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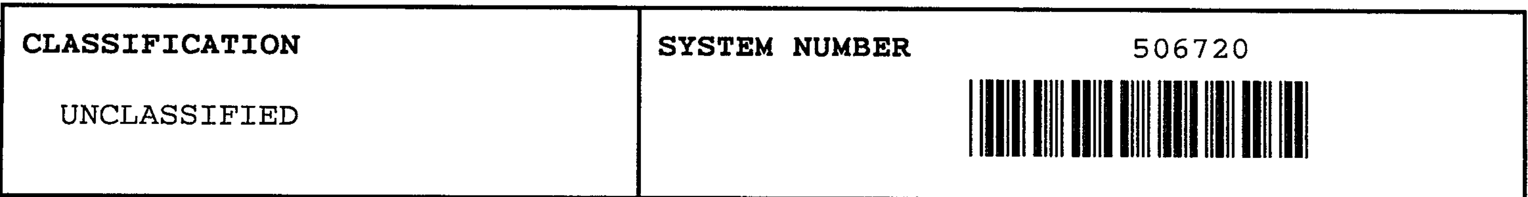

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TITLE

RADIATION DAMAGE AND IMMUNE SUPPRESSION IN SPELNIC MONONUCLEAR CELL POPULATIONS

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Radiation damage and immune suppression in splenic mononuclear cell populations

N. P. HARRINGTON*, K. A. CHAMBERS‡, W. M. ROSS*† & L. G. FILION‡ **Department of Physiology, University of Ottawa, †Defence Research Establishment, Ottawa, and ‡Department of Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada*

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SUMMARY

We have examined alterations in all of the major splenic mononuclear cell (SMNC) populations in C57Bl/6 mice following whole-body irradiation (0–700 cGy) in order to determine which populations may play a role in active immune suppression and/or haematopoietic recovery. A protocol has been established for characterization and differentiation by flow cytometric analysis (FCA) of the major MNC populations in the mouse spleen: T lymphocytes ($CD4^+$ and $CD8^+$ cells), B lymphocytes, natural killer (NK) cells, and monocytes/macrophages. Ionizing radiation caused decreased spleen cellularity and decreased ability of surviving SMNC to respond to mitogen. FCA revealed alterations in the relative composition of the constituent splenic cell populations following irradiation, reflecting differential radiosensitivity, with selective enrichment of NK cells (seven-fold) and $CD4^+$ T lymphocytes (three-fold). Enrichment developed during the 7-day post-irradiation period. In addition, some MNC became activated in a dose- and time-dependent fashion following whole-body irradiation, as indicated by expression of CD71, the transferrin receptor. These cells were $CD34^+$ and Thy1.2$^+$, but were $CD4^-$ or $CD8^-$as well as $CD45^-$ (B cell). The observed increase in NK cells corresponds with a previously reported increase in natural suppressor (NS) cells following total-lymphoid irradiation (TLI). The balance of recovery-inhibiting NK cells and recovery-enhancing $CD4^+$ T lymphocytes following irradiation may reflect or influence the degree of haematopoietic recovery, and may provide an indication of the extent of damage (biological dosimetry).

Keywords flow cytometry radiosensitivity immune suppression whole-body irradiation natural killer cells

INTRODUCTION

Exposure to ionizing radiation can render the immune system incompetent, resulting in susceptibility to opportunistic infection. In addition to cytoreduction of the lymphoid system, ionizing radiation has been demonstrated to cause active suppression through the induction of suppressor cells which non-specifically suppress lymphocyte proliferative responses [1,2]. Such cells have been termed natural suppressor (NS) cells, and were initially characterized in neonatal rodents but have since been reported among cells derived from spleen following total-lymphoid irradiation (TLI) [3,4], cyclophosphamide administration [5], chronic graft-*versus*-host disease (GVHD) [6], and tumour growth [7]. Lower levels of NS activity have also been observed in normal bone marrow [8]. NS cells have been shown to secrete soluble suppressor factors which block IL-2 synthesis and inhibit IL-2-dependent T cell proliferation in the presence of excess IL-2 [9,10]. The identity and mechanism of activation of cells expressing NS activity, however, are not clear. Various experimental models have suggested that non-specific suppression may be mediated by T lymphocytes [11], non-T lymphocytes [3], and cells of the monocytic lineage [12].

While NS cells may increase susceptibility to opportunistic infection, the ultimate survival of an animal following irradiation depends largely on the recovery of haematopoiesis. The haematopoietic capacity of the bone marrow, the organized lymphoid tissues, and individual small recirculating lymphocytes are all exquisitely radiosensitive. Since haematopoiesis provides for renewal of mature circulating blood cells, those cells killed by irradiation or used up performing their functions will not be replaced until haematopoietic recovery occurs. Haematopoietic recovery requires a sufficient number of surviving endogenous stem cells to proliferate, restore the stem cell pool, and provide specific progenitor cells which will differentiate into functionally mature cells. This process is regulated by a variety of cytokines which are produced by cells that constitute the haematopoietic environment, including stromal elements and monocytoid accessory cells from peripheral blood, such as T lymphocytes and macrophages [13–15]. Haematopoietic recovery in mice given

Correspondence: Dr Lionel G. Filion, Department of Microbiology and Immunology, University of Ottawa, 451 Smyth Rd., Ottawa, ON K1H 8M5, Canada.

