

# Radiation Effects on Cultured Human Monocytes and on Monocyte-Derived Macrophages

By E. Stephen Buescher and John I. Gallin

Prior to administration, leukocyte transfusions are commonly irradiated with up to 5,000 R to eliminate lymphocytes and thereby prevent graft-versus-host disease in the recipient. It has been widely believed that phagocytes are resistant to this irradiation. In a recent report, we noted that phagocyte oxidative metabolism was compromised during preparation of white cells for transfusion. As part of our effort to examine the basis for this inhibition of phagocyte function during white cell preparation, we assessed the effects of irradiation on the long-lived monocytes that have been shown to persist at inflammatory foci posttransfusion. Human monocytes were irradiated for up to 3 min, receiving 2,500–5,000 R. This irradiation damaged human monocytes, significantly decreasing their *in vitro* survival for the first 3 wk of culture ( $p < 0.02$ , irradiated versus control survival), and growth as assessed

by two-dimensional cell size measurements during the first 2 wk of culture ( $p < 0.01$ , irradiated versus control growth). Despite smaller cell size, total cell protein was significantly increased over time in irradiated cultures ( $p < 0.001$ , irradiated versus control total protein per cell). Extracellular release of lysozyme and  $\beta$ -glucuronidase per cell was not affected by irradiation, but extracellular lactate dehydrogenase (LDH) release was significantly increased after irradiation ( $p < 0.001$ , irradiated versus control LDH release). Irradiated monocytes killed *Listeria monocytogenes* at a slower rate than the nonirradiated controls ( $p < 0.05$ , irradiated versus control rate of killing). Thus, the data indicate that irradiation in doses used to prevent graft-versus-host disease in leukocyte transfusion recipients has a deleterious effect on *in vitro* human monocyte survival and function.

**H**UMAN PERIPHERAL BLOOD phagocytes have generally been considered to be resistant to irradiation.<sup>1</sup> A recent study has shown that radiation doses in excess of 10,000 R have deleterious effects on polymorphonuclear leukocyte (PMN) functions, but lesser doses have negligible effects on bacterial killing, chemotaxis, and superoxide production (after high dose stimulation).<sup>2</sup> On the basis of this information, and because fatal graft-versus-host disease has rarely been reported after leukocyte transfusion,<sup>3</sup> phagocytes collected for transfusion are commonly irradiated with up to 5,000 R prior to transfusion to eliminate radio-sensitive lymphocytes and thereby prevent graft-versus-host disease in the recipient. Because it has recently been shown that transfused phagocytes, especially transfused monocytes, can persist in the recipient for at least 2 days, and that transfused monocytes retain the ability to mature into macrophages *in vitro*,<sup>4</sup> we evaluated the effects of *in vitro* irradiation on human blood monocytes and monocyte-derived macrophages.

## MATERIALS AND METHODS

Human mononuclear cells were separated by the method of Boyum<sup>5</sup> from heparinized whole blood donated by normal volunteers. After separation, mononuclear cell fractions were washed

twice in sterile phosphate-buffered saline (PBS) or Hanks' balanced salt solution with calcium and magnesium (HBSS w  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) (Duchland Laboratories, Inc., Denver, PA) and then resuspended in RPMI 1640 media (Grand Island Biologicals, Grand Island, NY) with supplemental glutamine, penicillin, streptomycin, and pooled 5% human AB serum (RPMI 1640/PSG5). The mononuclear cell suspension was then divided into two equal aliquots, and one aliquot was irradiated with either 2,500 or 5,000 rads, administered over 1.5–3 min in a Gammator M (Radiation Machinery Corporation, Parsippany, NJ) irradiating machine; whereas the second aliquot was allowed to sit at room temperature. Irradiated and nonirradiated cell aliquots were then planted in duplicate 25 sq cm or 75 sq cm sterile tissue culture flasks (Falcon Plastics, Oxnard, CA) or Teflon jars (Savillex Corp., Minnetonka, MN) at cell densities of  $5\text{--}15 \times 10^6$  cells per flask. Cultures were maintained at 37°C, in 5%  $\text{CO}_2$  atmosphere, with 100% humidity. When necessary, the number of mononuclear phagocytes in the mononuclear cell suspensions was determined by nonspecific esterase staining using commercially available reagents (Technicon Instruments Corp., Tarrytown, NY).<sup>6</sup> Cultures were fed approximately every 7 days by decanting spent RPMI 1640/PSG5 media and replacing it with fresh media. Control experiments showed that this schedule for maintaining cultures was better than biweekly feeding or using media containing 20% AB serum.

