment (Fig. 3B) has shed some light on the relative radioresistance of individual $CD19^+$ lymphocyte subsets. As follows from subpopulation analysis (Fig. 3A), $CD27^+$ or $CD21^-$ B-cells

represent most promising single parameter biodosimetric candidates. Thus not surprisingly, when both the relative decrease in irradiated samples and the data variation have been taken into account, the $CD21^{-}CD27^{+}$, $CD27^{+}CD38^{-}$ and $CD21^{-}CD38^{+}$ immunophenotypes appear to be worth testing as radiation biomarkers. In addition, the CD21/CD27 double positive and double negative subsets bear some biodosimetric potential, too. Importantly, in the process of subdividing major B-cell subpopulations into subsets, the proportion of the cells of interest decreases and small number errors may begin playing a role. Moreover, because the overall cell numbers in irradiated samples significantly decrease with the dose and time of cultivation, experimental data may be negatively influenced when very small populations are analyzed. In this respect, the $CD21^{+}CD27^{+}$, $CD21^{-}CD27^{-}$ and $CD27^{+}CD38^{-}$ B-cell subsets with their relative size of 15 – 28 %, 15 – 22 % and 11 – 24 %, respectively, can provide more reliable data than the smaller $CD21^{-}CD38^{+}$ (8 – 16 %) and $CD21^{-}CD27^{+}$ (3 – 6 %) ones.

Interestingly, data from Ficoll-isolated PBMC have been confirmed by a pilot study of CD19 versus CD27 expression on B-cells in irradiated and sham-treated PBL preparations. Similar to IVNIR in PBMC (0,65 ± 0.05, see the left column in the middle of Fig. 3A), the values of 0.53 ± 0.05 have been obtained for IVNIR of $CD19^{+}CD27^{+}$ small intact lymphocytes in PBL preparations. This finding suggests that full blood cultivation *ex vivo* may represent a convenient alternative to more labored and time-consuming density gradient PBMC isolation.

The $CD27^{+}$ B-cell subset as a radiation dose indicator

A convenient biodosimeter should be able to distinguish between low (sublethal) and high (lethal) dose of irradiation as lethal versus sublethal case

discrimination is one of the most important parameters in terms of the treatment of an irradiated individual. We have thus tested the reporter capacity of $CD19^{+}CD27^{+}$ lymphocytes in 0, 3, 6 and 10 Gy-irradiated PBMC samples. We have also asked a question if it is specifically the CD27 surface glycoprotein that is sensitive to irradiation irrespective of its presence on the surface of B- or T-cells or the peripheral blood $CD27^{+}$ B-cells represent a radiation-sensitive population, the disappearance of which indicates previous exposure to γ-rays. Last but not least, we have considered that the practical assay should be as simple as possible for being performed on simple and inexpensive analyzers. We have therefore used a modified staining protocol with the cocktail of anti-CD27/FITC, anti-CD3/RPE and anti-CD19/PC5 moAbs, which is convenient for data acquisition on standard flow cytometers equipped with a single blue laser and three fluorescence channels. When the fourth channel is available it can be used for propidium iodide- or 7-aminoactinomycin D-based live/dead discrimination, although Fig. 1 shows that thorough gating in the FSC/SSC dot plot provides a relatively pure population of intact cells and PI exclusion need not be absolutely required for reliable biodosimetric data acquisition.

Dose dependent relative changes in major lymphocyte populations and their $CD27^{+}$ subpopulations in PBMC are illustrated in Fig. 4. As expected, peripheral B-cells are more radiosensitive than T-cells, which is reflected by the increasing T : B cell ratio (Fig. 4A). This, however, cannot be used as a reliable biodosimetric parameter due mainly to a relatively large data variation. On the other hand, the $CD27^{+}$ T-cell subpopulation does not change its relative size within the T-cell compartment after irradiation (Fig. 4B), which means that $CD27^{+}$ and $CD27^{-}$ T-cells are equally radiosensitive. Similarly, when $CD27^{++}$ T-cells (with CD27 expression higher than that on B-cells), and their $CD27^{+}$ counterparts (CD27 expression

