# Radiosensitivity of peripheral blood B cells in pigs

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ABSTRACT: Swine are here introduced to biodosimetry in an attempt to develop a large animal model allowing for comparison of *in vitro* experiments with the *in vivo* processes occurring after exposure to gamma radiation. This work investigates the radiosensitivity of the B cell compartment in peripheral blood. Four-week-old piglets were irradiated using the whole body protocol or full blood samples were irradiated *in vitro* in the dose range of 0–10 Gy. Relative radioresistance of B cell subpopulations and subsets was determined by measuring their relative numbers in leukocyte preparations at selected time intervals after irradiation using two color immunophenotyping and flow cytometry. Porcine B cells represent the most radiosensitive lymphocyte population in peripheral blood. Among B cell subpopulations and subsets investigated, the CD21+SWC7+ and CD21+CD1+ cells are highly radiosensitive and possess biodosimetric potential, at least in the range of low doses. Differences between cultures irradiated *in vitro* and lymphocyte dynamics in peripheral blood of irradiated animals clearly document the limits of *in vitro* data extrapolation in biodosimetry. We have shown that pigs can successfully be used in radiobiology and experimental biodosimetry due mainly to their availability, size and a relatively broad spectrum of available immunoreagents for lymphocyte classification.

Keywords: dosimetry-biological; B lymphocytes; radiosensitivity; pig

A good estimate of the received dose in individuals exposed to ionizing radiation is crucial as allogeneic bone marrow transplantation can decrease the mortality at doses between 4 and 10 Gy (Weisdorf et al., 2006) while supportive treatment by hematopoietic growth factor is often insufficient and incompatible grafting can have adverse effects due to the host reaction at lower sublethal exposures (Baranov et al., 1989). Victims of radiation accidents or possible targets of military or terrorist attacks by radionuclide-containing/producing weapons usually do not bear personal dosimeters and one of the major tasks of biodosimetry is to identify body-borne materials with biodosimetric marker potential that can be used for received dose determination (Straume et al., 1992; Pass et al., 1997; Mori et al., 2005; Marchetti et al., 2006). Peripheral blood leukocytes, with their accessibility

and relatively high radiosensitivity, have traditionally been used for biodosimetric purposes. The estimation of the level of irradiation in victims of the Chernobyl 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. Several biodosimetric assays including micronuclei formation (Lee et al., 2000), dicentric chromosome enumeration (Belloni et al., 2005; Gotoh 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. Such biodosimetric read

out systems have proved reliable and provided good dose estimates but they are labour-intensive and time consuming. Faster and easier approaches with sufficiently high reliability are thus constantly being sought.

The lymphoid compartment of peripheral blood leukocytes is famed for its high radiosensitivity (Harrington et al., 1997; Chambers et al., 1998) and has thus been investigated as a possible source of useful biomarkers (Gridley et al., 2002). Differences in the radiosensitivity of lymphocyte populations, subpopulations and subsets have been used for biodosimetric purposes (Crompton and Ozsahin, 1997; Harrington et al., 1997; Mori et al., 2005). The higher radiosensitivity of B cells over T cells has been 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). Surface immunophenotyping combined with Annexin V binding by apoptotic cells 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; Philippe et al., 1997; Kern et al., 1999). Hence, Schmitz et al. (2003) found B cells to be the most radiosensitive lymphocyte population in a large set of human PBMC samples and a higher radiosensitivity of CD19$^+$ lymphocytes (B cells) than their CD3$^+$ counterparts (T cells) was confirmed by Philippe et al. (1997). The higher radioresistance of CD4$^+$ T cells over CD8$^+$ cells has been documented by several groups (Seki et al., 1995; Wilkins et al., 2002; Schmitz et al., 2003). When T lymphocyte subsets were analyzed in more detail, CD4-positive and CD45RA-negative cells were more prone to apoptosis than their CD8-positive and CD45RA-positive counterparts (Philippe et al., 1997). Riggs et al. (1988) showed that, in irradiated mice, splenic B cells with the sIgM$^{++}$/sIgDdim surface phenotype are more radiosensitive than B cells bearing much less sIgM than IgD on the surface. An analogous observation was made in irradiated rats – at doses equal to or higher than 3.5 Gy the non-circulating marginal zone B cell population appeared more susceptible than circulating cells from the follicular compartment (Bazin and Platteau, 1986; Bazin et al., 1986).

We have recently reported on the existence of several subsets of human peripheral blood lymphocytes possessing a promising biodosimetric potential *in vitro*. The CD8$^+$ NK cell population has proven to be a sensitive and reliable biosimeter in the dose range of 3–20 Gy. Cultivation of *in vitro*-irradiated human peripheral blood mononuclear cells (PBMC) for 16 hr revealed that doses above 5 Gy induce an approximately 50% reduction in CD8$^+$ NK cells when compared to the control samples and this NK subset can apparently be used as a low versus high dose discriminator, at least under *in vitro* conditions. In addition, 48 h cultivation allowed the study of low dosage irradiation using the CD8$^+$ NK cell subset (Vokurkova et al., 2006). Within the B cell compartment of human peripheral blood lymphocytes the CD27$^+$ and CD21$^-$ subpopulations have proven to be sensitive and reliable enough for biodosimetric purposes in the dose range of 0–6 Gy (Rehakova et al., 2008). Importantly, this conclusion is valid not only for PBMC preparations but applies to full blood samples, too, which is more important from the practical point of view and also better resembles the naturally occurring situation. We have also identified several B cell subsets differing in CD21, CD27 and CD38 expression the biodosimetric potential of which is still under investigation.