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bone marrow transplants has been demonstrated to be enhanced by a distinct Thy-1$^+$ subpopulation which was resistant to radiation and cyclophosphamide [16]. The fact that such cells appeared to be relatively radioresistant and that a small population of radioresistant Thy-1$^+$ lymphocytes was found to survive *in vivo* in heavily irradiated animals [17] suggests that these cells may play a role in facilitating haematopoietic recovery. While the role of the CD4$^+$ T cell subset in haematoregulation has recently come under investigation [18–20], the relative radiosensitivities of the CD4 and CD8 subpopulations of T lymphocytes compared with other mononuclear cell (MNC) populations have not been well characterized *in vivo* after irradiation.

This study was undertaken, using a murine model, to examine alterations in the major splenic mononuclear cell (SMNC) populations and their state of activation following whole-body irradiation (WBI) in order to determine which populations may play a role in active immune suppression and/or haematopoietic recovery. We employed flow cytometric analysis (FCA) using MoAbs to characterize and differentiate the various cell types by their specific and unique cell surface markers (CD), including CD71, the transferrin receptor [21,22]. The working hypothesis was that the relative concentration of certain cells is altered by irradiation, and this leads to the occurrence of immune system dysfunction or failure. It was our aim to identify particular alterations in relative cell number that might correlate with the dose received, the extent of resultant injury, and the immune competent status of the animal.

Natural killer (NK) cells and a subpopulation of CD4$^+$ T cells may be two of the most important cell types to monitor. Although the absolute numbers of all cells declines with dose, the spleens may become proportionately enriched with some cells with respect to other cells. This could lead to an imbalance of cytokine production, release, or function. In addition, some MNC may become activated following WBI, suggesting that population may be involved in active immune suppression or in haematopoietic recovery. Some alterations may serve as indicators of damage in radiation accident victims or of recovery in radiotherapy patients (biological 'dosimetry').

MATERIALS AND METHODS

Mice

Female C57Bl/6 mice, 18–20 g and 6–8 weeks of age, were purchased from Charles River Labs (Montreal, PQ). The mice were kept 4–6 per cage at room temperature and supplied with Purina Mouse Chow Pellets and acidified tap water pH 2·5 *ad libitum*. All experiments received prior approval from the University of Ottawa Animal Care Committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care. The number of mice used in each experiment ranged from 2 to 10 and was dependent on the radiation dose.

Irradiation

Mice were placed in ventilated plexiglass containers and exposed bilaterally to gamma radiation (0–700 cGy) at a dose rate of 115 cGy/min from a ^{137}Cs radiation source (Gamma Cell-40; Nordion International Inc., Kanata, ON).

Isolation of MNC suspension

Mice were killed by cervical dislocation and briefly immersed in 70% alcohol. The spleen was removed, cleansed of adipose tissue, placed in a sterile Eppendorf vial with PBS at pH 7·2 (Gibco, Burlington, ON), then transferred to a sterile glass Petri dish containing 1 ml PBS. Incisions were made at the base and apex of the spleen, and the cells were gently massaged out of the capsule using sterile 16 G needles bent at a 90° angle. A single-cell suspension was obtained by repeated aspiration through a 23 G needle. A nucleated cell count was performed on a Coulter Zm Cell Counter. Cell viability was determined using 0·4% trypan blue dye exclusion in a haemocytometer. The cell suspension was diluted in PBS (1×10^7 cells/ml) and the MNC were isolated on Lympholyte-M (Cedarlane Labs, Hornby, ON) by gradient centrifugation for 20 min at 1500 *g* and 22°C. The resulting concentration of MNC at the interface (SMNC: lymphocytes, NK cells, and monocytes) was collected with a plastic 2-ml pipette connected to a Pipet-aid aspirator (Drummond Scientific, Broomall, PA). The cells were washed twice with PBS and resuspended in complete medium (Iscove's modified Dulbecco's medium (IMDM); Gibco) containing L-glutamine, 225 mM HEPES buffer, 20% heat-inactivated bovine calf serum (Gibco), 10 μg/ml gentamicin (Gibco), and 1% of 5×10^{-5} M 2-mercaptoethanol (Sigma, St Louis, MO). Cell counts and viability (%) were recorded.

