Oncogene (2001) 20, 7085–7095

www.nature.com/onc

Inflammatory-type responses after exposure to ionizing radiation *in vivo*: a mechanism for radiation-induced bystander effects?

Sally A Lorimore[1], Philip J Coates*[,1], Gillian E Scobie[1], Gordon Milne[1] and Eric G Wright[1]

[1]*Department of Molecular and Cellular Pathology, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, UK*

Haemopoietic tissues exposed to ionizing radiation are shown to exhibit increased macrophage activation, defined by ultrastructural characteristics and increased lysosomal and nitric oxide synthase enzyme activities. Macrophage activation post-irradiation was also associated with enhanced respiratory burst activities and an unexpected neutrophil infiltration. Examination of p53-null mice demonstrated that macrophage activation and neutrophil infiltration were not direct effects of irradiation, but were a consequence of the recognition and clearance of radiation-induced apoptotic cells. Increased phagocytic cell activity was maintained after apoptotic bodies had been removed. These findings demonstrate that, contrary to expectation, recognition and clearance of apoptotic cells after exposure to radiation produces both a persistent macrophage activation and an inflammatory-type response. We also demonstrate a complexity of macrophage activation following radiation that is genotype dependent, indicating that the *in vivo* macrophage responses to radiation damage are genetically modified processes. These short-term responses of macrophages to radiation-induced apoptosis and their genetic modification are likely to be important determinants of the longer-term consequences of radiation exposure. Furthermore, in addition to any effects attributable to immediate radiation-induced damage, our findings provide a mechanism for the production of damage via a 'bystander' effect which may contribute to radiation-induced genomic instability and leukaemogenesis. *Oncogene* (2001) **20,** 7085–7095.

Keywords: ionizing radiation; macrophage; inflammation; genetics; bystander

Introduction

Until recently, it has been generally accepted that the genotoxic and carcinogenic consequences of radiation exposure are due to the damage inflicted directly by the radiation, producing irreversible changes during DNA replication, or during the processing of the DNA damage by enzymatic repair processes. However, there is now considerable evidence that cells that were not themselves irradiated but were the progeny of cells exposed to ionizing radiation many cell divisions previously may express delayed gene mutations and a variety of chromosomal aberrations. These effects are generally referred to as radiation-induced genomic instability. Although the mechanism for these delayed effects of ionizing radiation is unclear, excessive production of reactive oxygen species has been implicated (reviewed by Wright, 1998; Iyer and Lehnert, 2000; Little, 2000). The paradigm of genetic alterations being restricted to direct DNA damage following radiation exposure has also been challenged by studies showing that nuclear damage can be observed after targeted cytoplasmic irradiation using the newly developed microbeam irradiators (Prise *et al*., 1998; Wu *et al*., 1999). Chromosomal damage has also been observed in cells that were not themselves irradiated but were in the neighbourhood of irradiated cells (Prise *et al*., 1998; Zhou *et al*., 2000) and cytotoxic effects can be observed in the medium of irradiated cells when the cell-free medium is subsequently transferred to non-irradiated cells (Mothersill and Seymour, 1997; Lehnert and Goodwin, 1997). Finally, irradiated cells secrete growth-inhibitory molecules both *in vitro* and *in vivo* in a p53-dependent manner (Komarova *et al*., 1998). These observations have given rise to the notion that ionizing radiation can induce so-called 'bystander effects' where the irradiated cells transfer a signal to non-irradiated cells. Again, the mechanism for such bystander-mediated effects are unclear, although reactive oxygen species have been implicated (Narayanan *et al*., 1997; Wu *et al*., 1999; Lyng *et al*., 2000). We have recently established a link between these two indirect radiation effects by demonstrating that genomic instability in haemopoietic cells can be induced by unexpected interactions between irradiated and non-irradiated cells i.e. by a bystander mechanism (Lorimore *et al*., 1998; Watson *et al*., 2000). These untargeted effects of radiation clearly pose a major challenge to current views of the mechanisms of radiation-induced DNA damage and radiation-induced malignancy.

Malignancies, and in particular myeloid leukaemias, are a major health consequence of exposure to ionizing radiation and interactions between the haemopoietic microenvironment and the target stem cells as well as

*Correspondence: PJ Coates; E-mail: p.j.coates@dundee.ac.uk
Received 9 March 2001; revised 31 July 2001; accepted 7 August 2001

the damage to the haemopoietic stem cells themselves have been implicated by both clinical and experimental observations. Thus, whilst uncommon, patients treated for haemopoietic disorders by bone marrow transplantation following preparative whole body irradiation may relapse with disease in the donor-derived cells (reviewed in McCann *et al*., 1993; Giralt and Champlin, 1994). Irradiation has also been shown to induce leukaemic transformation of non-irradiated stem cells transplanted into syngeneic mice (Duhrsen and Metcalf, 1990). These findings may reflect the altered characteristics of the stem cell microenvironment after irradiation, since irradiated haemopoietic stromal cells release mutagenic reactive oxygen species, produce different sets of adhesion molecules and growth factors, and alter the overall growth and phenotypic characteristics of co-cultured non-irradiated stem cells (Greenberger *et al*., 1996).

As with many responses to radiation, the development of radiogenic leukaemia is strongly influenced by genetic factors (Wright, 1998). In mouse model systems, CBA/Ca mice characteristically develop myeloid leukaemia after exposure to ionizing radiation and their haemopoietic cells are also susceptible to radiation-induced chromosomal instability. In contrast, C57BL/6 mice are resistant to both the development of radiation-induced myeloid leukaemia and to radiation-induced chromosomal instability in haemopoietic cells. Because interactions between tissue stroma and stem cells appear to be important in determining the overall consequences of radiation, we are investigating both the short- and long-term effects of radiation on haemopoietic tissues. Here we report unexpected macrophage activation combined with neutrophil infiltration following whole body irradiation, effects that persist long after the initial radiation insult. We also show that the degree of these responses is genotype dependent and their inflammatory nature suggests the potential for ongoing damage after the initial radiation insult.

Results

Macrophage ultrastructure following in vivo *irradiation*

Electron microscopical studies of macrophages before and 24 h after irradiation identified both increased cell membrane ruffling and increased lysosome number and size in the irradiated tissues, the classic ultrastructural features of macrophage activation (Adams and Hamilton, 1992) (Figure 1). Many of the lysosomal components of the cells were seen to be secondary and tertiary lysosomes, indicative of degradation of phagocytosed material.

Lysosomal and respiratory burst activity in macrophages following in vivo *irradiation*

In view of the morphological data suggesting macrophage activation post-irradiation, we investigated whether there was any evidence for increased enzyme activity in these cells. Quantitative spectrophotometric studies of lysosomal acid β-galactosidase activity in the spleens and bone marrow of three individual mice showed mean increases of fourfold (0.11 to 0.45) in the spleen and of threefold (0.13 to 0.4) in the bone marrow 24 h after irradiation with 4 Gy γ-rays. At this dose, there was a mean reduction in tissue cellularity of 82 and 74% in the spleen and bone marrow, respectively. Histochemical staining showed that the increase in acid β-galactosidase activity localized to cells with the distribution and morphological characteristics of acid phosphatase-positive macrophages (Figure 2a–d).