Spent media was saved from one series of experiments and frozen at  $-70^\circ\text{C}$  until enzyme assays were performed.

## Cell Counting

Cultures of human mononuclear cells were serially examined using a Zeiss ICM 405 inverted microscope linked to a computer-coupled television camera/image analyzer (Optomax ECV-1, Optomax Inc., Hollis, NH). Using the image analyzer, the number of cells in 7–15 fields in each flask were counted (fields typically containing 20–40 cells) at each time point. At the same sitting, the two-dimensional area of 20–30 cells selected at random in each flask was measured. Counts and measurements were made on the same flasks at 5–7 day intervals for up to 6 wk of culture. For some experiments, the two-dimensional area of fixed cells, flattened with a Cytocentrifuge (Shandon-Southern, Sewickley, PA), were measured using the Optomax ECV-1. In one experiment, the volume of live cells was

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measured using a fluorescent cell analyzer (Becton Dickinson FACS Systems, Sunnyvale, CA).

### Enzyme Studies

Lysozyme,  $\beta$ -glucuronidase, and lactate dehydrogenase (LDH) activities in spent media and whole cell lysates were determined by standard methods.<sup>7-9</sup> 5'-Nucleotidase activity on whole cell lysates was determined using the methods of Heppel and Hilmoe<sup>10</sup> to generate inorganic phosphate and the method of Ames and Dubin<sup>11</sup> to measure inorganic phosphate. Total cell protein was determined on whole cell lysates using the method of Lowry.<sup>12</sup> For data analysis, all enzyme activities and total cell protein values were expressed on a per cell basis.

### Cell Lysates

Cells adherent to the culture flask were rinsed twice with PBS, and after counting by the method outlined above, were lysed by 3 cycles of freeze/thawing. Cell lysates were kept frozen at  $-70^{\circ}\text{C}$  until assayed.

### Killing Assays

For monocyte killing assays, mononuclear cells were separated from whole heparinized blood, washed, and irradiated as described above. Cells were then placed in sterile Teflon jars and cultured as described above. After 0–7 days of culture, the cells were gently detached from the Teflon by pipetting, washed twice in sterile PBS, and the percentage of nonspecific esterase-positive cells determined. The number of nonspecific esterase-positive cells in the paired irradiated and nonirradiated cultures was adjusted to  $10^7$  cells/ml. *Listeria monocytogenes* was grown the night before in RPMI 1640 media, and on the morning of the experiment, organisms were harvested and washed 3 times in sterile ice-cold PBS. Organisms were adjusted to  $4 \times 10^7$ /ml. The killing assay was a modification of standard methods.<sup>13,14</sup> Briefly, to 0.5 ml of esterase-positive cells at  $10^7$ /ml was added 0.1 ml of bacterial suspension, 0.1 ml of fresh human AB serum, and 0.3 ml of HBSS, and triplicate samples were tumbled at  $37^{\circ}\text{C}$  for 210 min. At 20, 45, 90, 150, and 210 min,  $10\text{-}\mu\text{l}$  aliquots were removed from each tube and lysed in 10 ml sterile distilled water. One hundred microliters of the distilled water lysate was planted in warm agar in 35 mm  $\times$  10 mm disposable petri dishes (Falcon Plastics, Oxnard, CA) and the dishes placed in a  $37^{\circ}\text{C}$  incubator. After 48 hr of incubation, the number of bacterial colonies per plate was counted using the computer-coupled image analyzer. Killing experiments were carried out using paired irradiated and nonirradiated samples of cells.