Experimental models currently used in biodosimetry have several limitations and suffer from a major drawback, which is the missing link between *in vitro* experiments and *in vivo* observations. Since peripheral blood is the most convenient material for retrospective dose estimates in accidentally irradiated individuals, laboratory rodents do not represent an ideal approach as repeated blood collection is difficult to perform in these small animals and other tissues and cells like spleen and splenocytes have been mostly studied, which is not convenient for biodosimetry in humans for practical and ethical reasons. *In vitro* irradiation of human blood or blood-born cells have provided useful information on the biodosimetric potential of plasma and peripheral leukocytes but the *in vitro* observations need not parallel the situation *in vivo* especially due to the absence of circulation and material exchange between the blood and the rest of the body. *In vivo* observations in normal human population can be made after irradiation accidents only, and are therefore random and scarce and blood elements from therapeutically irradiated patients can barely be considered a representative sample. It is thus apparent that an additional approach using a large animal model that would provide a tool for direct *in vivo* and *in vitro* comparison is very desirable. Pigs

may represent a good choice for this due to their availability, cost effectiveness and the existence of a relatively large portfolio of specific research tools like immunoreagents and other molecular probes. In our attempt to introduce swine as an experimental model in radiobiology we have compared the radiosensitivity within the B cell compartment in the circulation of whole body irradiated piglets and in peripheral blood preparations irradiated *in vitro*.

## MATERIAL AND METHODS

### Animals, irradiation and sample collection

16 clinically healthy, 28-day-old Large White piglets were purchased from a private owner. All experimental procedures with the animals were made under agreement of the Ethical Commission of the Faculty of Military Health Sciences, Hradec Kralove and of the Commission for Animal Welfare of the Ministry of Defence of the Czech Republic. Before whole-body irradiation, 10 ml of venous peripheral blood was collected from the neck region (*vena carotida externa*) into a heparin-containing (final concentration of 20 IU/ml) syringe; 1 ml was used as a reference sample and the rest was dispensed into 24-well polystyrene cultivation plates (Falcon, Fisher Scientific, Ottawa, Ontario) in 1 ml aliquots for the *in vitro* part of the study. One plate was sham treated (non-irradiated control *in vitro* samples); the other plates were individually exposed to a selected dose (2, 4, 6 or 10 Gy) of $^{60}$Co γ radiation for five (2 Gy) to twenty-five (10 Gy) min. The plates were subsequently transferred into a humidified incubator and samples from individual wells were harvested 8, 24 and 48 h after irradiation. For each selected dose (2, 4, 6 and 10 Gy) of whole-body $^{60}$Co γ irradiation, four piglets under intramuscularly-led anesthesia (Narkamon 5% a.u.v., Rometar 2% a.u.v., 1 ml per 10 kg each; Spofa, Prague, Czech Republic) were used, *in vivo* samples were collected 1, 8, 24 and 48 h after the beginning of irradiation and immediately processed for flow cytometric (FCM) analysis.

Peripheral blood leukocytes (PBL) were isolated both from freshly collected and *in vitro* cultivated blood samples with the EasyLyse™ (Dako, Glostrup, Denmark) lysing solution according to the manufacturer instructions. PBL were washed once in phosphate-buffered saline (PBS), once more in the

Washing and Staining Buffer (WSB, PBS containing 0.2% gelatin from cold water fish skin and 0.1% sodium azide, all reagents from Sigma-Aldrich, Brooklyn, NY), resuspended in WSB, counted and the suspension density was set to $2–5 \times 10^6$/ml. All procedures after erythrocyte lysis were performed in an ice bath or a refrigerator in the presence of 0.1% sodium azide to prevent apoptosis progression before data acquisition.

### Lymphocyte immunophenotyping and data acquisition

Two-colour indirect immunophenotyping of pig PBL suspensions was used as described elsewhere (Sinkora et al., 1998). Briefly, after incubating the cells with a pair of isotype/subisotype mismatched mouse anti-pig monoclonal antibodies (MoAb) the cells were washed, Fc-receptors were blocked with PBS containing 10% heat inactivated goat non-immune serum and binding of primary MoAb was visualized by an appropriate cocktail of isotype/subisotype specific goat anti-mouse polyclonal antisera conjugated to FITC and RPE (Southern Biotechnology Associates, Inc., Birminghan, AL). Cells were washed again, propidium iodide (PI, Sigma-Aldrich, Brooklyn, NY) was added to a final concentration of 5 μg/ml and the samples were then subjected to FCM analysis. The following mouse anti-pig monoclonal antibodies were used: anti-IgA (27.7.16b), anti-IgG (27.9.1), anti-IgM (clone 28.4.1, generous gift from R. Zwart, Lelystad), anti-CD1 (IgG2a, 76-7-4, IgG2a, SBA), anti-CD2 (MSA4, generous gift from J. Lunney, Beltsville), anti-CD21 (clone BB6-11C9, IgG1, SBA) and anti-Swine Workshop Cluster 7 (SWC-7, clone 2F6/8, generous gift from J. Dominguez, Madrid) in the following cocktails: anti-IgM/anti-CD2, anti-CD21/anti-SWC7 and anti-CD21/anti-CD1. Anti-pig isotype specific MoAb were used for surface Ig visualization and subsequent counterstaining with the APC-conjugated anti-human CD79α MoAb HM57 cross-reacting with the pig CD79α orthologue (Jones et al., 1993; Faldyna et al., 2007) in samples fixed and permeabilized by Intrastain (Dako, Glostrup, Denmark) according to the manufacturer's instructions.

FCM data were acquired and analyzed on the CyAn ADP™ flow cytometric analyzer (Beckman Coulter) using the Summit™ 4.3 acquisition/analysis software. Based on excitation light scattering

and PI fluorescence a population of small intact lymphoid cells was identified as PI⁻ leukocytes with low forward and side scatter characteristics and a region tightly-fitting this population was designed as described elsewhere (Rehakova et al., 2008) to exclude lymphocytes in intermediate-to-late stages of apoptosis from analysis (Vokurkova et al., 2006). Detector voltages were optimized according to the background signal from control samples stained with secondary antisera only. FITC and RPE emission spectra overlap was compensated using single stained samples and the automatic compensation utility in Summit 4.3. For each MoAb combination and every single sample at least 20 000 small intact lymphocytes were included in the analysis and the relative proportions of single and double positive cell subpopulations and subsets were recorded using quadrant statistics.