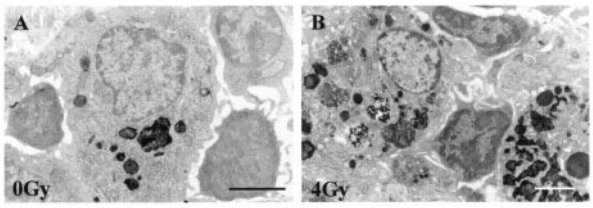

Figure 1 Electron micrographs of spleen from C57BL/6 control mice (**a**) or 24 h post-irradiation with 4 Gy (**b**) Macrophages are shown in both micrographs. Note the presence of larger numbers of secondary and tertiary lysosomes containing electron dense material, together with extensive membrane ruffling in the irradiated macrophages

To investigate the time at which increased lysosomal enzyme activity is first seen, we measured enzyme activities spectrophotometrically in spleen cell suspensions from three individual mice at various times after 4Gy irradiation. Mean levels of enzyme activity were similar to control unirradiated levels at times up to and including 4 h post-irradiation, but were significantly raised by 6 h. Enzyme levels continued to increase, reaching more than four times the control level at 24 h post-irradiation (Figure 3a). Interestingly, in control mice positive cells were seen at the margins of the red and white pulp and these represented only a small percentage of the total macrophage content as determined by acid-phosphatase staining. At 6 and 9 h post-irradiation, the proportion of macrophages staining positively for acid β-galactosidase had increased, and the macrophages were seen to be increased in size by acid phosphatase staining (Figure 2a,b). Unlike control spleen, acid β-galactosidase staining was observed in both red and white pulp 6 to 9 h post-irradiation (Figure 2c,d) and acid β-galactosidase-positive macrophages in the white pulp contained multiple pyknotic nuclei, indicating that these macrophages had recently ingested apoptotic cells (Figure 2g). By 24 h the staining was again predominant in red pulp areas. Similarly, in unirradiated bone marrow, only a small proportion of the acid phosphatase-positive macrophages showed acid β-galactosidase activity, but after irradiation most macrophages became strongly positive for acid β-galactosidase (data not shown).

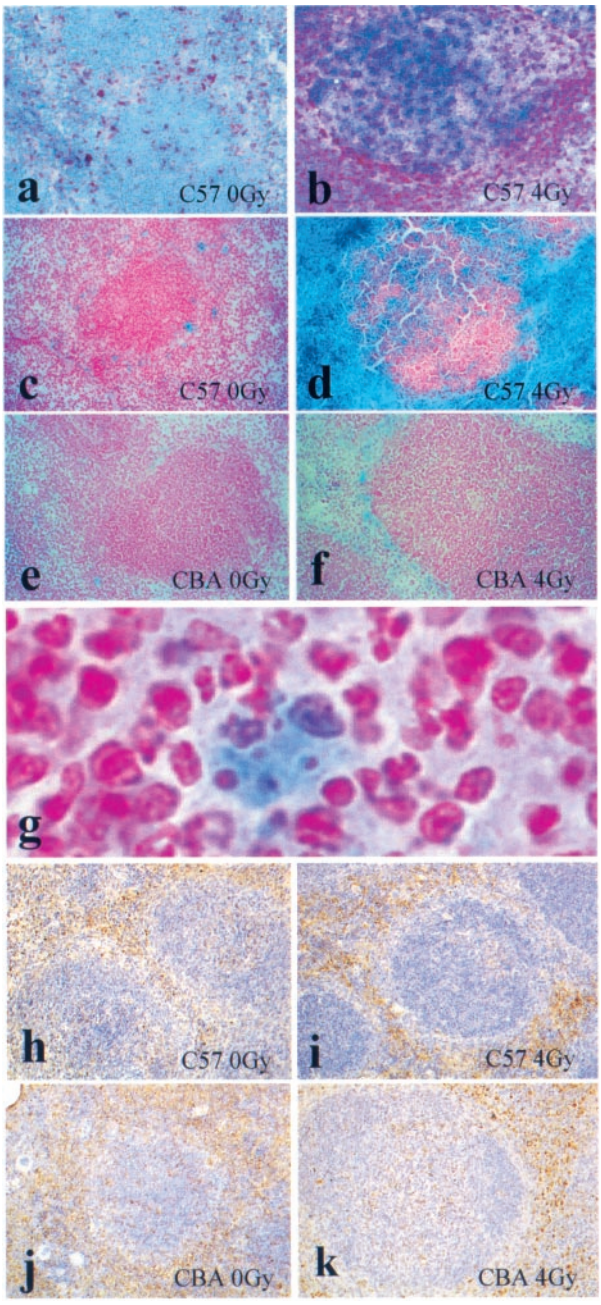

Figure 2 Increased macrophage enzyme activities following 4 Gy γ-irradiation. (**a**) Acid phosphatase staining of spleen sections before and (**b**) 9 h after irradiation. (**c**–**f**) show acid β-galactosidase staining of C57BL/6 and CBA/Ca spleen before and 9 h post-irradiation. (**g**) A high power magnification of a single acid β-galactosidase-positive macrophage 9 h post-irradiation containing pyknotic nuclei. (**h**–**k**) Nitrotyrosine staining in C57BL/6 and CBA/Ca spleen before and 24 h post-irradiation

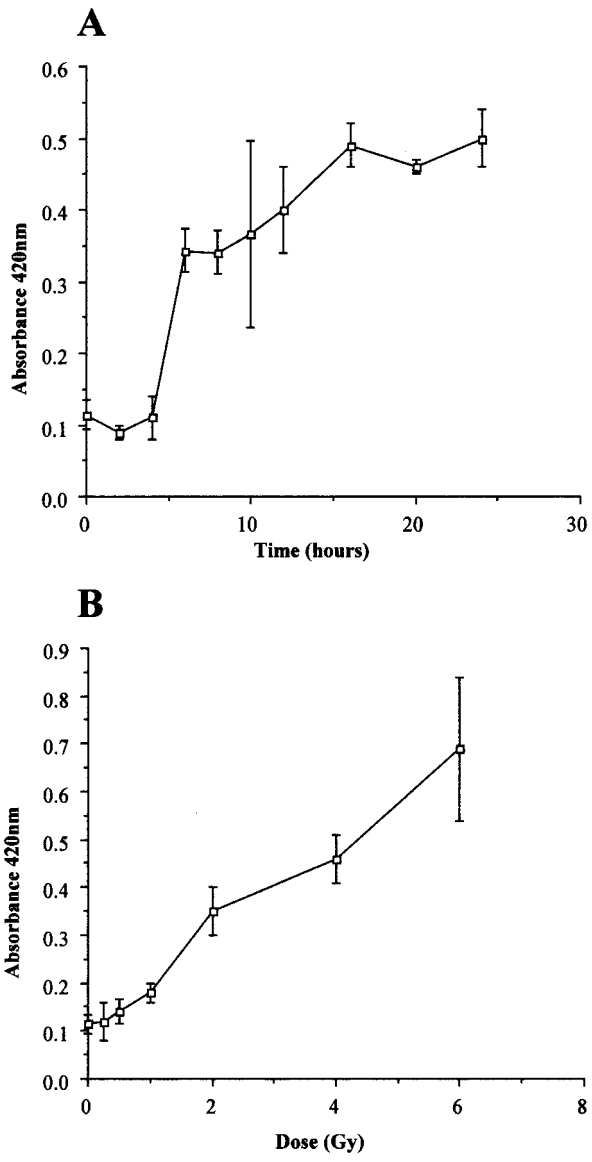

Figure 3 (**a**) Time course of acid β-galactosidase activity after 4 Gy radiation. Enzyme activity was significantly raised at 6 h, continued to increase and reached more than four times the control level 24 h post-irradiation. (**b**) Dose response measurements show increased acid β-galactosidase activity in spleen cell suspensions 24 h after radiation

We also exposed triplicate mice to differing doses of γ-irradiation and measured levels of acid β-galactosidase in spleens 24 h later. There was no significant increase above the control enzyme activity after exposure to 0.25 or 0.5 Gy (36 and 48% mean reduction in tissue cellularity respectively). Significantly increased activity was evident at doses equal to or greater than 1 Gy and for the relatively small decrease in tissue cellularity from 65% at 1 Gy to 73% at 2 Gy, a 2.5-fold increase in enzyme activity was measured (Figure 3b).