### Statistics

Standard errors were used throughout as the estimate of variance, and the significance of differences between means was determined by Student's *t* test unless otherwise indicated.

## RESULTS

### Radiation Effects of Monocyte Survival

Following irradiation with either 2,500 or 5,000 rads, monocytes grown as adherent cells were shown to survive less well than paired nonirradiated controls over 6 wk of observation (Fig. 1). There was no difference between the survival of cells receiving 2,500 rads ( $n = 4$ ) or 5,000 rads ( $n = 7$ ) (not shown). Irra-

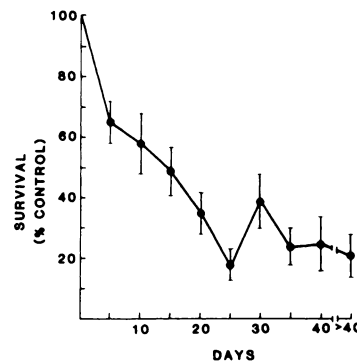


Fig. 1. In vitro monocyte survival after irradiation. Abscissa shows time in culture in days. Ordinate shows irradiated cell survival as percent of control cell survival. Symbols show mean  $\pm$  SE of 11 experiments, 7 with 5,000 rads, 4 with 2,500 rads.

diated cell numbers decreased most rapidly versus control during the first 3 wk following irradiation, being significantly less than control by 1 wk ( $p < 0.02$ ) and subsequently stabilized at approximately 30% of control for the remainder of the time observed. In one experiment examining survival of suspension grown nonadherent cells,  $65.3\% \pm 11.2\%$  (mean  $\pm$  SE) of monocytes survived in the irradiated cultures after 3–4 wk ( $p < 0.05$ , nonirradiated versus irradiated survival, paired sample *t* test). Similar decreased survival was seen in a second experiment, using elutriation-purified monocytes (80% esterase-positive) grown in Teflon jars. After 2 wk of culture, 23.2% of control monocytes and 5.7% of irradiated monocytes survived.

### Radiation Effects on Monocyte Growth

Adherent monocyte two-dimensional growth was inhibited equally by 2,500 rads and 5,000 rads of radiation (data not shown). Irradiated adherent monocytes increased their two-dimensional size more slowly than controls during the first 2 wk of culture (Figs. 2 and 3). In adherent cultures, both irradiated and control cell size stabilized after 2–3 wk, with the irradiated cells remaining approximately one-third smaller than control cells. In two experiments in which the two-dimensional area of nonadherent monocytes was examined after 3 wk of culture, similar differences were seen ( $1,529 \pm 82 \text{ sq } \mu$  versus  $1,075 \pm 45 \text{ sq } \mu$ ,  $841 \pm 65 \text{ sq } \mu$  versus  $502 \pm 68 \text{ sq } \mu$ ,  $p < 0.01$ , control monocytes versus irradiated monocyte areas). In one experiment, elutriation-purified monocytes, grown in Teflon jars, were examined at 7 and 14 days. After 7 days, 33.6% of the surviving irradiated monocytes increased their volume (versus 63.4% of control cells), whereas after 14 days, 29.9% of surviving irradiated monocytes had increased their volume (versus 37.1% of control cells).

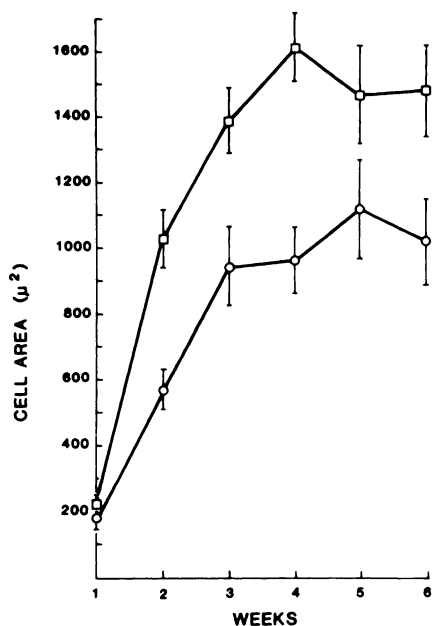


Fig. 2. In vitro monocyte growth after irradiation. Abscissa shows time in culture in weeks. Ordinate shows two-dimensional cell area in square microns. Symbols ( $\square$  control cells;  $\circ$  irradiated cells) show mean  $\pm$  SE ( $p < 0.01$ , irradiated versus nonirradiated mean cell size, paired sample t test).