## Data analysis

To evaluate relative radiosensitivity of lymphocyte subpopulations and subsets we have recently invented a dose- and time-dependent variable called the Irradiated Versus Non-Irradiated Ratio (IVNIR; Rehakova et al., 2008), which is calculated by dividing the relative number of cells with the selected immunophenotype in an irradiated sample by its respective value in its non-irradiated control. We have defined porcine B cell subpopulations using one of the traditional B-lineage restricted markers (sIgM or CD21); their subsets were characterized by the co-expression of another B-lineage restricted (SWC7) or promiscuous (CD1, CD2) surface markers. Therefore, IVNIR values for peripheral blood sIgM⁺ B cell subpopulations *in vivo* were obtained as ratios of the sIgM⁺ lymphocyte percentage in samples collected after irradiation and the percentage of the corresponding subpopulation among circulating lymphocytes before the treatment. For subset analysis, the proportion of a subset (e.g. CD21⁺CD1⁺ lymphocytes) within the parental subpopulation (CD21⁺ B cells in this case) was the number used for IVNIR calculation. In cultivated samples, two approaches to IVNIR calculation are possible. The relative number of a selected B lymphocyte subpopulation or subset in the irradiated sample can be divided by either its corresponding number at the beginning of cultivation or the proportion in the non-irradiated control sample cultivated for the same time period. To be able to

compare *in vivo* and *in vitro* data shown in this paper with as yet unpublished *ex vivo* experiments (*in vivo* irradiation followed by *in vitro* cultivation) where no relevant non-irradiated control is available, we have decided to use the former approach to IVNIR calculation. Spontaneous apoptosis in non-irradiated *in vitro* samples was then quantified similarly to IVNIR calculation: the percentage of the studied population in the cultivated sample was divided by the corresponding number at the beginning of cultivation. We are aware of the fact that the acronym IVNIR is inaccurate in this case but the resulting number reflecting the capability of spontaneous survival of the selected population *in vitro* is essential for radioresistance data interpretation. IVNIR generally provides radiobiological data that are quite insensitive to intra-group differences in lymphocyte compartment composition, which is a good basis for reasonable statistical analysis. In conclusion, for both of the experimental approaches (*in vivo* and *in vitro*), each irradiation dose and time interval and every single B cell population defined by surface phenotype, IVNIR was calculated in the first step. Subsequently, mean values and standard deviations were calculated for IVNIR data from individual piglets within the experimental group and the results were graphically demonstrated. Generally, IVNIR values above 1.0 report on the radioresistance, which is higher than the average within the parental population while IVNIR below one correspond to more radiosensitive lymphocyte subpopulations and subsets. *In vitro*, the results from irradiated samples must be correlated with the "IVNIR" of non-irradiated samples reflecting the time course of spontaneous apoptosis.

## RESULTS

### Irradiation induced lymphocyte depletion *in vivo* and *in vitro*

Absolute counts of lymphocytes were determined in all samples by CytoCount™ absolute counting beads (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The lymphoid population was identified by excitation light scattering and surface marker expression and only small intact lymphocytes were counted as survivors and included in analysis.

*In vivo*, whole-body exposure to the lowest dose (2 Gy) resulted in a small increase in circulating

lymphocytes 1 h after irradiation, which was followed by a decrease to 10% of the original (before irradiation) values 8, 24 and 48 h after irradiation (Zarybnicka et al., in preparation). At higher doses (above 4 Gy), an immediate drop in circulating lymphocyte counts was observed already 1 h after irradiation (down to 70% in 4 or 6 Gy irradiated animals and 30% after irradiation with 10 Gy), which was followed by further reduction in circulating lymphocytes to about 1/5 and below 1/10 of the original numbers in 4 Gy irradiated piglets and the piglets treated with lethal doses above 6 Gy, respectively, 7 h later. Importantly, the lymphocyte count reduction recorded between hour 1 and 8 represented the most dramatic decrease during the observation period *in vivo*. 24 and 48 h after irradiation only statistically insignificant changes in absolute lymphocyte numbers occurred when compared to the situation after the most pronounced decrease during the first 8 h.

The decrease of lymphocyte numbers in *in vitro* irradiated samples did not closely parallel the situation *in vivo*. 1 h after irradiation, no changes in absolute lymphocyte counts were recorded. 7 h later, a decrease to 50% of the original values were observed in all irradiated samples in the whole range of doses (2–10 Gy). Importantly, a significant reduction (down to 70%) in absolute lymphocyte counts was also observed in the non-irradiated controls. One and two days after irradiation, the

absolute counts of intact lymphocytes dropped down to 5% and 1%, respectively and the effect was dose-independent. In comparison, the absolute lymphocyte counts in non-irradiated controls decreased to 30% and 10% of the original values during 24 h and 48 h incubation, respectively. Taken together, absolute lymphocyte counts were good enough to distinguish between irradiated and non-irradiated samples both *in vivo* and *in vitro* 8 h after irradiation and later. However, the only significant dose-dependent differences could be observed *in vivo* 1 h after irradiation. In conclusion, absolute lymphocyte numbers cannot be used as a biodosimetric indicator in pigs.