Additional evidence for functional activation of macrophages in both the spleen and bone marrow after irradiation was provided by a standard luminescence assay for the production of superoxide (characteristic of the respiratory burst that occurs in phagocytic cells) in the absence of exogenous stimulation. Luminescence was measured from three mice in duplicate over a period of 1 h in bone marrow or spleen suspensions. Values represent the amount of

light emitted at each time point measured and are given in arbitrary units (a.u.). Without any radiation exposure, phagocytes from C57BL/6 mice show a low level of constitutive respiratory burst activity that is increased from 125 to 250 a.u. 24 h after 4 Gy γ-irradiation. After stimulation with PMA cells from unirradiated control mice showed a greater respiratory burst when compared to cells removed from mice exposed to 4 Gy γ-irradiation 24 h previously (910 vs 740 a.u. respectively).

Increased nitrotyrosine following in vivo *irradiation*

Nitric oxide (NO) production is a characteristic feature of activated macrophages, which induce nitric oxide synthase 2 (NOS2, or iNOS) as part of the inflammatory process. Nitrotyrosine is a product of the reaction between reactive nitrogen species and peptides or proteins, and formation of nitrotyrosine *in vivo* is associated with the expression of NOS2 (Wink *et al*., 1998). By immunohistochemistry we found nitrotyrosine to be present in a small percentage of cells in unirradiated spleen with the distribution and cellular morphology of macrophages. After irradiation, both the number of positive cells and the intensity of the reaction product were greatly increased (Figure 2h,i). These data indicate that exposure to ionizing radiation induces the expression of NOS2 in tissue macrophages *in vivo*, providing further evidence for radiation-induced macrophage activation.

Radiation-induced macrophage activation is associated with neutrophil infiltration

Further investigations of the tissue response to apoptosis revealed an unexpected accumulation of neutrophils in the red pulp of irradiated spleens. Neutrophils were identified by the characteristic morphology of these cells and neutrophil infiltration was confirmed by immunostaining for myeloperoxidase (Figure 4a–f). Neutrophil accumulation was first seen 6 h after radiation exposure, and the number of neutrophils increased up to 24 h post 4 Gy γ-irradiation. Neutrophils were seen both within the red pulp and also at the margins of the blood vessels in the red pulp. This latter feature of neutrophil margination is a classic sign of an acute inflammatory response, during which neutrophils are recruited from the bloodstream to the affected site due to the local production of chemoattractant molecules. Consequently, these observations clearly indicate an ongoing neutrophil infiltration into the spleen following irradiation, the timing of which coincides with the increased macrophage activity we have also demonstrated.

Increased macrophage activation following irradiation is associated with p53-dependent apoptosis

The data above indicate that increased macrophage activity is a consequence of radiation exposure. The observations that macrophages which contained multi-

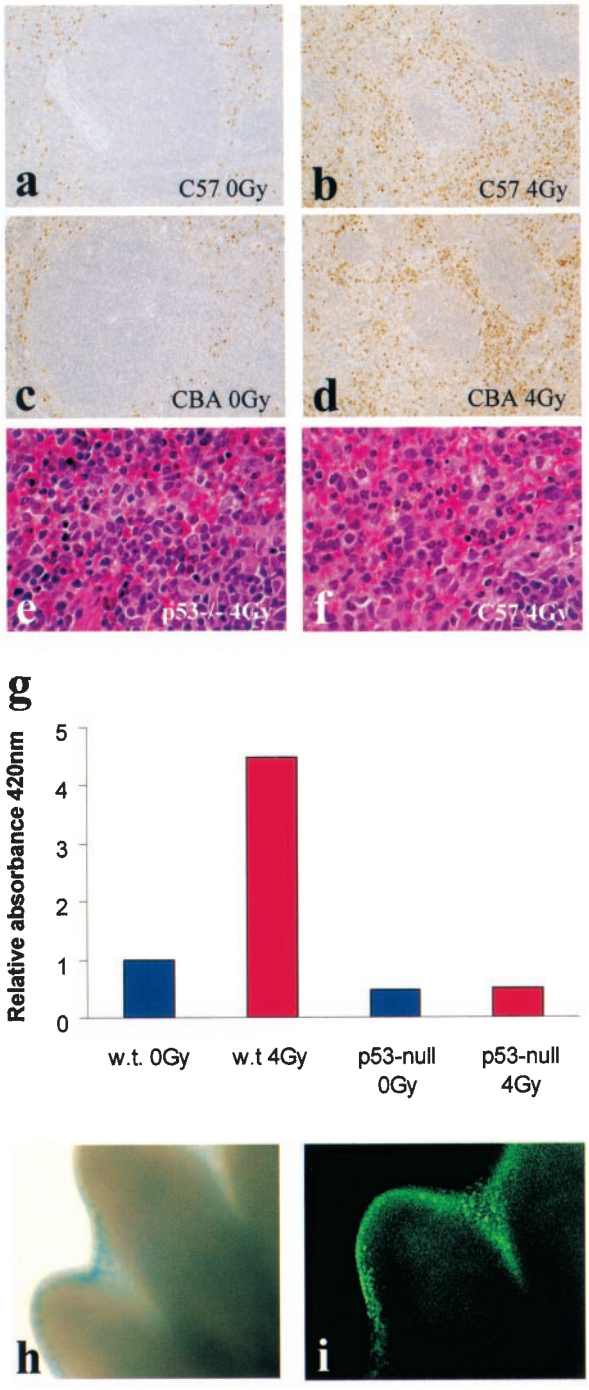

Figure 4 Neutrophil infiltration demonstrates inflammation post-irradiation. (**a**–**d**) Myeloperoxidase immunocytochemistry of spleen from C57BL/6 and CBA/Ca before and 24 h post-irradiation. (**e**, **f**) Haematoxylin and eosin stained spleens from p53-null and C57BL/6 24 h after irradiation shows a lack of neutrophil infiltration in p53−/− mice. (**g**) Increased acid β-galactosidase activity is only observed in p53 wild type and not p53-null mice 24 h post-irradiation. (**h**, **i**) Acid β-galactosidase-positive macrophages (**h**) correlate with areas of acridine orange-positive apoptotic cells (**i**) in the developing footplate of unirradiated p53-null mice, demonstrating that increased acid β-galactosidase in macrophages associated with apoptosis is p53 independent

ple intracellular apoptotic bodies were both β-galactosidase-positive and contained detectable amounts of nitrotyrosine, together with the finding that enzyme activities begin to rise concurrently with the peak number of apoptotic cells suggested that there might be a link between macrophage activation and apoptosis, rather than activation being a direct effect of radiation exposure. To test whether macrophage activation is a direct effect of irradiation exposure, we measured enzyme activities in the spleens of three individual p53−/− mice, where radiation-induced apoptosis is absent due to the lack of p53 function. In these mice, acid β-galactosidase was not increased above control 24 h after 4 Gy irradiation, although a mean 4.5-fold increase was seen in three identically treated p53+/+ mice of the same genetic background (Figure 4g). Similarly, immunohistochemical examination of nitrotyrosine in spleens from p53-null mice showed no detectable increase in nitrotyrosine immunoreactivity after radiation.