comparable to B-cells) were analyzed separately no difference in radiosensitivity between $CD27^{++}$, $CD27^{+}$ and $CD27^{-}$ T-cell subpopulations was observed within the range of doses studied (data not shown). In striking contrast, the $CD19^{+}CD27^{+}$ lymphocyte subpopulation has confirmed its biodosimetric potential (Fig. 4C) in a dose-dependent manner. Irradiation with Co^{60} γ-rays followed by *in vitro* cultivation of PBMC for 16 hr has resulted in about 25 % and 50 % reduction of IVNIR at 3 and 6 Gy, respectively. The differences in IVNIR in the range of 0 - 6 Gy were highly statistically significant. No significant differences in the relative size of the $CD19^{+}CD27^{+}$ lymphocyte subpopulation were observed between 6 and 10 Gy-irradiated samples. The IVNIR data variation indicate that the relative decrease shown in Fig. 4C could provide a reliable estimate of the received dose in the range of 0 – 6 Gy in *in vitro* cultures.

Discussion

Differences in the response to ionizing radiation between and within white lineages of hematopoiesis have been documented by many authors (Schwartz *et al*. 1983, Ashwell *et al.* 1986, Koopman *et al.* 1994, Hertveldt *et al*. 1997, Philippé *et al*., 1997, Seki *et al.* 1995, Kern *et al*. 1999, Wilkins *et al.* 2002, Schmitz *at al.* 2003, Mori *et al.* 2005). In contrast to relatively radioresistant myelomonocytic cells (granulocytes and monocytes) lymphocytes represent highly radiosensitive blood leukocytes (Dutreix *et al.,* 1987, Mori and Desaintes 2004, our unpublished observations *in vitro* and *ex vivo*) and, in general, belong to most radiosensitive quiescent cells within the mammalian body. Thus not surprisingly, lymphocytes have traditionally been regarded as possible biodosimetric markers both for retrospective determination of the received dose (Gale *et al.* 1993, Crompton and Ozsahin 1997,

Menz et al. 1997) and as indicators of individual's radioresistance (Crompton and Ozsahin 1997, Crompton *et al.* 1999, Ozsahin *et al.*, 2005). Although some authors originally concluded that major lymphocyte populations, their subpopulations and subsets were equally prone to radiation-induced apoptosis (Dutreix *et al.* 1987, Clave et al. 1995), other groups have observed that the impact of *in vivo* or *in vitro* γ-irradiation on a lymphoid cell strongly depends both on its type and differentiation status. Therefore, B-lymphocytes have generally been considered the most radiosensitive lymphocyte population (Anderson *et al.* 1977, Durum and Gengozian 1978, Schwartz *et al*. 1983, Ashwell *et al.* 1986, Philippé *et al.* 1997, Pecaut *et al.* 2001) and, in the more radioresistant T-cell compartment, $CD4^+$ T-cells appeared less radiosensitive than their $CD8^+$ counterparts (Seki *et al.* 1995, Wilkins *et al.* 2002, Schmitz *et al.* 2003) although some groups have concluded the opposite (Farnsworth *et al.* 1988). Most controversial data exist about the radiosensitivity of NK-cells. While some authors (Ban and Cologne, 1992, Clave et al., 1995, Philippé *et al.* 1997, Louagie *et al.* 1999, Pecaut *et al.* 2001) described NK-cells as a relatively radioresistant population, other groups (Farnsworth *et al.* 1988, Rana *et al.* 1990 and Seki *et al.* 1995) have emphasized NK-cell radiosensitivity. Experiments focused on the radioresistance of B- and T-cell subsets are scarce and provide only fragmentary information about well-defined populations. Rather surprisingly, even though high radiosensitivity and differential radioresistance within the lymphoid compartment appear to offer an opportunity to design fast and relatively simple biodosimetric protocols, only a few groups have successfully used lymphocyte subpopulations and subsets as biodosimeters (Crompton and Ozsahin, 1997, Menz *et al.* 1997, Vokurková *et al.* 2006).

In our opinion, the difficulties with identifying convenient biodosimeters in the lymphoid compartment have originated from the focus on major lymphocyte populations and their most popular subpopulations like $CD4^+$ or $CD8^+$ T-cells (Clave *et al*. 1995, Philippé *et al.* 1997, Crompton and Ozashin, 1997, Kunosoki *et al.* 1994) or subsets expressing different isoforms of CD45 (Clave *et al.* 1995, Philippé *et al.* 1997). The individual's radioresistance has also been expected to represent a bottleneck in biodosimetric studies within the outbred human population (Terzoidi *et al*. 2006).