FCA of SMNC subsets

Cells to be analysed by FCA were diluted with PBS/0·1% Na azide to a concentration of 1×10^7 cells/ml. Aliquots of 100 μl were placed into 7-ml polystyrene conical test tubes which contained 100 μl of rat immunoglobulin (10 μg/ml) to block non-specific Fc receptor binding to SMNC, and incubated for 10 min at 22°C. Fluorescently labelled MoAbs, the optimal concentrations of which had been previously determined (results not shown), were added to each tube. Appropriate isotypic controls were also run with each sample to negate non-specific antibody binding to MNC. These included IgG2a, IgG2b, IgG1 and IgM. The tubes were vortexed gently and incubated at room temperature for 15 min. Cells were washed with 2 ml PBS/0·1% Na azide and centrifuged at 1500 *g* for 5 min at 22°C, then fixed with 2% paraformaldehyde (Sigma) for 10 min, rewashed in PBS/0·1% Na azide, and resuspended to a final volume of 300 μl. Table 1 lists the antibodies used to identify specific SMNC. FCA was performed using the Coulter Epics Profile II (Coulter Electronics, Hialeah, FL); the argon laser

Table 1. Monoclonal antibodies used in this study*

Antibody†	Cell reactivity
Thy 1.2-PE	T cells
CD45R/B220-FITC	B cells
CD4-FITC	$T_{H/Ind}$
CD8-FITC	$T_{S/C}$
NK-FITC	Natural killer cells
CD71-PE	Transferrin receptor
Ia-PE	Class II MHC
Mac-PE or FITC	Monocyte/macrophages
CD34-PE	Stem cell

*A summary of CD markers is given by Clark and Lanier [47], with more details provided by Schlossman *et al.* [22].

† All MoAbs were obtained from Pharmingen (San Diego, CA), except F4/80-PE (Caltag, San Diego, CA).

was operated at 15 mW and 488 nm. Standard Brite Flow Cytometric Fluorescence Intensity Standardization Beads (Coulter) were used to calibrate the fluorescence signals for fluorescence 1 (green) and fluorescence 2 (red). By ensuring that the peak for each fluorochrome fell within the established range (±1 channel), as defined by mean channel number, it was possible to compare samples that had been run days or even months apart. For each sample, 5–10 000 cells were analysed, and the resulting histograms analysed using the Epics Elite Flow Cytometry Workstation software (Coulter). Gates were set on the forward scatter (FS) *versus* side scatter (SS) dot plots to focus analysis on only MNC, excluding granulocytes and non-cellular debris. The number of cells of a particular subset was determined by multiplying the number of cells per spleen (as determined by Coulter cell counts) by the proportion of cells registered with that phenotype.

Statistical analysis

Data are presented as mean ± s.e.m., or s.d. where indicated. The effects of WBI on the phytohaemagglutinin (PHA) response for different SMNC were compared by two-way factorial analysis of variance (ANOVA). If the results from this test were significant ($P<0.05$), a multiple comparison test (Bonferroni) was applied.

RESULTS

Effect of WBI on cellularity

Mice were given WBI (0–700 cGy) and after 1, 4, and 7 days their spleens were removed and a single-cell suspension was made for each group (pooled). Figure 1 shows that all radiation doses resulted in significantly fewer nucleated cells than 0 cGy (sham) at all time durations ($P<0.05$), and 400 and 700 cGy resulted in significantly fewer cells than 100 cGy ($P<0.05$). However, there was no significant difference between 400 and 700 cGy on any given day. There was no significant difference in cellularity after any given dose for any sampling time.

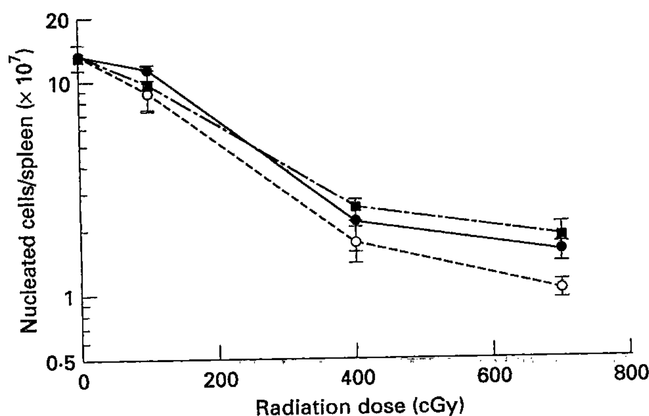

Fig. 1. Decline in the total number of nucleated cells after irradiation. Mice were irradiated with 0–700 cGy and their spleens were removed on days 1 (●), 4 (O) and 7 (■) post-irradiation. Data represent the mean and s.e.m. of five experiments with cells pooled from the spleens of 2–10 mice per experiment depending on the radiation dose used: two mice were used per experiment for doses of 0 and 100 cGy, eight mice for 400 cGy, and 10 mice for 700 cGy. A two-way ANOVA determined that there was a significant decline in cell number with all doses of radiation and at all time points *versus* control mice. There were significantly fewer cells after 400 and 700 cGy compared with 100 cGy ($P<0.05$), but there was no significant difference between 400 and 700 cGy.