These data are compatible with our hypothesis that macrophage activation following radiation exposure is due to radiation-induced apoptosis, but might also indicate a direct p53-mediated pathway of macrophage activation. To test whether the increased macrophage activity is dependent on p53 function, we examined acid β-galactosidase activity in a classical situation of physiological apoptosis during embryonic tissue remodelling. Previous studies have shown that apoptosis in the developing footplate of the mouse can be easily demonstrated *in vivo* by acridine orange staining, and the apoptotic cells are contained within F4/80-positive macrophages (Wood *et al*., 2000). The forelimbs of unirradiated wild type and p53-null E13.5 mouse embryos each demonstrated a clear temporal and spatial association between high apoptosis (acridine orange-positive cells) and intense acid β-galactosidase staining in individual cells within the interdigital zones of the footplate (Figure 4h,i). In contrast, before (E12.5) or after (E14.5) the extensive apoptotic remodelling phase of footplate development, both acridine orange and acid β-galactosidase showed minimal staining in both p53-null and wild type animals. These footplate data clearly demonstrate that increased macrophage acid β-galactosidase activity occurs in the presence of high numbers of apoptotic cells and is independent of p53 function.

Taken together with the nitrotyrosine data, these observations indicate that increased macrophage activity is induced by apoptosis, rather than being a direct effect of radiation. Additionally, because neutrophil infiltration was not apparent in p53−/− irradiated spleens, inflammation after irradiation is also not a direct effect of radiation exposure (Figure 4e).

Macrophage activation is a genetically modified process

We wanted to determine whether there was any evidence for genetic modification of the responses we had found, because we have previously reported genetic differences in radiation-induced chromosomal instability in two inbred strains of mice, C57BL/6 and CBA/Ca (Watson *et al*., 1997). The results described above had all been obtained using the C57BL/6 strain. When measured using the quantitative spectrophotometric assay, mean levels of acid β-galactosidase activity were higher in spleen and bone marrow from C57BL/6 animals than in CBA/Ca mice, the levels in spleen cell suspensions being induced fourfold for C57BL/6 compared to 2.5-fold for CBA/Ca 24 h after whole body irradiation with 4Gy γ-irradiation. One possible explanation for these results was that there are higher percentages of macrophages in the C57BL/6 strain, thereby giving higher average levels of lysosomal enzyme activity. To investigate this, we performed acid phosphatase staining together with immunohistochemistry and FACS analysis using a monoclonal antibody against the mouse macrophage marker, F4/80. By acid phosphatase staining and immunohistochemistry of paraffin sections, the distribution and numbers of macrophages were indistinguishable between the two genotypes. By quantitative FACS analysis of cell suspensions, the percentages of F4/80-positive macrophages were also seen to be similar in the two genotypes, with or without prior exposure to radiation (6.2% for both CBA/Ca and C57BL/6 without irradiation; 1.6% for C57BL/6 and 1.2% for CBA/Ca 24 h after 4 Gy γ-irradiation). Because F4/80 measures monocytic precursors as well as resident macrophages, we quantitated macrophage numbers in bone marrow morphologically by electron microscopy, which showed that after irradiation the two strains contained similar numbers of macrophages (26 ± 0.4 per grid unit area for C57BL/6 versus 22.5 ± 0.87 per grid unit area for CBA/Ca). Taken together, these data show that the relatively higher levels of acid β-galactosidase in lysates of spleen and bone marrow cell suspensions of C57BL/6 mice are not simply due to the presence of increased numbers of macrophages in these samples, but must instead be due to an increased level of enzyme activity per cell. Histochemical staining for acid β-galactosidase on frozen sections of spleen or bone marrow taken from C57BL/6 and CBA/Ca mice showed only a small proportion of macrophages positive in unirradiated tissues with more stained cells present in C57BL/6 than in CBA/Ca mice. At 9 h post-irradiation with 4 Gy, the number of positively stained cells and the intensity of staining was increased in both strains and was greater in C57BL/6 mice than in CBA/Ca (Figure 2c–f). This genotypic difference in staining was maintained up to 24 h post-irradiation.

We have previously demonstrated genetic differences in PMA-induced superoxide generation between unirradiated C57BL/6 and CBA/Ca bone marrow cells (Watson *et al*., 1997). We have now extended that study to assess superoxide generation in spleen and bone marrow cells with and without radiation and in the presence or absence of exogenous stimulation with PMA. Unlike the results in C57BL/6 mice, in the absence of exogenous stimulation with PMA phagocytic cells from unirradiated CBA/Ca bone marrow and spleen had no detectable superoxide activity. Further-

more, even after radiation (which induces a further increase in the respiratory burst of C57BL/6 mice) there was no detectable superoxide generation in CBA/Ca animals. In contrast to the undetectable endogenous respiratory burst activity of CBA/Ca mice, exogenous stimulation of the cells with PMA led to a greater respiratory burst than that seen in C57BL/6 mice, in both normal and irradiated CBA/Ca tissues (Figure 5).

We also studied the *in vivo* production of NO and the accumulation of neutrophils in the two genotypes by immunohistochemical staining for nitrotyrosine or myeloperoxidase in sections of spleen. We noted that whilst both strains showed an induction of nitrotyrosine-modified proteins after radiation exposure, the staining was less intense in irradiated CBA/Ca animals compared to C57BL/6 mice, although the distribution of staining was similar (Figure 2h–k). Neutrophil accumulation in the two strains was quantitated by counting the number of myeloperoxidase-positive polymorphs in the red pulp with or without exposure to 4 Gy γ-irradiation 24 h previously. Again, neutrophil accumulation was seen to occur in both strains, and there was no significant difference between the strains (29.2 ± 1.2 and 32.0 ± 1.9, $P = 0.1098$, increasing to 82.1 ± 3.6 and 88.4 ± 4.1, $P = 0.0786$; for C57BL/6 and CBA/Ca respectively).

These data indicate complex genotypic differences in radiation-induced macrophage activation and neutrophil infiltration in these two inbred strains of mice. Since we have shown that induction of macrophage activities is correlated with radiation-induced apoptosis, and it is known that the level of apoptosis after radiation exposure varies between different mouse strains (Nomura *et al*., 1992), one possible explanation for these results would be that there are higher levels of apoptosis in C57BL/6 mice than in CBA/Ca mice, leading to higher enzyme activities in the former. To investigate this possibility, we counted apoptotic cells at various times after irradiation in the spleens of duplicate animals. In both strains, radiation-induced apoptotic cells are first seen 2 h after radiation, their numbers peak at 6 h and have begun to decrease by 9 h post-irradiation, reaching near to control values by 24 h. Quantitative analysis showed there to be an approximately 1.5-fold higher level of apoptosis in C57BL/6 mice after radiation (C57BL/6; 911 ± 42 and CBA/Ca; 619 ± 40 apoptotic cells per high power field, 6 h after 4 Gy). This resulted in a greater reduction in tissue cellularity at 24 h for C57BL/6 than for CBA/Ca; 82 and 72% respectively.