## Radiosensitivity of circulating sIgM⁺ B cells

B cells in pigs have traditionally been visualized using anti-immunoglobulin antisera or MoAb due partially to the lack of immunoreagents directed against true B-lineage markers like CD45R (B220) in mice or CD19 and CD20 in humans. Immunodetection of plasma membrane-anchored immunoglobulin as an integral part of the clonally distributed B cell receptor by pig Ig isotype-specific MoAb has revealed that more than 95% of all circulating porcine B cells are positive for surface IgM (sIgM⁺) while approximately only one out of seven peripheral blood B cells bear IgA or IgG on their
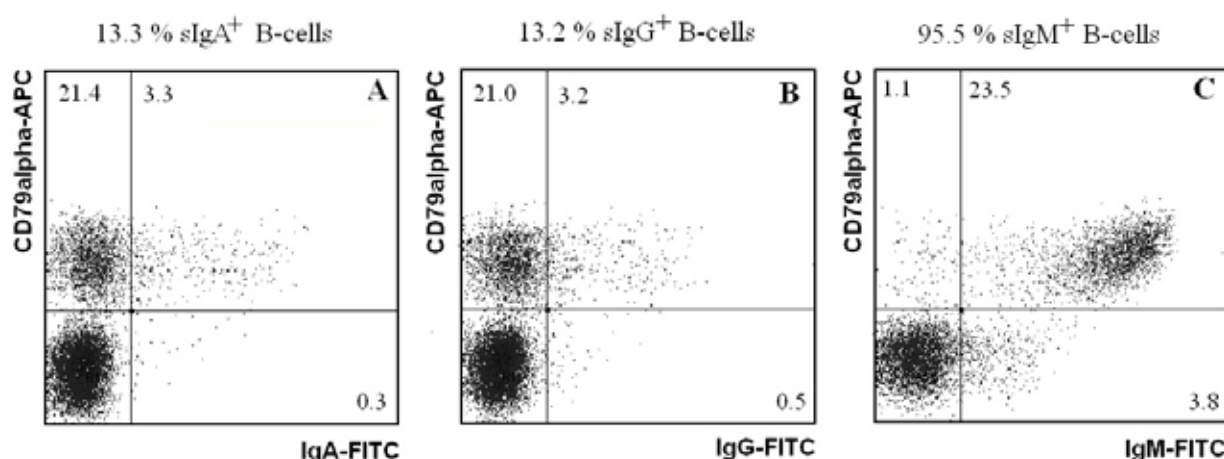


Figure 1. Surface IgA (A), IgG (B) and IgM (C) expression on peripheral blood lymphocytes in a 6-week-old piglet. Surface binding of pig Ig isotype-specific MoAb was visualized using FITC-conjugated goat anti-mouse IgG1 polyclonal antiserum, which was followed by intracellular staining with APC-conjugated anti-CD79α MoAb. A typical expression profile with more than 95% of sIgM⁺ and between 10 and 15% of both sIgA⁺ and sIgG⁺ B-cells is shown; a significant proportion of circulating B cells thus bear at least two Ig isotypes on their surface. CD79α-negative events weakly positive for one of the Ig isotypes studied (lower right quadrants in the dot plots) are lymphocytes with autoreactive antibodies or Fc-receptor-bound Ig on their surface
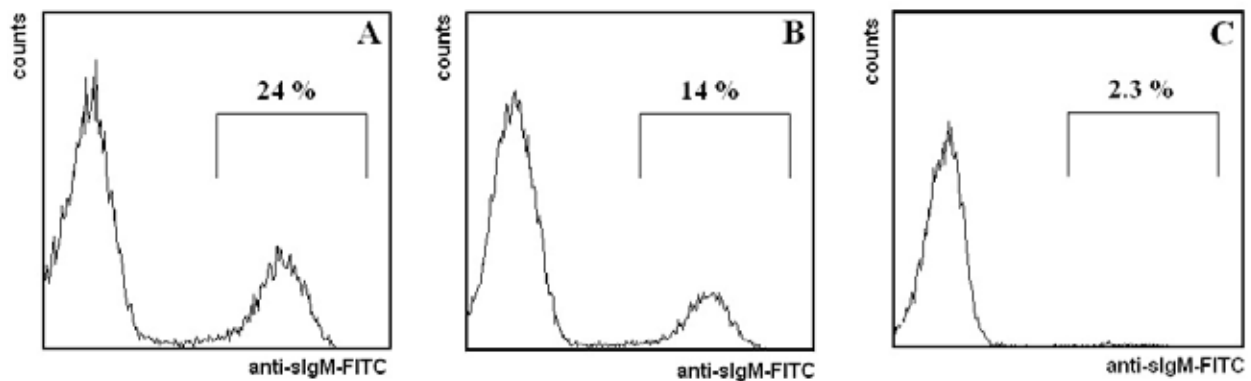
Figure 2. Surface IgM expression on lymphocytes in a PBL preparation from a piglet either before (A) or one and eight hours after (B and C, respectively) whole body irradiation with the dose of 6 Gy. The histogram region has been set to include only sIgM$^{hi+}$ events into statistics

surface (Figure 1). We have thus chosen anti-IgM MoAb as a convenient tool for studying the overall radioresistance of porcine peripheral blood B cells in pigs both *in vivo* and *in vitro*. As low staining intensity of CD79α-negative non-B cell lymphocytes with anti-IgM MoAb occurs due to naturally occurring autoreactive antibodies and/or Fc-receptor mediated binding (lower right quadrant in Figure 1C), we have decided to neglect B lymphocytes with low sIgM expression and only the population with high sIgM (sIgM$^{hi+}$) expression involving the vast majority of all circulating B cells (Figure 1C) have been included in the analysis.