#### Radiation Effects on Cell-Associated Proteins

Total cell protein was significantly greater in irradiated cultures than in nonirradiated cultures ( $p < 0.02$ , irradiated versus nonirradiated), with the ratio of irradiated to nonirradiated total cell-associated protein increasing with time in culture (Fig. 4). At 6 wk of culture, there was approximately fivefold more protein in the irradiated versus nonirradiated cells. In 2 experiments, no significant difference in cell-associated lysozyme,  $\beta$ -glucuronidase, or 5'-nucleotidase activity per cell could be found at 2 and 4 wk of culture, comparing paired irradiated and nonirradiated cul-

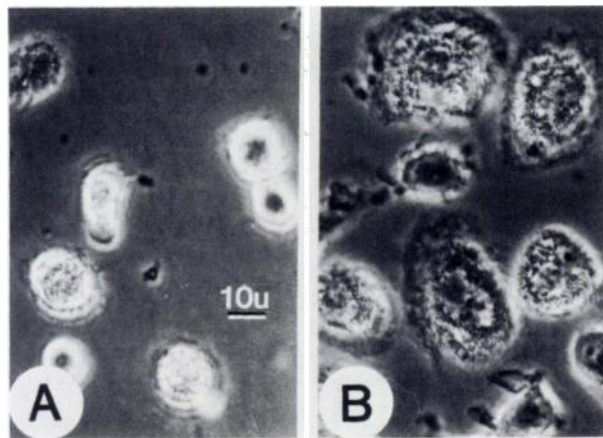


Fig. 3. Photomicrographs of 10-day-old human monocytes in culture. (A) Irradiated cells. (B) nonirradiated control cells.

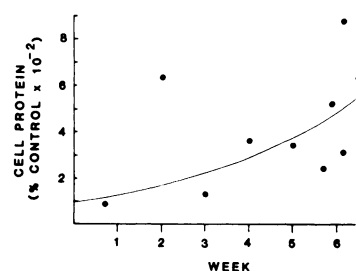


Fig. 4. Total cell-associated protein in irradiated monocytes. Abscissa shows time in culture in weeks; ordinate shows micrograms of protein per cell as percent of nonirradiated control values. The curve through the points is the linear regression of the logged data, demonstrating a significant increase in total protein over time, irradiated versus nonirradiated controls ( $p < 0.001$ , t test).

tures (data not shown). Similarly, radiation did not affect the quantity of extracellularly released  $\beta$ -glucuronidase or lysozyme per cell (Fig. 5). Although there was a tendency toward more lysozyme release per cell at 2, 3, and 5 wk in irradiated cultures, the differences were not significant. In contrast, the extracellular release of the cytosolic enzyme lactate dehydrogenase was increased in the irradiated cultures ( $p < 0.001$ , paired sample t test, irradiated versus nonirradiated cultures at all time points), reflecting cell death.

#### Radiation Effects on Killing of *Listeria monocytogenes* by Monocytes

In 10 experiments, irradiated monocytes were found over a 210-min period to kill *Listeria monocytogenes* less rapidly than paired unirradiated control cells (Fig. 6). Although the difference in killing was numerically small and in 3 experiments no inhibition of killing was seen, as a group the effect of radiation on the rate of monocyte killing was statistically significant, ( $p < 0.05$ , irradiated versus nonirradiated, one-tailed Student's t test).