These data might suggest that the genotypic differences in radiation-induced macrophage activation we had observed were due simply to differing levels of radiation-induced apoptosis. To investigate the relationship between cell death and macrophage activation we turned to the DBA/2 strain, which is known to be particularly resistant to radiation-induced apoptosis (Nomura *et al*., 1992). Spectrophotometric measurements in spleen lysates from these mice 24 h after 4 Gy irradiation showed that the levels of acid β-galactosidase were approximately twofold higher than the corresponding CBA/Ca level. In contrast, measurements of apoptosis showed twofold lower levels of cell death in DBA/2 mice. Therefore, genotypic differences in the induction of macrophage lysosomal enzyme activity following irradiation are not directly related to genotypic differences in apoptosis.

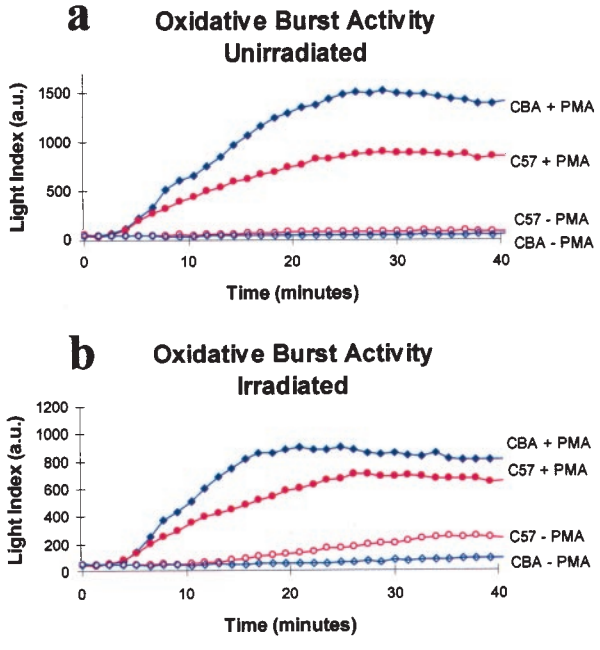

Figure 5 Respiratory burst activity following radiation. C57BL/6 or CBA/Ca mice were exposed to 0 Gy or 4 Gy radiation and superoxide production was measured in bone marrow cells from each animal by chemiluminescence 24 h later. (**a**) shows the production of superoxide in unirradiated animals. (**b**) shows the respiratory burst activity in cells from mice exposed to radiation

Discussion

We have shown that macrophage activation and inflammatory-type responses in the haemopoietic system are early consequences of exposure to ionizing radiation *in vivo*. This conclusion is based on the finding of tissue neutrophil infiltration, the ultrastructural characteristics of tissue macrophages, their increased lysosomal and NOS enzyme activities and the enhanced respiratory burst activities indicating increased phagocytic cell activity. Dose response and time course studies, together with the absence of these responses in irradiated p53-null mice, indicated that the mechanism for this response is the recognition and phagocytosis of radiation-induced apoptotic cells.

Our electron microscopic investigations of unirradiated haemopoietic tissues demonstrated a bimodal distribution of macrophages in which cells had few or many secondary lysosomes, but all cells showed minimal membrane ruffling. Those cells with many secondary lysosomes may reflect the ongoing phagocytic clearance of apoptotic cells as a part of normal cell

turnover in the rapidly proliferating haemopoietic system. Indeed, phagocytosis of apoptotic cells is a feature of normal steady-state haemopoiesis (Necas *et al*., 1998), with physiological levels of apoptosis quantitatively similar to those associated with apoptosis after exposure to the doses of radiation used in our studies. However, steady-state apoptosis *in vivo* is inconspicuous and difficult to measure because of the extremely rapid phagocytosis of the dead cells and their degradation beyond histological identification. Twenty-four hours after irradiation all macrophages had larger and increased numbers of secondary lysosomes together with very extensive membrane ruffling, reflecting the increased activity of tissue phagocytes and demonstrating that essentially all macrophages in haemopoietic tissues are involved in the recognition and phagocytosis of radiation-induced apoptotic cells. The distributions of nitrotyrosine and acid β-galactosidase positive cells confirms the presence of macrophage heterogeneity in control tissues and shows that following radiation exposure the majority of these cells induce these enzyme activities. The morphological identification of macrophage activation and increased macrophage enzyme activities were also associated with increased phagocytic activity (measured by respiratory burst assays), and with neutrophil infiltration.

The time and dose response experiments and irradiation of p53-null mice indicate that increased macrophage activities and neutrophil infiltration are a function of the apoptotic process, rather than being a direct consequence of radiation exposure. However, although there are significant reductions in tissue cellularity at doses up to 0.5 Gy, lysosomal enzyme activities were within the control range and significant increases in acid β-galactosidase activity were seen only at doses of 1 Gy and above. Furthermore, the greatest enzyme activity was not coincident with the initial increase in apoptosis (first seen at 2 h post-irradiation) and increased up to 24 h, during which time apoptosis had declined almost to basal levels. Thus, acid β-galactosidase activity is not simply related to either radiation dose or resultant cell loss. Neutrophil infiltration of the splenic red pulp was also observed at 6 h (coincident with the first increases in macrophage enzyme activity) and increased up to 24 h post-irradiation. As the resolution of inflammation is known to occur by apoptosis and rapid phagocytosis of neutrophils (Giles *et al*., 2000), the continued increase in enzyme activity in the red pulp might be due to ongoing phagocytosis of infiltrating apoptotic neutrophils. However, electron micrographs of tissues at 24 h post-irradiation showed only a few macrophages contained identifiable apoptotic bodies whereas many had secondary lysosomes containing degraded cellular material; this is not consistent with the phagocytosis of recently produced apoptotic cells, but the data are compatible with phagocytosis of apoptotic cells produced by the initial radiation damage resulting in persistent macrophage activation and neutrophil accumulation in the red pulp, a conclusion supported by a recent report of neutrophil infiltration in the thymus after irradiation (Uchimura *et al*., 2000). Whilst it would be expected that the haemopoietic cell death resulting from irradiation requires rapid phagocytic clearance, the increase in enzyme activity after phagocytosis, the length of time that activated macrophages persist in bone marrow and spleen and the significant neutrophil infiltration would not be expected (reviewed by Giles *et al*., 2000; Gregory, 2000).

The simplest interpretation of our findings is that the recognition and engulfment of large numbers of apoptotic cells post-irradiation by macrophages (and possibly other resident non-professional phagocytes that are recruited to deal with the increase in apoptosis) induces a cascade of signalling mechanisms within the phagocytic cells that in turn lead to the features of macrophage activation and neutrophil infiltration we have identified. This hypothesis is supported by the time course of acid β-galactosidase and nitrotyrosine accumulation. At 6 h post-irradiation only a few macrophages showed detectable staining, and these contained multiple apoptotic bodies. The proportion of positively stained macrophages increased over time and by 24 h, when very few apoptotic bodies were visible by light microscopy, there were further increases in positively staining macrophages, indicating considerable temporal heterogeneity in the increase in enzyme activities after phagocytosis of apoptotic cells post-irradiation.