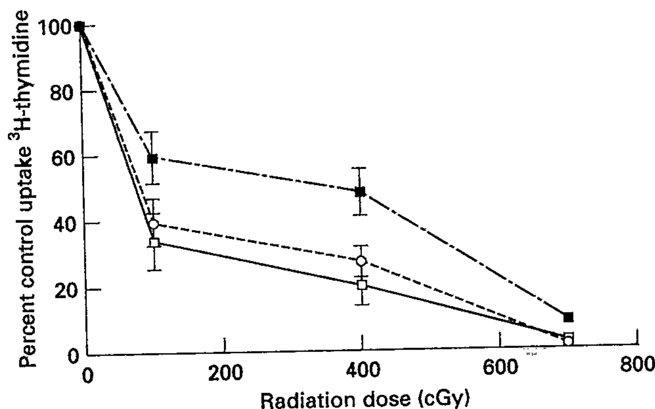

Fig. 2. Phytohaemagglutinin (PHA) response of splenic mononuclear cells (SMNC) isolated from mice exposed to whole-body irradiation (WBI). Mice were exposed to WBI up to 1 week before removing spleens and isolating SMNC, which were cultured (2×10^6 cells/ml) and stimulated with an optimal dilution of PHA for 72 h. The amount of ^{3}H-TdR uptake in cultures from irradiated mice is expressed as a mean percentage (±s.e.m.) of corresponding control uptake. Data represent the pooled results of four experiments. The mean values of uptake by unstimulated and stimulated control cultures were 1095 and 28 787 ct/min, respectively. A two-way ANOVA determined that there was a significant decline in the proliferative capabilities of SMNC with each increase in radiation dose ($P<0.05$). SMNC from mice which received doses of 100 and 400 cGy and isolated 7 days post-WBI showed significantly greater proliferation than SMNC isolated on days 1 and 4 ($P<0.05$). □, 1 day; O, 4 days; ■, 7 days.

Effect of irradiation on PHA response of SMNC

Not only was there decreased cellularity of the mouse spleen following irradiation, but the surviving SMNC also had a decreased ability to respond to mitogen (PHA). This was demonstrated after 100, 400 and 700 Gy by ^{3}H-TdR incorporation (Fig. 2). The dose–response indicated a clear radiation-induced depression of SMNC proliferative capabilities over this range. The proliferation of SMNC isolated on days 1, 4, and 7 showed a significant reduction with each increase in radiation dose ($P<0.05$). After 100 or 400 cGy, proliferation of SMNC isolated on day 7 post-WBI was significantly greater than SMNC on days 1 or 4 ($P<0.05$).

FCA of murine SMNC

Fresh cells. Experiments were performed to characterize the major MNC populations of the spleen of C57Bl/6 mice by FCA. The mean and s.d. of each cell subset, as a percentage of the total SMNC population ($n=5$), are shown in Fig. 3 as controls. Any cell expressing a marker above background (non-binding isotype control levels and autofluorescence) was designated positive.

In single-labelling experiments, B cells comprised about half of the total SMNC in non-irradiated mice [23]. T cells, labelled with anti-Thy1.2 MoAb, made up about one-third of the total SMNC. NK cells were labelled using anti-NK1.1 antibody [24], and were found to be very small in number (≈4%). Monocytes/macrophages were labelled using anti-F4/80 antibody, accounting for 9% of SMNC. Class II MHC-expressing cells (including B cells, monocytes/macrophages, and other antigen-presenting cells) made up