We have recently tested the hypothesis that cultivation of irradiated PBMC and appropriate lymphoid subset selection can provide a clue for successful biodosimetry *in vitro*. We have confirmed that it is neither major lymphocyte populations (T- and B-cells) nor the most popular subpopulations (e. g. $CD4^+$ or $CD8^+$ T-cells) that would represent reliable biodosimetric markers (data presented here, Vokurková *et al.* 2006). This is due mainly to their heterogeneity – even a relatively simple compartment like peripheral blood where essentially no cycling, differentiating or apoptotic cells are present under normal conditions consists of numerous stages of T, B and NK cell development ranking from naïve recent emigrants from primary centers to fully differentiated and effectors stages including memory, cytolytic and antibody secreting lymphocytes. The selection of a uniform lymphocyte subset and careful data analysis have recently revealed highly radiosensitive $CD3^-CD8^+$ NK-cells, the disappearance of which after irradiation provided us a qualitative and (semi)quantitative tool for retroactive determination of the received dose. 16 and 24 hr after irradiation, the relative proportion of $CD3^-CD8^+$ NK-cells decreases in cultivated PBMC in a dose dependent manner (Vokurková *et al.* 2006). 48 hr after irradiation the decrease of the proportion of intact $CD3^-CD8^+$ small lymphocytes

allows for distinguishing between samples irradiated with low (< 3 Gy) and high (> 3 Gy) doses.

We show here that subpopulations and subsets with similar characteristics also exist in the B-cell compartment. In contrast to T-cell subpopulations differing in CD27 expression, the proportions of which do not appear to change in the broad range of doses used in our experiments, the CD27-positive subpopulation of peripheral blood B-cells has proved highly radiosensitive as its relative size was significantly reduced by irradiation and subsequent cultivation. In this respect, $CD27^+$ B cells resemble the $CD8^+$ NK-cells; a decrease of about 25 % relatively to controls was observed in 3 Gy-irradiated samples in 16 hr (Vokurková *et al.* 2006). The $CD3^-$ $CD8^+$ and $CD19^+CD27^+$ lymphocyte subpopulations appear to have the highest informative value at sublethal doses (below 6 Gy) followed by 16 hr cultivation when the total number of lymphocytes does not drop below the 2/3 of the starting value (data not shown). It is worth noticing that the CD21-negative B-cell subpopulation has a biodosimetric potential similar to $CD27^+$ B-cells. Our focus on the $CD27^+$ phenotype has been based on higher relative numbers of $CD27^+$ than $CD21^-$ B-cells in PBMC samples and the lower standard deviation of $CD19^+CD27^+$ IVNIR.

Importantly, the comparison of experiments with full blood and PBMC has confirmed the equivalence of the approaches, which offers a possibility to considerably simplify the experimental protocol. In addition, observations performed after full blood cultivation can be regarded as an *ex vivo* assay with cells maintained under more natural conditions and as such they may represent a link between the situation *in vitro* and *in vivo*. Extensive dose and cultivation time dependence experiments are running now in our laboratory to calibrate the $CD27^+$ B-cells and $CD8^+$ NK-cells as *ex vivo* biodosimetric indicators.

Subset analysis within the $CD19^{+}PI^{-}$ lymphoid compartment in 10 Gy irradiated PBMC confirmed the usefulness of the $CD27^{+}$ and $CD21^{-}$ subpopulations as biodosimetric indicators. The $CD38^{+}$ and $CD38^{-}$ subsets of $CD27^{+}$ B-cells were negatively affected by irradiation but relatively high data variation made them less convenient for biodosimetric purposes. On the other hand, the $CD21^{-}CD38^{+}$ subset seemed to be fully responsible for radiation-induced reduction of $CD21^{-}$ B-cell numbers and as such it qualifies for more detailed investigation. When CD21 expression was studied versus CD27, the existence of one radioresistant ($CD21^{+}CD27^{-}$) and three radiosensitive ($CD21^{-}CD27^{-}$, $CD21^{+}CD27^{+}$ and $CD21^{-}CD27^{+}$) subsets became apparent.