A more conventional explanation for our findings would be that macrophage activation and neutrophil infiltration result either from direct effects of ionizing radiation on macrophages and/or from a failure to efficiently clear apoptotic cells leading to the presence of secondarily necrotic cells. These necrotic cells would accumulate to appreciable levels only when the phagocytic capacity of the tissue had been overwhelmed, such as when a large number of apoptotic cells were produced within a short time frame, as is the case following radiation exposure. Necrosis, unlike apoptosis, is well known to produce an inflammatory response, and this difference is one of the most commonly quoted characteristics that distinguish necrotic cell death from spontaneous apoptotic cell death *in vivo* (Wyllie *et al*., 1980). Studies *in vitro* have shown that phagocytosis of necrotic cells causes a pro-inflammatory response, whereas phagocytosis of apoptotic cells results in the production of both pro- and anti-inflammatory cytokines, providing a molecular mechanism to account for these differences (Giles *et al*., 2000; Gregory, 2000). In our experiments performed *in vivo*, analysis of apoptotic cells shows them to be clustered and within macrophages, but our histological studies cannot rule out that a few necrotic cells may be present. If increased macrophage activities were caused solely by phagocytosis of necrotic cells, macrophage activation and neutrophil infiltration should be directly related to the number of cells killed by irradiation for the equivalent number of macrophages. Analysis of different genetic backgrounds indicates that this is not the case, since the levels of

induced acid β-galactosidase in DBA/2 mice are intermediate between those of CBA/Ca and C57BL/6 mice, even though DBA/2 mice have the lowest apoptotic index of the three strains. Furthermore, there is a similar degree of neutrophil infiltration but different levels of apoptosis between C57BL/6 and CBA/Ca mice. These genotypic differences in apoptosis, induced macrophage activity and neutrophil infiltration demonstrate that the *in vivo* inflammatory-type response to radiation is not simply related to the number of apoptotic cells and therefore cannot be explained solely on the basis of secondary necrotic cells accumulating as a result of a macrophage clearance deficit.

The influence of genetic factors on macrophage responses following *in vivo* exposure to ionizing radiation is an additional important finding of our study. Phagocytes from C57BL/6 mice showed a low constitutive superoxide production that was not observed in cells obtained from CBA/Ca mice and this genotype difference was more marked after irradiation. However, phagocytes from CBA/Ca mice produced a greater rate and total amount of inducible superoxide both with and without irradiation than those from C57BL/6 mice. In both strains, prior irradiation reduced the maximal ability of cells to respond to PMA. In addition, acid β-galactosidase activity was higher in C57BL/6 mice, and was more induced in this strain after radiation. Furthermore, although both C57BL/6 and CBA/Ca mice showed increased nitrotyrosine staining after radiation, the levels were higher in C57BL/6 than in CBA/Ca, compatible with higher induction of NOS2 activity in the former strain. Previous studies *in vitro* have shown that irradiation potentiates the production of NO by macrophages when they are subsequently stimulated by lipopolysaccharide or interferon-gamma, by a process involving induction of tumour necrosis factor alpha (McKinney *et al*., 1998). NO is pleiotropic and its effects depend on concentration and target cell. Thus, NO can be either pro- or anti-apoptotic, and can either downregulate or upregulate p53 activity (Brune *et al*., 1996; Brockhaus and Brune, 1999). NO can also directly induce the DNA-dependent protein kinase, a key enzyme for repair of DNA damage resulting in enhanced protection to the direct toxic effects of NO and also protecting from subsequent DNA damaging agents (Xu *et al*., 2000). Finally, NO is well known as an immunoregulatory molecule and can be either pro- or anti-inflammatory (Nathan and Shiloh, 2000). Whilst it has been suggested that high dose irradiation may directly induce NO production by macrophages (Gorbunov *et al*., 2000), our data indicate that macrophage activation as a consequence of cell death contributes significantly to the production of NO *in vivo*.

The precise pathway(s) responsible for the observations of an inflammatory-type response following irradiation are unclear at the present time, but presumably result from the activation of macrophages following recognition and phagocytosis of apoptotic cells and the consequent production and release of cytokines and other stimulatory molecules. The recognition of apoptotic cells by macrophages is highly complex, and involves at least seven distinct molecular families. The outcome for the macrophage of apoptotic cell recognition is further complicated by cells being at different stages of apoptosis and by variation of phagocytic responses in different sub-populations of macrophages (Giles *et al*., 2000). Cellular interactions of macrophages involve adhesion molecules, cytokines, prostaglandins and glucocorticoids (Giles *et al*., 2000; Gregory, 2000) and there are multiple pathways by which macrophages may be activated, many markers used to define activation and an activated macrophage may not necessarily exhibit all such markers (Adams and Hamilton, 1992; Handel-Fernandez and Lopez, 2000). As haemopoietic tissues are complex due to the variety of cell types and heterogeneity of radiation responses, dissecting the cellular interactions and responses underlying our findings by the analysis of cytokine production and target cell response would present a major challenge. Furthermore, the conventional hypothesis that secondary necrosis or direct effects of ionizing radiation may induce macrophage activation cannot be ruled out. However, since genetic factors markedly influence macrophage responses, the solution to this problem is best investigated *in vivo* by genetic linkage analysis. Indeed, preliminary observations of (C57BL/6xCBA/Ca) F_1 animals have shown that the F_1 mice have an acid β-galactosidase response that is intermediate between the two parental strains, but is more similar to C57BL/6 than to CBA/Ca. Our ongoing analysis of N_2 animals (CBA/CaxF_1) has revealed a complex pattern of responses, with less than 10% of the animals showing an F_1-like response. These data indicate that at least three genetic loci probably contribute to the overall phenotypic response (Silver, 1995). In contrast, preliminary studies of the increased respiratory burst following radiation show that this phenotype is dominant for the CBA/Ca strain, indicating that the two responses to irradiation are controlled by different sets of genes. Studies using other inbred mouse strains also indicate that increased superoxide production and increased lysosomal enzyme activity 24 h post-irradiation in haemopoietic tissue are not linked. Whilst the respiratory burst is generally seen in conjunction with phagocytosis, dissociation of phagocytosis from the respiratory burst can be observed (Yamamoto and Johnston, 1984) and the data indicate that the strain-dependent differences in phagocytic cell function, as a consequence of apoptosis, reflect the complexity of factors that regulate macrophage biology.