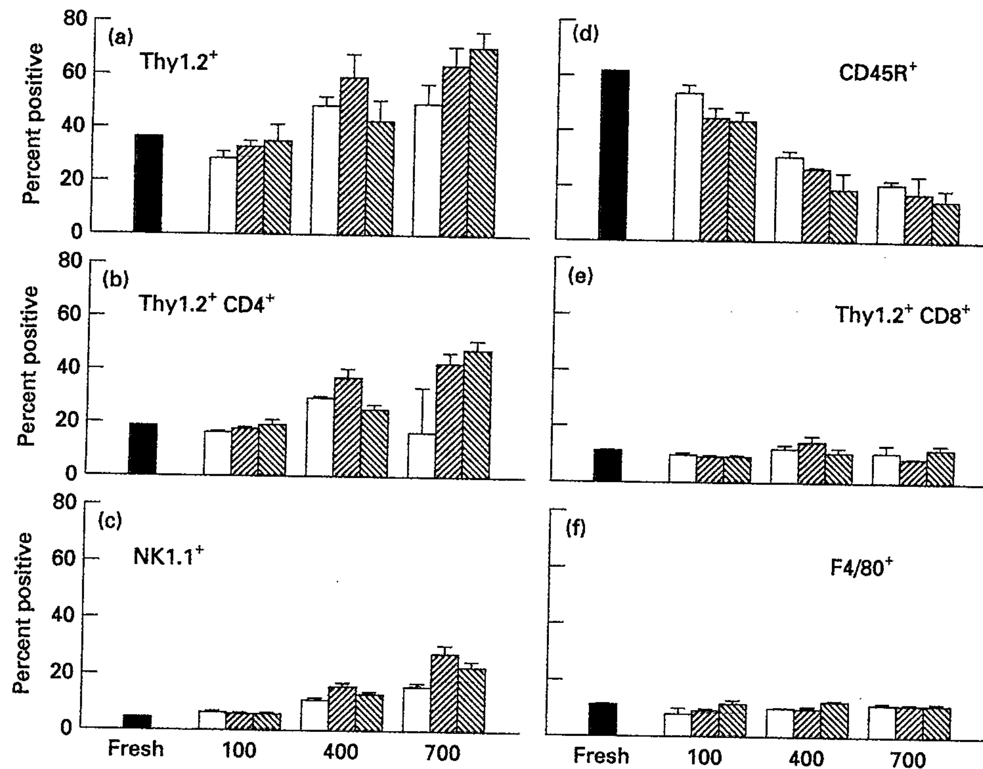

Fig. 3. The relative proportion of various splenic mononuclear cell (SMNC) populations after irradiation. For 1 week after irradition, SMNC were analysed by flow cyotmetry. The relative proportion is the number of positive cells as a percentage of the total number of SMNC for (a) total T lymphocytes, (b) $CD4^+$ T lymphocytes, (c) natural killer cells, (d) total B lymphocytes, (e) $CD8^+$ T lymphocytes, and (f) monocyte/macrophages. Each bar represents the mean of 3–10 animals (three mice with control and 100 cGy, eight mice with 400 cGy, and 10 mice with 700 cGy) in four separate experiments. The error bars indicate s.e.m.

about 55% of SMNC, and CD71, the transferrin receptor, was found on only 3·4%.

In double-labelling experiments T lymphocyte subsets were characterized using anti-Thy1.2 MoAb combined with anti-CD4 or anti-CD8 antibodies. Thy^+CD4^+ cells (including T helper/inducer) made up 16·6% of the SMNC population, while Thy^+CD8^+ cells (including T cytotoxic/suppressor cells) comprised 9·6%. The percentage of B cells expressing the class II MHC molecule was 56·6% (Fig. 3).

Cells from irradiated mice. Using FCA, the effects of radiation dose and time after exposure on the major SMNC populations were examined. Although radiation caused a large decline in the total number of spleen cells, as shown in Fig. 1, the degree of cytoreduction varied among the different cell types. C57Bl/6 mice were irradiated with 100, 400 and 700 cGy and SMNC were analysed after 1, 4 and 7 days. The relative proportion of each cell type, expressed as a percentage of the total number of SMNC, is depicted in Fig. 3a–f. The non-specific binding of the antibodies was assessed with the use of isotype controls, as outlined in Materials and Methods. The proportion of T cells increased with radiation dose (Fig. 3a); 400 and 700 cGy resulted in significant increases in the proportion of T lymphocytes on days 1–7 (Fig. 3a,b) compared with control mice ($P<0{\cdot}05$). The $CD4^+$ and $CD8^+$ T lymphocyte subsets, however, demonstrated differential radiosensitivity; the proportion of $CD4^+$ T cells increased two-fold with radiation doses of 400 and 700 cGy ($P<0{\cdot}05$) (Fig. 3b), whereas the proportion of $CD8^+$ T cells remained unchanged (Fig. 3e). The proportion of $CD45^+$ B cells declined from 53·6% in controls to 26·4% by day 4 after 700 cGy. Doses of 400 and 700 cGy resulted in significantly lower levels of B cells than in control mice at all time periods post-irradiation ($P<0{\cdot}05$) (Fig. 3d). In contrast, a significant increase (seven-fold) in the proportion of NK cells (Fig. 3c) was seen with increasing dose, from 3·9% in controls to 26% of total SMNC on day 4 after 700 cGy. The relative proportion of $F4/80^+$ monocytes/macrophages was not significantly different than in controls (Fig. 3f).

Figure 4a shows the decline by day 4 in the surviving fraction of each population with increasing dose, providing a concise overall comparison of the differential effects of WBI on the various MNC populations in the mouse spleen. Although all cell populations declined, they did so at different rates. In increasing order of radiosensitivity were: NK cells ($D_0 = 4{\cdot}8 \pm 0{\cdot}8$), $CD4^+$ T cells ($D_0 = 3{\cdot}1 \pm 0{\cdot}6$), $CD8^+$ T cells ($D_0 = 2{\cdot}2 \pm 0{\cdot}4$), monocytes/macrophages ($D_0 = 1{\cdot}7 \pm 0{\cdot}1$), and B cells ($D_0 = 1{\cdot}5 \pm 0{\cdot}1$). Figure 4b shows the proportion of each population of SMNC expressed as a percentage of that cell type in unirradiated control mice. The proportion of NK cells displayed the greatest increase to seven-fold control values, whereas the more radiosensitive B cells declined to 33% of controls. Macrophages and $CD8^+$ T lymphocytes remained near control values, whereas the $CD4^+$ T lymphocytes increased almost three-fold. The results were similar for all doses and times studied post-WBI.