We do not show here the results of three-dimensional subset analysis of $CD19^{+}$ B-cells as further subdivision makes some populations too small to prevent small number errors and detailed description of individual subset radioresistance has not been the purpose of our work. Moreover, we feel that we do not have enough experimental data to make any general conclusions concerning relative radioresistance of peripheral blood B-cell subsets, although our results showing high radiosensitivity in the $CD27^{+}$ compartment of activated/memory peripheral blood B-cells (Klein *et al.* 1998) is in a good agreement with lessons learnt from animal models (Bazin and Platteau, 1984, Bazin and Platteau, 1986, Bazin *et al*., 1986). At this level of understanding we have concluded that the CD19 versus CD27 immunophenotypic comparison of freshly isolated peripheral blood lymphocytes with the samples cultivated for 16 hr may represent a convenient, fast and simple biodosimetric assay, especially when applied to full blood samples when neither density gradient isolation nor humidified atmosphere during cultivation is absolutely required.

Figure description

Fig. 1 Gating for small intact B-cells. Region settings and gate logic used for immunophenotypic analyses in the compartment of small intact B-cells are demonstrated on an example of 10 Gy-irradiated and 16 hr cultivated PBMC. R2 region in the FSC/SSC dot plot (**A**) contains mononuclear cells with characteristic low SSC values. When cells from R2 were analyzed for their size (FSC) versus PI fluorescence (**B**) or CD19 expression (**C**), a FSC channel (indicated by the vertical line in all dot plots) was selected, below which both an increase of PI (**B**) signal and lower CD19 expression (**C**) were apparent, which is typical for cells in the intermediate and later stages of apoptosis. This FSC channel has been chosen as the left margin of the R1 region close-fitting to a dominant population of small mononuclear cells clearly visible in **A**. R3 and R4 regions were designed in dot plots **B** and **C,** to include only PI-negative and CD19-positive events into analysis. The population of small intact B-cells has then been defined as the events within the R1+R3+R4 logical gate. For better visualization of R1 and R2, only 2000 dots are shown in **A**; dot plots **B** and **C** contain 20 000 events from R2. **B**: Numbers in quadrants in **B** show relative numbers of cells.

Fig. 2 B-cell subpopulation and subset definition. An example of CD27 versus CD21 (A and D), CD38 versus CD21 (B and E) and CD38 versus CD27 (C and F) two dimensional expression profiles on small intact B-cells ($CD19^{+}PI^{-}$ events in R1 in Fig. 1) is shown in sham-treated (**A**, **B**, **C**) or 10 Gy-irradiated (**D**, **E**, **F**) PBMC cultivated for 16 hr. Spectral overlap between FITC and RPE was compensated using single stained controls. A limited number of 3 000 events are shown in all dot plots to

optimize visualization of differences in cell subset proportions between the irradiated and non-irradiated sample. For selected marker combinations, the quadrant statistics in individual dot plots define the double positive (upper right quadrant), double negative (lower left quadrant) and two single positive (upper left or lower right quadrants) subsets, the relative numbers of which are given.

Fig. 3: Radiosensitivity of B-cell subpopulations and subsets. For each subpopulation or subset of small intact B-lymphocytes, the irradiated (10 Gy) versus non-irradiated ratio (IVNIR) was calculated by dividing its proportion in the irradiated and 16 hr cultivated sample by the respective number in the sham-treated, control sample. Then the IVNIR average value and standard deviation for all seven PBMC preparations was calculated and plotted in **A** (B-cell subpopulations) and **B** (B-cell subsets). The values above 1 report on a higher proportion of the analyzed phenotype in the irradiated sample than in its non-irradiated control. Similarly, more radiosensitive subpopulations and subsets tend to lower IVNIR values. The CD27/CD38 (grey bars), CD21/CD38 (black bars) and CD21/CD27 (empty bars) subsets in **B** are thus shown in the ascending order in terms of their radiosensitivity.

Fig. 4: Dose dependence of γ-radiation-induced relative changes of the T : B-cell ratio and CD27 expression on T- and B-cells in 16 hr cultivated PBMC preparations. **A**: In sham-treated and 3, 6 or 10 Gy irradiated samples the relative number of T-cells was divided by the B-cell proportion, IVNIR was calculated for every dose and mean values ± standard deviations were plotted against the irradiation dose. Analogously, dose dependence of IVNIR calculated for $CD3^{+}CD27^{+}$ and

$CD19^+CD27^+$ small intact lymphocytes are shown in **B** and **C**, respectively. Statistically significant differences are shown.

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