In addition to demonstrating that an inflammatory-type response is an unexpected part of the response to ionizing radiation, our data are important for a fuller understanding of the long-term effects of radiation on haemopoietic and other tissues. Inflammatory responses can contribute to the development of leukaemia and may be particularly important in the development of radiation-induced leukaemias (Walburg

et al., 1968; Yoshida *et al*., 1993). There is also evidence that radiation-induced genomic instability can be induced by an indirect mechanism (Lorimore *et al*., 1998; Watson *et al*., 2000) and that in both haemopoietic tissue (Watson *et al*., 1997) and mammary epithelium (Ponnaiya *et al*., 1997), there is genotype-dependent expression of the instability phenotype. The finding of a more efficient apoptotic response to DNA damage in C57BL/6 than in CBA/Ca haemopoietic tissues is consistent with our previous findings of genotype-dependent apoptotic responses in irradiated urinary epithelium (Mothersill *et al*., 1999). Taken together, the data support the hypothesis that there is an inverse relationship between the more effective recognition of damage and expression of an instability phenotype. Our findings also provide a plausible bystander mechanism for the unexpected interactions between irradiated and unirradiated haemopoietic cells producing genomic instability both *in vitro* and *in vivo* (Lorimore *et al*., 1998; Watson *et al*., 2000) as activated macrophages are known to produce clastogenic factors via the intermediacy of superoxide and NO, and are able to produce gene mutations, DNA base modifications, DNA strand breaks, and cytogenetic damage in neighbouring cells, all features associated with radiation-induced chromosomal instability (Wright, 1998).

Materials and methods

Animals and irradiation

CBA/Ca, C57BL/6, DBA/2 and p53+/+ or −/− mice (Jacks *et al*., 1994) were used in this study in accordance with the guidance issued by the Medical Research Council and Home Office Project Licence Number PPL 30/1272. All mice were bred and housed on site. Mice were γ-irradiated at 0.45 Gy min^{-1} using a CIS bio international IBL 637 Caesium irradiator. Most experiments employed a whole body dose of 4 Gy γ-rays, a non-lethal but effective leukaemogenic dose (Mole *et al*., 1983).

Preparation of cell suspensions

Whole spleen cell suspensions were produced by mechanical dissociation through a steel gauze with 2.5 ml ice cold 10 mM phosphate buffered saline, pH 7.4 (PBS), followed by syringing through a 21G needle three times. Bone marrow suspensions were prepared by removal of both femurs, which were cut at either end to allow collection of cells and flushed three times using a 21G needle into a total volume of 2 ml cold HBSS containing 0.1% BSA. Tissue cellularity was assessed by diluting bone marrow and spleen suspensions in 3% acetic acid and counting nucleated cells with a haemocytometer. Reduction in tissue cellularity represents the decrease in total cell number of irradiated tissues compared to unirradiated controls.

Electron microscopy

Spleens were removed, divided into pieces of 0.2 cm maximum dimension and fixed in 3% glutaraldehyde for at least 1 h. Bone marrow plugs were prepared by expelling the bone contents by flushing. Tissues were post-fixed in osmium tetroxide before embedding in epoxy resin according to standard procedures. Ultrathin sections were stained with lead citrate and uranyl acetate and were examined using a Phillips EM208S transmission electron microscope. Macrophages were identified by their characteristic ultrastructural morphology and quantitated by counting the number of macrophages per unit area.

Histochemical detection of lysosomal enzyme activities

Acid phosphatase positive cells were identified in frozen sections of spleen and bone marrow plugs. Sections were dried at room temperature for 1 h or longer and fixed in 65% acetone; 8% formalin in citrate buffer, pH 3.6 for 30 s at room temperature, followed by washing in distilled water. Slides were incubated in a solution of napthol AS-B1 phosphoric acid and freshly diazotized fast garnet GBC in 0.1 M acetate, pH 5.2 (Sigma, UK), washed in distilled water and counterstained with haematoxylin. Acid β-galactosidase activity was identified by fixing frozen sections of adult tissues or whole embryonic fore-limbs in ice cold 0.5% gluteraldehyde in PBS for 15 min. Samples were washed in PBS containing 2 mM $MgCl_2$ and in detergent solution (40 mM citric acid/sodium phosphate, pH 4.0; 0.01% v/v NP-40; 0.01% w/v $C_{24}H_{39}O_4Na$; 100 mM NaCl; 2 mM $MgCl_2$), then incubated at 37°C in detergent solution containing 1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$, washed and counterstained with neutral fast red.

Quantitative analysis of acid β-galactosidase activity

An equal volume of red cell lysis buffer pH 7.4 (155 mM NH_4C1; 11.9 mM $NaHCO_3$; 0.1 mM EDTA) was added to 5×10^6 nucleated splenic or bone marrow cells suspended in PBS and left for 10 min on ice. The cells were washed twice in PBS and nucleated cells lysed on ice for 10 min in 1 ml 0.1% Triton X-100 in water. After spinning at 14 000 *g* for 10 min at 4°C, 800 μl of lysate was added to 200 μl of 5× assay buffer (40 mM citric acid/sodium phosphate, 100 mM NaC1; 2 mM Mg $C1_2$; pH 4.0). Two hundred μl of either 4 mg/ml ortho-nitrophenyl-β-D-galactopyranoside (ONPG) in 1× assay buffer or 1× assay buffer alone was then added and the samples were incubated for 1 h at 37°C before 300 μl 1 M sodium carbonate was added to stop the reaction. Samples were read in a spectrophotometer at 420 nm and the control value (minus ONPG) subtracted from the readings of the test samples to give the measured absorbance due to acid β-galactosidase activity.

Measurement of superoxide generation (respiratory burst)

Wells of a chemiluminescence microtitre plate (Dynatech Laboratories, Billinghurst, Sussex, UK) were coated with 200 μl of Hanks' buffered saline solution containing 20 mM HEPES, pH 7.4 and 0.1 g/100 ml BSA (HBSS/BSA; Life Technologies, Paisley, UK) overnight at 4°C then washed three times with HBSS. Bone marrow or spleen cells were suspended at 2×10^6/ml in HBSS/BSA and 100 μl of each sample was added to each of four wells. Fifty μl of luminol (Sigma, Dorset, UK) (134 μg/ml in HBSS/BSA) was added to each well. Finally, 50 μl HBSS/BSA ± 10 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) was added and chemiluminescence measured in each of the duplicate wells with or without PMA at regular intervals for up to 1 h in a Microlumat LB96P luminometer.

FACS and immunohistochemistry

Assessment of macrophage numbers was performed by FACS analysis of spleen and bone marrow cell suspensions. The F4/80 macrophage-specific rat monoclonal antibody (AMS Biotechnology, Oxon, UK) was diluted 1/10 in PBS containing 5% normal rabbit serum and 5% normal mouse serum (Harlan Sera-Lab Ltd, Leicestershire, UK) and 10 μl were added to 1×10^6 cells in PBS plus rabbit and mouse sera. Following three washes, FITC-conjugated affinity purified anti-rat immunoglobulins, pre-absorbed with mouse immunoglobulins (Vector Laboratories, Peterborough, UK) diluted 1/100 in PBS plus sera were added and incubated for 30 min. Cells were analysed by flow cytometry using a FACSVantage SE (Becton Dickinson, UK). Cells incubated in the absence of the primary F4/80 monoclonal antibody were used as a negative control to gauge levels of endogenous fluorescence and non-specific binding of the fluorochrome.