Activation markers. MoAbs were also used to identify activated populations of cells. Expression of CD71, the transferrin

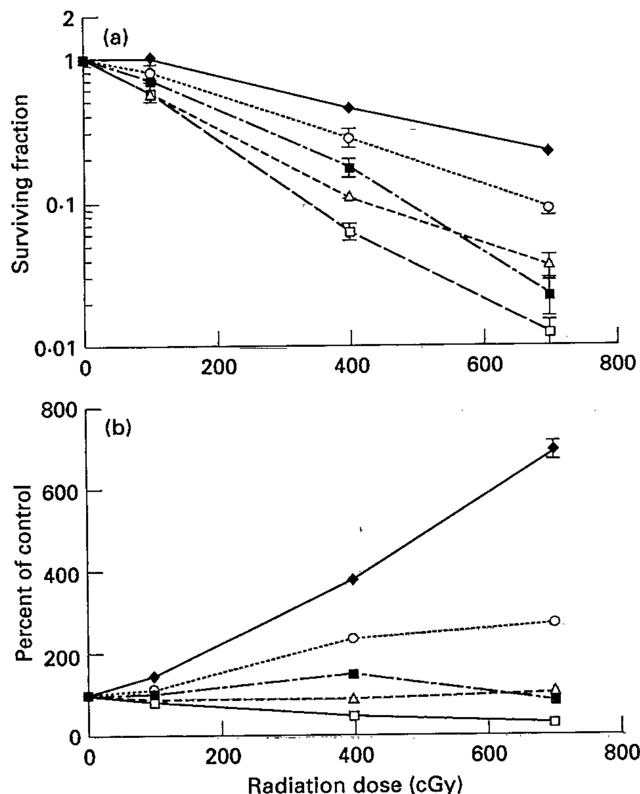

Fig. 4. Effect of irradiation on the absolute and relative numbers of splenic mononuclear cells (SMNC). SMNC isolated 4 days after 0–700 cGy revealed that (a) the surviving fraction delined for all cell types as dose increased (ratio of number of positive cells after radiation to the total number of positive cells in unirradiated control mice); and (b) the proportions of certain SMNC subpopulations actually increased, expressed as a percentage of unirradiated control mice. Data represent the mean and s.e.m. for four experiments with SMNC pooled from eight mice for each experiment. ◆, Natural killer; ○, CD4; Δ, MAC; ■, CD8; □, B.

receptor, was found to be very low in fresh SMNC (controls), as can be seen in the one-parameter histogram in Fig. 5. Only 3–4% of spleen cells from unirradiated mice were found to stain positively for the marker (results not shown). All doses of radiation (100–700 cGy) failed to increase the percentage of positive cells on day 1 post-WBI. However, in mice given 100 cGy, the percentage of transferrin-expressing cells increased to 9% by day 4, and to 11% by day 7 post-irradiation ($P<0{\cdot}05$) (Fig. 5). A higher dose of WBI (400 cGy) did not produce any increase in CD71 expression until 7 days post-irradiation, but the increase was greater than after 100 cGy, reaching 21% of all SMNC ($P<0{\cdot}05$). With the highest dose (700 cGy), expression of CD71 did not increase at all over the 7-day post-irradiation period of study. In order to identify which SMNC population was expressing the CD71 molecule and hence was 'activated', double-labelling experiments were carried out using anti-CD71 in combination with the T cell markers Thy1.2, CD4 and CD8, the pan B cell marker CD45R/B220, the macrophage marker F4/80$^+$, the NK cell marker NK1.1 and the stem cell marker CD34. There was no evidence of a double-stained population for cells stained with antibodies to CD4, CD8, CD45R/B220 (B cell) or NK1.1 (NK). However, 13% of the CD71 cells stained for CD34 and 43% stained for Thy1.2.

DISCUSSION

Suppressor cell activation and severe damage to the bone marrow stem cell population are two adverse effects resulting from exposure to ionizing radiation [3,25,26] which increase susceptibility to opportunistic infections. In the present study we examined the relative radiosensitivity and state of activation of SMNC populations following WBI. Such alterations may be indicative of a role for a particular subpopulation in active immune suppression or in haematopoietic recovery, and may be useful as an early biological marker for severity of damage to the critical immune system, and thus as an effective prognostic indicator.

WBI of mice produced an exponential decline in cell survival of splenic cells (Fig. 1), as has been previously demonstrated both *in vitro* and *in vivo* [27]. Irradiation was also found to be capable of depressing the response of surviving SMNC to mitogen. There was a gradual decline in the proliferative response of SMNC to PHA with dose (Fig. 2). Thus, following the doses of radiation used in this study, animals became immuno-incompetent both as a result of the reduction in the number of viable SMNC (due to destruction and lack of replacement) and the decreased ability of the surviving SMNC to respond properly to challenge (i.e. to proliferate). While the decreased ability of lymphocytes to respond to mitogen is due largely to reproductive cell death [28], it may also be partly due to the effects of an activated suppressor cell population. TLI [3,25] and WBI [26,29] have been shown to produce or activate NS that can non-specifically suppress mixed-leucocyte reactivity and mitogenic responses, and block the generation of cytotoxic cells.

In our experiments, following WBI the spleen was similarly hypocellular (Fig. 1), and the relative proportions of the MNC subsets were dramatically changed (Fig. 3a–f). We examined changes in the major SMNC populations, including B lymphocytes, the CD4$^+$ and CD8$^+$ subsets of T lymphocytes, monocyte/macrophages, and NK cells. Although all cell populations declined, they did so at different rates. In increasing order of radiosensitivity were the NK cells, CD4$^+$ T cells, CD8$^+$ T cells, monocytes/macrophages, and B cells (Fig. 5). The extreme radiosensitivity that we report for B cells in the spleen has been known since 1973, when Nossal & Pike [30] demonstrated that 1 day after WBI exposure to 8 Gy, the number of B cells fell by a factor of over 200, while the total cellularity decreased by only a factor of 10. In this study, the NK cells were the most strikingly radioresistant cell population of the SMNC (Fig. 3e). In control mice, NK cells made up only a small fraction of the total population (3%), whereas 7 days after 700 cGy, NK cells comprised almost one-quarter of the total SMNC.