#### DISCUSSION

Peripheral blood phagocytes have classically been considered to be relatively radioresistant human tissues.<sup>1,15</sup> Little doubt exists that they are more resistant than the cells of the lymphocytic series.<sup>15</sup> In vitro experiments using human PMN have demonstrated this resistance to functional damage at radiation doses of 10,000–25,000 rads delivered as a single dose.<sup>2,16</sup> Other human studies examining effects of in vivo irradiation on in vitro leukocyte function and metabolism could show no abnormality in intracellular metabolism<sup>17</sup> or bacterial killing<sup>18</sup> acutely. In contrast, some in vivo animal studies have demonstrated deranged microbial killing after single-dose, total animal irradiation.<sup>19,20</sup> Although all these studies addressed the effect of irradiation on the granulocyte or a mixed

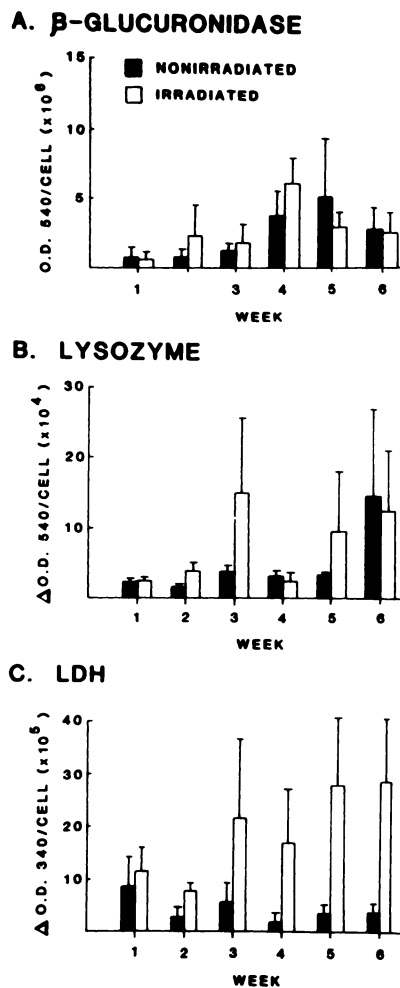


Fig. 5. Lysozyme,  $\beta$ -glucuronidase, and LDH release by irradiated and nonirradiated monocytes. Abscissa shows time in culture in weeks. Ordinate shows enzyme activity per surviving cell as  $\Delta$ OD<sub>540</sub> × 10<sup>4</sup> for lysozyme, OD<sub>540</sub> × 10<sup>8</sup> for  $\beta$ -glucuronidase, and  $\Delta$ OD<sub>340</sub> × 10<sup>5</sup> for LDH. Data are expressed as mean ± SE. Differences are not statistically significant for  $\beta$ -glucuronidase and lysozyme, but are significant for LDH release, irradiated versus nonirradiated cells ( $p < 0.001$ , paired sample  $t$  test).

leukocyte preparation, little has been said about the radiosensitivity of the human monocyte, including the reticuloendothelial system macrophage, except that the radioresistance of the monocyte is similar to that of the mature granulocyte.<sup>15</sup>

Recently, we reported that viable phagocytes, especially monocytes, transfused as therapy for a life-threatening infection in a patient with chronic granulomatous disease, could be found in the inflammatory exudate 2 days after administration.<sup>4</sup> In addition, we noted that PMN prepared by accepted methods for transfusion to this patient had a remarkable variability of their oxidative metabolism as assessed by nitroblue tetrazolium dye reduction, and the question was raised as to whether the irradiation of the leukocyte transfusion units before administration could partially

account for the noted variability in oxygen metabolism. Therefore, we thought it important to evaluate the effects of radiation in doses used to prevent graft-versus-host disease in leukocyte transfusion recipients on the survival, growth, and function of human monocytes and monocyte-derived macrophages.