For immunohistochemistry, bone marrow plugs or bisected spleens were fixed in neutral buffered 10% formalin or in Methacarn solution (60% methanol; 30% chloroform; 10% glacial acetic acid), processed to paraffin wax and 4 μm sections cut for immunocytochemistry. Sections were incubated overnight at 4°C in the appropriate primary antibody. Macrophages were identified in Methacarn tissues using F4/80 diluted 1/100 in 5% normal rabbit serum. Nitrotyrosine was identified using an immunoaffinity purified rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA) diluted to 1 μg/ml in PBS containing 3% BSA. Neutrophils were identified using a polyclonal rabbit serum to myeloperoxidase (Dako, UK) diluted 1/2000 in 5% normal swine serum. Following washes in PBS, antigens were detected using biotinylated rabbit anti-rat or swine anti-rabbit immunoglobulins (Dako Ltd, UK) diluted 1/500 in PBS containing 3% BSA, followed by a pre-formed complex of avidin-biotin-peroxidase (Dako) each for 30 min at room temperature. Immunoreactive sites were revealed with hydrogen peroxide in the presence of diaminobenzidine and cell nuclei were counterstained with haematoxylin.

Quantitation of apoptosis and neutrophil infiltration

Apoptotic cells were quantitated in 4 μm sections of formalin fixed, paraffin processed tissues stained with haematoxylin and eosin. Apoptosis was identified by the characteristic morphological features of nuclear condensation, condensed chromatin margination at the nuclear periphery, cytoplasmic blebbing and nuclear fragmentation (Wyllie *et al*., 1980). Neutrophils were identified in haematoxylin/eosin stained paraffin sections by their characteristic nuclear morphology and by myeloperoxidase immunocytochemistry. Quantification of apoptotic cells or neutrophils was performed by counting the number of cells present in a minimum of 15 high power fields (at least 10 000 apoptotic or myeloperoxidase-positive polymorphonuclear cells) with the aid of an eyepiece graticule. Results are presented as the mean number per high power field $\pm$ the standard error of the mean. For acridine orange staining embryonic forelimbs were incubated in 1 μg/ml acridine orange in PBS for 30 min at 37°C washed in PBS and viewed by confocal microscopy (Wood *et al*., 2000).

Acknowledgements
The authors thank R Mitchell, S Macfarlane, J Gibbs, N Kernohan, M Boylan and R Pleass. The research was supported by grants from the Medical Research Council, the European Commission and the Leukaemia Research Fund.

References

Adams DO and Hamilton TA. (1992). *The Macrophage,* Lewis CE and McGee JOD (eds). Oxford University Press: Oxford, pp. 75–114.
Brockhaus F and Brune B. (1999). *Oncogene,* **18,** 6403–6410.
Brune B, Golkel C and von Knethen A. (1996). *Biochem. Biophys. Res. Commun.* **229,** 396–401.
Duhrsen U and Metcalf D. (1990). *Blood,* **75,** 190–197.
Giles KM, Hart SP, Haslett C, Rossi AG and Dransfield I. (2000). *Br. J. Haematol.,* **109,** 1–12.
Giralt SA and Champlin RE. (1994). *Blood,* **84,** 3603–3612.
Gorbunov NV, Pogue-Geile KL, Epperly MW, Bigbee WL, Draviam R, Day BW, Wald N, Watkins SC and Greenberger JS. (2000). *Radiat. Res.,* **154,** 73–86.
Greenberger JS, Epperly MW, Jahroudi N, Pogue-Geile KL, Berry LA, Bray J and Goltry KL. (1996). *Acta Haematol.,* **96,** 1–15.
Gregory CD. (2000). *Curr. Opin. Immunol.,* **12,** 27–34.
Handel-Fernandez ME and Lopez DM. (2000). *Macrophages A Practical Approach*. Paulnock DM (ed). Oxford University Press, pp .1–30.
Iyer R and Lehnert BE. (2000). *Arch. Biochem. Biophys.,* **376,** 14–25.
Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT and Weinberg RA. (1994). *Curr. Biol.,* **4,** 1–7.
Komarova EA, Diatchenko L, Rokhlin OW, Hill JE, Wang ZJ, Krivokrysenko VI, Feinstein E and Gudkov AV. (1998). *Oncogene,* **17,** 1089–1096.
Lehnert BE and Goodwin EH. (1997). *Cancer Res.,* **57,** 2164–2171.
Little JB. (2000). *Carcinogenesis,* **21,** 397–404.
Lorimore SA, Kadhim MA, Pocock DA, Papworth D, Stevens DL, Goodhead DT and Wright EG. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 5730–5733.
Lyng FM, Seymour CB and Mothersill C. (2000). *Br. J. Cancer,* **83,** 1223–1230.
McCann SR, Lawler M and Bacigalupo A. (1993). *Leuk. Lymphoma,* **10,** 419–425.
McKinney LC, Aquilla EM, Coffin D, Wink DA and Vodotz Y. (1998). *J. Leukoc. Biol.,* **64,** 459–466.
Mole RH, Papworth DG and Corp MJ. (1983). *Br. J. Cancer,* **47,** 285–291.
Mothersill C and Seymour C. (1997). *Int. J. Radiat. Biol.,* **71,** 421–427.
Mothersill CE, O'Malley KJ, Murphy DM, Seymour CB, Lorimore SA and Wright EG. (1999). *Carcinogenesis,* **20,** 2273–2278.
Narayanan PK, Goodwin EH and Lehnert BE. (1997). *Cancer Res.,* **57,** 3963–3971.
Nathan C and Shiloh MU. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 8841–8848.
Necas E, Sefc L, Sulc K, Barthel E and Seidel HJ. (1998). *Stem Cells,* **16,** 107–111.
Nomura T, Kinuta M, Hongyo T, Nakajima H and Hatanaka T. (1992). *J. Radiat. Res.,* **33** (Suppl.): 109–123.

Ponnaiya B, Cornforth MN and Ullrich RL. (1997). *Radiat. Res.,* **147,** 121–125.
Prise KM, Belyakov OV, Folkard M and Michael BD. (1998). *Int. J. Radiat. Biol.,* **74,** 793–798.
Silver LM. (1995). *Mouse Genetics. Concepts and Applications*. Oxford University Press: Oxford, pp. 195–263.
Uchimura E, Watanabe N, Niwa O, Muto M and Kobayashi Y. (2000). *J. Leukoc. Biol.,* **67,** 780–784.
Walburg HE, Congrove GE and Upton AC. (1968). *Int. J. Cancer,* **3,** 150–154.
Watson GE, Lorimore SA, Clutton SM, Kadhim MA and Wright EG. (1997). *Int. J. Radiat. Biol.,* **71,** 497–503.
Watson GE, Lorimore SA, Macdonald DA and Wright EG. (2000). *Cancer Res.,* **60,** 5608–5611.
Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW and Mitchell JB. (1998). *Carcinogenesis,* **19,** 711–721.
Wood W, Turmaine M, Weber R, Camp V, Maki RA, McKercher SR and Martin P. (2000). *Development,* **127,** 5245–5252.
Wright EG. (1998). *Int. J. Radiat. Biol.,* **74,** 681–687.
Wu L-J, Randers-Pehrson G, Xu A, Waldren CA, Geard CR, Yu ZL and Hei TK. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 4959–4964.
Wyllie AH, Kerr JFR and Currie AR. (1980). *Int. Rev. Cytol.,* **68,** 251–306.
Xu W, Liu L, Smith GC and Charles IG. (2000). *Nature Cell Biol.,* **2,** 339–345.
Yamamoto K and Johnston RB. (1984). *J. Exp. Med.,* **159,** 405–416.
Yoshida K, Nemoto K, Nishimura M and Seki M. (1993). *Leuk. Res.,* **17,** 437–440.
Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ and Hei TK. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 2099–2104.