The identity of cells expressing NS activity is not clear. Various experimental models have implicated T lymphocytes [11], non-T lymphocytes [3], and cells of the monocytic lineage [12]. Early studies by Oseroff *et al.* [3] found NS to be phenotypically null, i.e. they did not readily fall within the T lymphocyte, B lymphocyte, or macrophage lineages. The NS made up only a small proportion of spleen cells, but increased dramatically after TLI. The appearance of suppression followed the disappearance of the null cells, and was only present when the spleens were predominantly haematopoietic organs with few mature T and B lymphocytes [3].

NS cells are known to have several features in common with NK cells. Both cell types are present before antigenic challenge, both show lack of antigen specificity, and both have the appearance of large granular lymphocytes. However, no direct link between

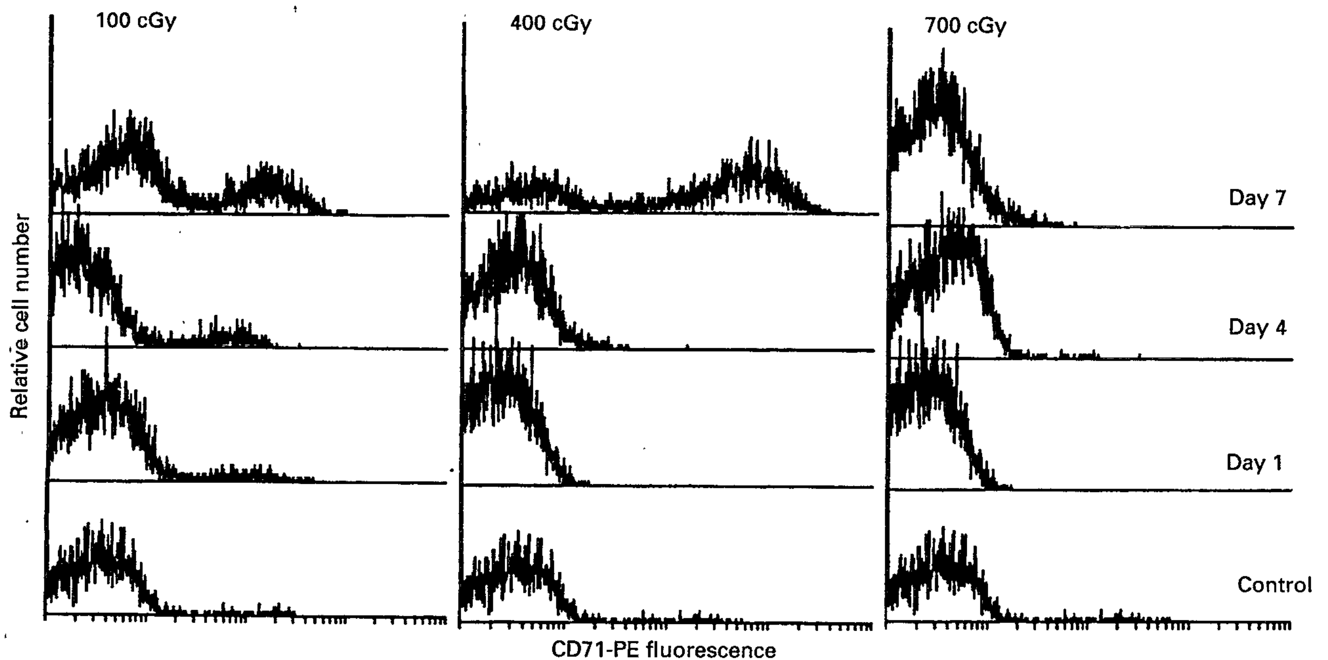

Fig. 5. Induction of CD71 expression after whole-body irradiation (WBI). Flow cytometric analysis of cell surface CD71 expression on splenic mononuclear cells (SMNC) revealed that the percentage of $CD71^+$ SMNC increased after exposure to ionizing radiation, apparently in a dose-related manner. There was a significant increase in the percentage of $CD71^+$ cells with 100 cGy on days 4 and 7 post-WBI and with 400 cGy on day 7 post-WBI ($P < 0{\cdot}05$), but not after 700 cGy.

the two cell types has been found. Much progress has been made since the early studies on NS cells in the characterization and identification of cells based upon the expression of phenotypic markers. In the present study, we did not find a 'null' cell population which was transiently expressed. In contrast, virtually all cells of the spleen were identified with the panel of MoAbs used. No attempt has been made to determine whether null NS cells express NK cell markers. It is possible therefore that the previously reported null cells may be equivalent to the transiently observed increase in NK cells reported here, the introduction of more specific MoAbs having made such a distinction possible. Interestingly, it has recently been reported that lymphoid irradiation of Hodgkin's disease patients results in an expanded NK cell compartment which is thought to play a role in the long term immunosuppression observed in these patients [31].