Figure 2 shows an example of a decrease with time of circulating IgM$^+$ B cells in a piglet exposed to whole body irradiation with a lethal dose of 6 Gy. In this case, the relative number of circu-

lating lymphocytes with high expression of sIgM decreased nearly to one half of the original value one hour after the beginning of irradiation. Seven hours later, only a few sIgM$^{hi}$ lymphocytes could be found in the peripheral blood; less than 3% of events with the light scatter characteristics of lymphoid cells possessed the high surface density of sIgM. When the total reduction of lymphocytes is taken into account (in 6 Gy irradiated animals down to 60% and 10% of the original absolute numbers one and eight hours after irradiation, respectively) it becomes apparent that, similar to other species, porcine lymphocytes represent a relatively radiosensitive leukocyte population with B cells being one of the most radiosensitive leukocyte subpopulations of all (Anderson et al., 1977; Bazin and Platteau, 1986).
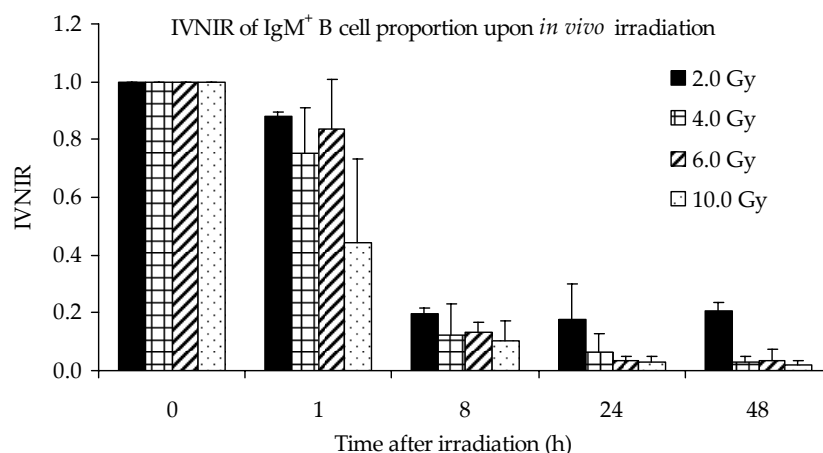


Figure 3. IVNIR representation of relative sIgM$^{hi+}$ B cell numbers within the lymphoid population in PBL isolated from pigs after whole-body irradiation with the doses of 2, 4, 6 and 10 Gy (black, checked, hatched and dotted bars, respectively) at selected time intervals after treatment. Mean values and standard deviations for four animals per group are given
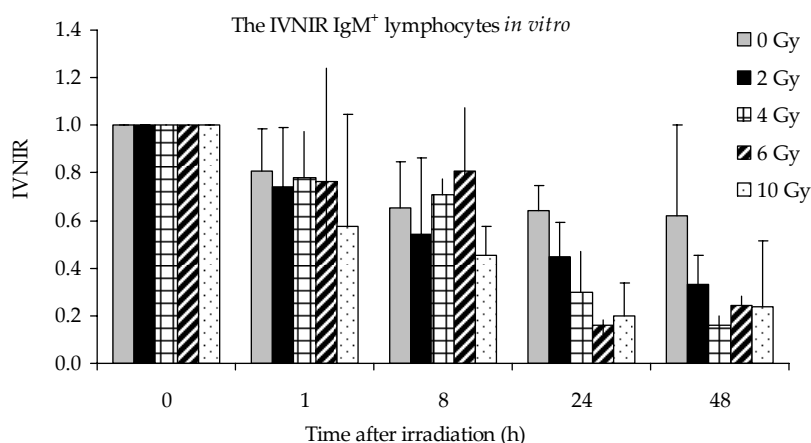
Figure 4. IVNIR representation of relative sIgM$^{hi+}$ B cell numbers within the lymphoid population in PBL isolated from pig full blood irradiated *in vitro* with the doses of 2, 4, 6 and 10 Gy (black, checked, hatched and dotted bars, respectively) at selected time intervals after treatment. The non-irradiated controls are shown by grey bars. Mean values and standard deviations of four blood samples from different pigs are given

Figure 3 shows the time course of circulating sIgM$^+$ B cell disappearance in pigs exposed to different doses in IVNIR representation. These *in vivo* data clearly show that a decrease in the relative numbers of sIgM$^+$ lymphocytes can be observed as early as 1 h after the beginning of irradiation; even at the lowest dose used, when a transient increase of absolute lymphocyte numbers is typical, a decrease in B cell proportion is apparent. Irradiation with doses equal to or below 6 Gy resulted in minor (about 20%) reductions in sIgM$^+$ B cell proportions among circulating lymphocytes; 10 Gy whole body irradiation caused a more pronounced decrease in sIgM$^+$ B cell IVNIR down to the average value of 0.5 with a relatively large variation in the experimental data. Seven hours later, B cell IVNIR was reduced to values below 0.2 and the relative B cell numbers continued to decrease with time with the exception of the lowest dose used; in 2 Gy irradiated piglets a plateau (IVNIR ~ 0.2) was reached 8 after irradiation.

*In vitro* experiments (Figure 4) show a different profile. Similar to the situation *in vivo*, a small decrease of relative sIgM$^{hi+}$ cell numbers can be observed 1 h after irradiation. However, the data variation is large and the differences are not significant. 8 h after irradiation a trend of a decreasing B cell proportion among lymphocytes was apparent. Once again, unsatisfactory data variation does not allow discrimination between samples irradiated with different doses and the non-irradiated control. The most interesting situation was in *in vitro* cultures one day after irradiation when a dose-

dependence of the relative B cell reduction became clear and it was possible to discriminate between samples treated with low (< 4 Gy) and high (> 6 Gy) doses of gamma rays. Culturing for another day did nothing to improve data quality and longer cultures do not appear to be convenient for B cell radiosensitivity studies in pigs. In general, our *in vivo* and *ex vivo* experiments show that, similarly to other species, porcine circulating B cells represent the most radiosensitive lymphocyte populations and it is thus worth attempting to identify relatively homogenous B cell subpopulations and subsets with radiobiological potential.

CD2$^+$ and CD2$^-$ subsets of porcine sIgM$^+$ lymphocytes belong to best characterized B cell subsets in pigs. The CD2$^-$ population of B lymphocytes is essentially absent in pig fetuses and germ-free piglets (Sinkora et al., 1998) and immunophenotyping of cells derived from mucosa-associated lymphatic tissue has recently revealed that essentially all Peyer's patch CD79α$^+$ lymphocytes in young piglets are CD2$^-$ (Sinkorova et al., in preparation). The recirculating sIgM$^+$CD2$^-$ lymphocytes may thus represent a functionally separated mucosa-associated B cell pool recirculating from inductive (e.g. Peyer's patches) to effector (e.g. intestinal *lamina propria*) sites. We have thus tested the hypothesis that the two potentially independent B cell pools, sIgM$^+$CD2$^+$ and sIgM$^+$CD2$^-$ lymphocytes, possess different radiosensitivity. Figure 5 shows that highly variable data have been obtained from studies on the two B cell subsets both *in vivo* and *in vitro*. In
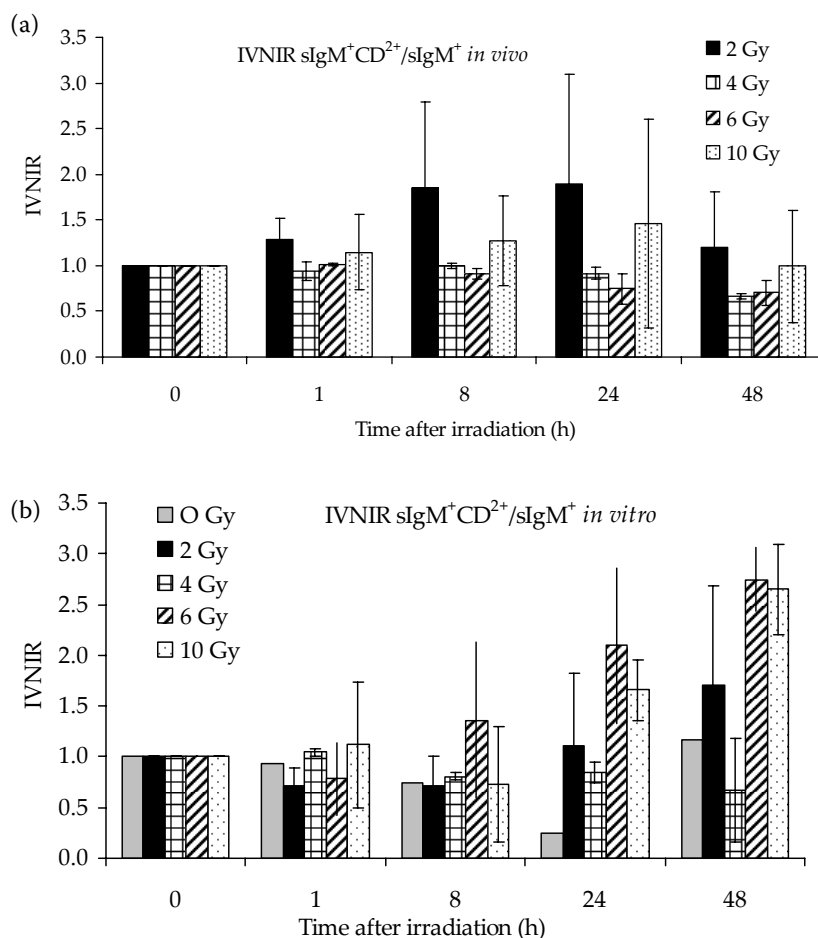
Figure 5. In PBL isolated from full blood samples collected at selected time intervals either after whole body irradiation (*in vivo* experiments, (**a**) or after *in vitro* irradiation (**b**), the proportion of the CD2^{+} subset within the sIgM[hi+] B cell subpopulation was calculated by dividing the percentage of sIgM[hi+]CD2^{+} events by the percentage of sIgM[hi+] small intact lymphocytes. IVNIR for such sIgM[hi+]CD2^{+}/sIgM[hi+] ratio was calculated for doses of 2, 4, 6 and 10 Gy (black, checked, hatched and dotted bars, respectively). IVNIR of non-irradiated *in vitro* controls is shown by grey bars in B. Mean values and standard deviations of four blood samples from different pigs are shown

contrast to the *in vivo* data where no time- or dose-dependence could be found, a statistically significant increase in CD2^{+} B cells within the sIgM^{+} B cell subpopulation can be observed at higher (8 and 10 Gy) doses 24 and 48 h after irradiation. However, as quite a few B cells could be found in *in vitro* cultures 24 and 48 h after irradiation, especially within the range of high doses, such results are not reliable enough to make any conclusions on the differential radiosensitivity of CD2^{+} and CD2^{−} B cell subsets.

## Radiosensitivity of CD21^{+} B cell subsets

CD21^{+} lymphocytes represent a prominent population of circulating B cells in pigs. Like in other species, immature recent bone marrow emigrants are CD21-negative or weakly CD21-positive (Sinkorova et al., in preparation) while the majority of circulating B cells bear much higher amounts of CD21 on their surface. Using a panel of MoAb directed against lymphocyte markers in pigs we have recently revealed the heterogeneity of the B cell compartment in pigs (Sinkorova et al., in preparation). Based on the overall high radiosensitivity of the B cell compartment we have tested the assumption that the relatively large and clearly visible SWC7^{+} and CD1^{+} subsets of the CD21^{+} B cell subpopulation can be used as biodosimetric candidates in terms of the received dose estimate in pigs. We have chosen the sublethal dose of 2 Gy and studied the time dependence of IVNIR in the
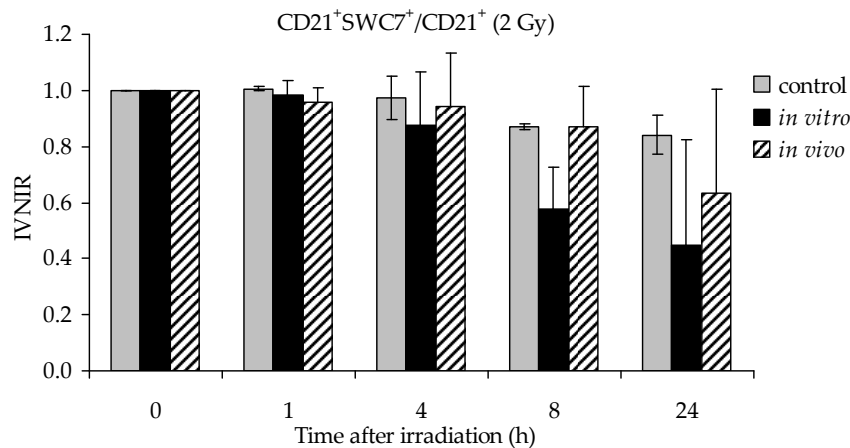
Figure 6. IVNIR time course of the CD21+SWC7+/CD21+ ratio (calculated from the proportions of CD21+SWC7+ and CD21+ lymphocytes) either in blood circulation in pigs treated according to the 2 Gy whole body irradiation protocol (hatched bars) or peripheral blood irradiated with 2 Gy *in vitro* (black bars). Non-irradiated *in vitro* controls are shown in grey. Samples from four different pigs were used