The high sublethal doses of radiation used in these studies induce haematopoietic depletion which is followed by vigorous recovery initiated from endogenous stem and progenitor cells that survive the irradiation [27]. *In vitro* studies have demonstrated that different lymphoid subsets have opposing effects on haematopoietic cell growth [33,34]. The haematopoietic recovery of an irradiated animal may then be influenced by the proportions of surviving recovery-enhancing and recovery-inhibiting cell types. T lymphocytes have been found to consist of two distinct subpopulations: a radioresistant population which can enhance haematopoietic recovery, and a radiosensitive population which can suppress it [16,35]. The relationship of such subpopulations to the immunoregulatory $CD4^+$ helper and $CD8^+$ suppressor T lymphocytes, however, has not been determined, but it has been recently reported that $CD4^+$ cells are stimulators of normal haematopoiesis and recovery following WBI [19,36]. The results reported here are in agreement with Williams *et al.* [19], in that the murine spleen contains a population of radioresistant $CD4^+$ T cells. As a result of the radioresistance of these cells, the spleens of irradiated mice became proportionately 'enriched' with $CD4^+$ T cells. By 7 days post-700 cGy WBI, $CD4^+$ lymphocytes made up almost 50% of all SMNC, compared with only 15% in control mice (Fig. 3b). The different radiosensitivities of $CD4^+$ and $CD8^+$ T lymphocytes observed in the spleen (Fig. 4a) are comparable to the radiosensitivities of the different types of T cells that exert helper and suppressor effects on haematopoietic recovery [35].

The proliferation and differentiation of stem and progenitor cells are known to be regulated by a variety of cytokines produced by cells which constitute the haematopoietic microenvironment. $CD4^+$ cells produce extensive quantities of a wide range of cytokines [37] such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), IL-3 and IL-6, all of which are known to be stimulators of haematopoiesis [38]. The finding *in vivo* that antibody-mediated ablation of $CD4^+$ cells before irradiation reduces haematopoietic recovery [36], taken together with the finding of residual radioresistant $CD4^+$ cells which become 'enriched' following irradiation, is suggestive of the physiological relevance of $CD4^+$ cells in haematopoietic recovery. A possible mechanism to explain the interaction between haematopoietic progenitors and $CD4^+$ cells would be the release of stimulatory cytokines such as IL-3 or GM-CSF directly after radiation injury or subsequent antigen stimulation. The enrichment of the spleen with NK cells post-irradiation may also be significant to the outcome of haematopoietic recovery. Activated NK cells have previously been shown to play a role in suppression of haematopoiesis after irradiation [39]. *In vitro* growth inhibition of haematopoietic cells has been consistently shown to emanate from NK and lymphokine-activated killer (LAK) cells with a

similar range of activity directed against stem cells (multipotent colony-forming units (CFU-GEMM)) [40], progenitor cells committed to granulocyte-macrophage differentiation (granulocyte-macrophage colony-forming units (CFU-GM)) [41], and erythropoietic progenitors (burst-forming units erythroid (BFU-E) and colony-forming units erythroid (CFU-E) [42]). NK and LAK cells produce or release interferon-gamma (IFN-γ) [43,44], as well as tumour necrosis factor-alpha (TNF-α) and TNF-β [45], all of which are well known growth inhibitors [46]. Although the number of $CD4^+$ T lymphocytes and NK cells was quite small compared with that of control animals, due to an overall decreased cellularity, a selective enrichment developed and persisted during the first 7 days post-WBI. The balance between haematopoietic recovery-enhancing $CD4^+$ T cells and recovery-inhibiting NK cells may be a key factor in the survival of irradiated animals. Furthermore, manipulating the ratio (or activation state) of these two cell types may prove useful in enhancing haematopoietic recovery.

Analysis of activation markers on SMNC following irradiation demonstrated a dose- and time-dependent expression of CD71, the transferrin receptor. At a dose of 100 cGy, increased CD71 expression was seen as early as 4 days post-WBI (9%), whereas at the higher dose of 400 cGy, increased expression was delayed until 7 days post-WBI and a greater proportion of SMNC were positive (22%) (Fig. 5). A whole-body dose of 700 cGy did not result in any increase in CD71 expression over the 7-day time period in our study, but may at a later time after irradiation. The delayed expression may be dose-dependent.

The type of cells expressing CD71 was demonstrated to be $CD34^+$ and Thy1.2, but not $CD4^+$, $CD8^+$, $CD45R/B220^+$ or $NK1.1^+$. This result suggests that CD71 is expressed on early progenitor cells and may be an indicator for early haematopoietic activity. CD34 and Thy1.2 account for 56% of $CD71^+$ cells. The remaining cells may be other linaege-specific cells. The absence of T cell subset markers (CD4 or CD8) also suggests that the $CD71^+$ cells are immature T cells that appear in the periphery after radiation.

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