two selected B cell subsets within the time interval of two days after irradiation. Figure 6 shows *in vivo* as well as *in vitro* results including those obtained by CD21+SWC7+ subset analysis. While a small and insignificant decrease in the SWC7+ B cell subset within the CD21+ subpopulation occurs 1 and 4 h after irradiation *in vitro*, a lower relative size of the SWC7+ subset is clearly visible in cultures 8 h after the beginning of irradiation. One day after irradiation the variance of the experimental data becomes large and the difference between CD21+SWC7+ and CD21+SWC7− subset raidosensititvity was no longer significant, due probably to the small num-

bers error. The situation *in vivo* is less clear as the IVNIR of the CD21+SWC7+ subset did not change significantly during the observation period.

Among the B cell immunophenotypes studied the CD1+ subset of CD21+ lymphocytes represents the most promising B cell population from the radio-biological point of view. Figure 7 shows the *in vivo* (hatched bars) and *in vitro* (black bars) results for CD21+CD1+/CD21+ ratio in IVNIR representation. 2 Gy-induced significant differences between the relative proportions of the CD1+ and CD1− subsets of CD21+ B cells were observed as early as 4 hr after irradiation *in vitro* and this higher radiosensitivity
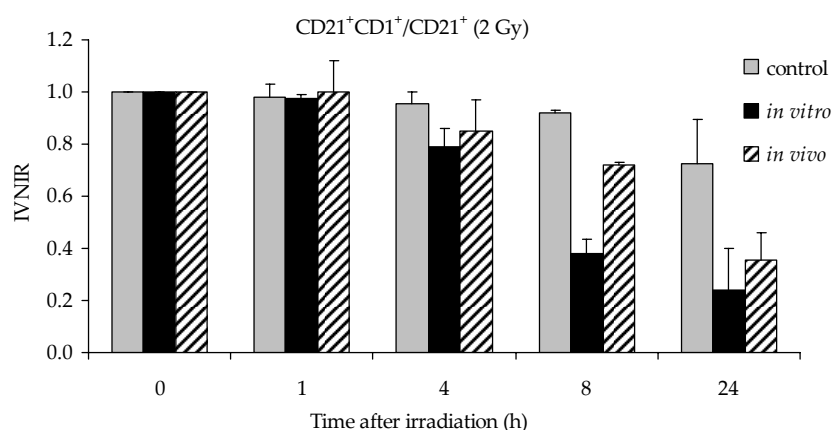


Figure 7. IVNIR time course of the CD21+CD1+/CD21+ ratio (calculated from the proportions of CD21+CD1+ and CD21+ lymphocytes) either in blood circulation in pigs treated according to the 2 Gy whole body irradiation protocol (hatched bars) or peripheral blood irradiated with 2 Gy *in vitro* (black bars). Non-irradiated *in vitro* controls are shown in grey. Samples from four different pigs were used

of CD21$^+$CD1$^+$ B cells was even more pronounced 4 h later when the CD21$^+$CD1$^+$/CD21$^+$ IVNIR dropped below 0.5. One day after irradiation the situation is similar although the data suffer from a low number of total B cells in the sample. The observations *in vivo* parallel the *in vitro* experiments. Although 4 h after 2 Gy irradiation the difference between the sample and its control collected before irradiation is minor, 8 h after irradiation the IVNIR of the CD21$^+$CD1$^+$/CD21$^+$ ratio decreased to 0.7 and the low variation of the data set makes the results highly significant. Another reduction in the CD1$^+$ subset among CD21$^+$ B cells could be observed 1 day after irradiation when the IVNIR of the CD21$^+$CD1$^+$/CD21$^+$ ratio decreases and reaches the value of 0.5 that can be observed in *in vitro* samples from 8 h of cultivation onwards.

## DISCUSSION

We and others have used different approaches in attempting to identify highly sought after biodosimetric indicators capable of reporting on the level of irradiation damage in victims of nuclear attacks and individuals accidentally exposed to ionizing radiation. Leukocytes and especially lymphocytes as relatively radiosensitive and easily accessible somatic cells have traditionally been in the spotlight and many labs have investigated their biodosimetric potential. Although some skepticism regarding the ability of lymphocyte subpopulations and subsets to report on the received dose exists due mainly to, in some regard, controversial results published by different groups, we have recently identified several candidate subsets of human peripheral blood lymphocytes (CD8$^+$ NK cells, CD21$^-$ and CD27$^+$ B cells) with promising biodosimetric potential *in vitro*. These *in vitro* observations, however, are almost impossible to recapitulate *in vivo* and we have thus been seeking a new approach that would optimally mimic the situation in the human population.

In this study we present the first experimental data from pigs, a newly-introduced large animal model in biodosimetry. Similarly to other species, porcine lymphocytes have proven a highly radiosensitive population and a profound decrease in absolute counts within the lymphoid compartment has been clearly demonstrated. As expected, the kinetics of lymphocyte depletion *in vitro* did not parallel the situation *in vivo*. Several mechanisms may contribute to the observed differences. Namely

the existence of very efficient scavenger mechanisms within the body can explain the much faster removal of cells within the very early stages of radiation-induced apoptosis than in cell cultures where limited amounts of phagocytes are present, and where conditions are unfavorable. On the other hand, radiation-induced recruitment of tissue-borne lymphocyte populations and cell replenishing from a relatively radioresistant precursor pool could be behind the higher absolute counts of circulating lymphocytes 24 and 48 h after irradiation when compared to the corresponding *in vitro* cultures. Our results show that lymphocyte numbers are negatively affected by exposure to gamma (γ) rays and the received dose can be estimated very early after irradiation *in vivo* (Zarybnicka et al., in preparation). However, it is questionable whether or not absolute counts can have any practical biodosimetric relevance in pigs due to the fact that already 8 h after irradiation no dose dependence could be observed. From the biodosimetric point of view the situation *in vitro* was even less encouraging. Although irradiated samples could be distinguished from non-irradiated controls, no dose dependence in absolute lymphocyte numbers has been observed and the increased apoptosis due to irradiation was the only conclusion we could make without subpopulation analysis.

Not surprisingly, the peripheral B cell compartment in pigs has proved highly radiosensitive both *in vivo* and *in vitro* and B lymphocytes apparently represent the most radiosensitive lymphoid population. The fast disappearance of sIgM$^{hi}$ cells from the circulation of irradiated piglets and their relative decrease among lymphocytes in samples exposed to γ rays *in vitro* strongly suggest that B cells are the most radiosensitive lymphocyte population; our as yet unpublished data on T lymphocytes and NK cells confirm this observation. Despite a transient increase in total lymphocyte numbers in piglets irradiated with a sublethal dose of 2 Gy the relative proportion of sIgM$^{hi}$ cells begun decreasing as soon as the first *in vivo* samples were analyzed and this process progressed rapidly. Unfortunately IVNIR variation within the experimental group suggests that total B cell number dynamics in irradiated piglets has a low significance in terms of dose-dependence. We can only conclude that piglets subjected to 10 Gy irradiation can be identified 1 h after irradiation by a more dramatic decrease in sIgM$^{hi}$ cells in circulation when compared to their counterparts irradiated with doses of 6 Gy or

lower but this is not sufficient even for lethal versus sublethal dose discrimination. More importantly, 48 h after irradiation the sublethal dose of 2 Gy has differentiated from low-to-high lethal doses (above 4 Gy) by the persistence of significant numbers of sIgM$^{hi}$ cells in circulation. Such a finding can prove important in selecting B cells as a long-term indicator of received dose sublethality in our *in vivo* experimental model with some implications for the human system. *In vitro* results provided unsatisfactory results. Spontaneous apoptosis in full blood B cell compartment was huge and irradiation with lower doses did not significantly increase its extent. It is worth mentioning that data variation in non-irradiated controls was quite high and we have to take into account the possibility that also factors other than irradiation, such as anesthesia, stress or the collection procedure played a role in this phenomenon. In addition, the differences between non-irradiated and high-dose-irradiated samples were minor. In consequence, the bulk B cell population does not appear worth studying in *in vitro* irradiated cultures.

The best characterized porcine B cell subpopulations – CD2$^+$ and CD2$^-$ B cells – were not shown here to differ significantly in their radiosensitivity both under *in vivo* and *in vitro* conditions due mostly to the high IVNIR variation within experimental groups (although a radioresistant subset of sIgM$^{++}$CD2$^+$ appears to selectively survive higher irradiation doses, see the sIgM$^{++}$CD2$^+$/sIgM$^{++}$ IVNIR in Figure 5B in 6 and 10 Gy-irradiated samples 24 and 48 h after irradiation). However, in contrast to this some interesting results from the biodosimetric point of view were found within the CD21$^+$ B cell subsets. A higher radiosensitivity of the CD21$^+$SWC7$^+$ subset than CD21$^+$SWC7$^-$ B cells was observed after 8 h cultivation of *in vitro* irradiated samples. This finding was not confirmed *in vivo* further demonstrating that simple extrapolation of *in vitro* data to the *in vivo* situation is inadequate. Most importantly, the analysis of the CD21$^+$ lymphoid compartment in the peripheral blood has revealed that at least one subset of porcine B cells are worth studying as a biodosimetric indicator candidate. As the lowest γ ray dose used in our study (2 Gy) induced the significant decrease in the CD1$^+$ subset size among the CD21$^+$ B cells *in vitro* as early as 4 h after irradiation and this process progressed within the following 4 h we have concluded that the CD21$^+$CD1$^+$ B lymphocytes may represent the population of interest for received dose estimate in pigs, specifically within the suble-

thal range. Interestingly, this *in vitro* observation was accompanied by a similar *in vivo* effect where a higher radiosensitivity of CD21$^+$CD1$^+$ B cells over their CD21$^+$CD1$^-$ counterparts was reflected by a decrease in the IVNIR of the CD21$^+$CD1$^+$/CD21$^+$ ratio to 0.7 and below 0.4 eight hours and one day after whole body irradiation with 2 Gy, respectively.

Taken together, we have identified pig B cells as the most radiosensitive peripheral blood lymphocyte population in pigs, which marks them as a good candidate for short time and low dose biodosimetric studies. Among several B cell subsets studied we have identified highly radiosensitive cells with the CD21$^+$CD1$^+$ surface phenotype with promising potential as received dose indicators in this newly introduced large animal model. Together with our as yet unpublished data on T and NK cell subpopulations and subset radiosensitivity we conclude that, similarly to humans, the peripheral blood lymphocytes in pigs represent a promising compartment for biodosimetric studies including the comparison of the situation *in vivo* and *in vitro* so frequently missing in other experimental approaches.

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