The data clearly show that in vitro survival of adherent irradiated monocytes is significantly less than nonirradiated controls. Experiments done on suspension cultured monocytes showed similar decreased survival, making it unlikely that the decreased survival seen in adherent cultures was due to cell detachment rather than cell death. The number of irradiated cells decreased continuously over the first 3 wk following radiation, whether cells were adherent or in Teflon jar cultures. Because human blood monocytes do not multiply in culture, this decreased survival represented cell death rather than decreased cell multiplication. Similar decreased survival in vitro after irradiation<sup>21</sup> as well as decreased phagocytic activity<sup>22</sup> have been reported in mouse peritoneal exudate cells. Irradiated adherent cells did not increase their two-dimensional area at the same rate as control cells. In control experiments, we evaluated suspension cells to address whether the decreased size of irradiated cells reflected decreased spreading or decreased growth. Both the area and the volume of irradiated suspension-grown cells were found to be less than control cells, suggesting that the size differences seen were due primarily to decreased irradiated cell growth. After 3 wk, adherent irradiated cells maintained their size at approximately two-thirds the size of the nonirradiated controls. It is possible that the effects of irradiation on monocyte growth and survival were due to enrichment of cultures with radioresistant lymphocytes that suppressed subsequent monocyte growth, or to depletion in cultures of

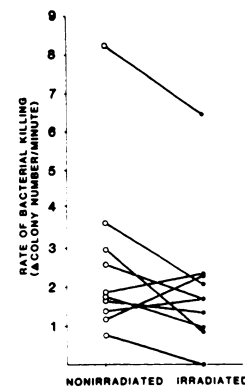


Fig. 6. Depression of rate of microbial killing (*L. monocytogenes*) by irradiation of human monocytes. Abscissa shows irradiated and nonirradiated test pairs. Ordinate shows log (slope of killing curve) as the rate of killing of *L. monocytogenes*. Decreased microbicidal activity due to irradiation is statistically significant ( $p < 0.05$ , one-tailed Student's  $t$  test) ( $n = 10$ ).

radiosensitive lymphocytes that stimulate growth. These possibilities were assessed using monocytes prepared by elutriation. Similar effects on cell growth and survival were seen with elutriated cells, indicating that the impaired growth of irradiated cells was unlikely to be due to depletion of a subset of lymphocytes. However, in these experiments, the cultured cell fraction still contained 20% lymphocytes, and therefore, with currently available technology, we cannot exclude the possibility that enrichment with radioresistant lymphocytes contributes to decreased monocyte growth and survival.

Because of the observed deleterious effects of radiation on the survival and growth of monocyte-derived macrophages, we evaluated the effects of radiation on other cellular parameters. Unexpectedly, the total protein per cell was greater in the smaller irradiated cells than in the controls at all time points, except the earliest one examined (5 days). The percentage increase in total cell protein rose with increasing time in culture to 500%–800% of control values at 6 wk in culture. To approach the question of whether the observed increases in total protein could be explained by increases in specific proteins, both the extracellular release and cellular content of lysozyme and  $\beta$ -glucuronidase, two granule-associated proteins in monocytes, were studied. The extracellular activities of these two enzymes were not significantly different on a per-cell basis in irradiated versus nonirradiated cultures. Additionally, in two experiments, the intracellular content of these two enzymes and 5'-nucleotidase was examined at 2 and 4 wk and was not found to be significantly different. Thus, the increased cell-associated protein levels found in the irradiated cultures were not explainable by differences in the granule enzymes lysozyme and  $\beta$ -glucuronidase or the surface ectoenzyme 5'-nucleotidase. As there are many different structural and functional proteins in monocytes besides lysozyme,  $\beta$ -glucuronidase, and 5'-nucleoti-

dase, we cannot say from these studies which proteins are responsible for the increased total cell proteins seen in our irradiated cultures. However, the increasing quantities of total protein per cell over time suggest persistent damage to the surviving cells after radiation. Supporting this concept of persistent damage postirradiation is the finding of significantly increased extracellular LDH activity over time in irradiated cultures. Control experiments showed that LDH was not degraded by the culture conditions between media changes, suggesting that the extracellular accumulation of this enzyme was due to persistent damage.

Although decreased *in vitro* growth, survival, and cytosolic enzyme leakage present a convincing argument for the deleterious effects of radiation on monocytes and monocyte-derived macrophages, they do not necessarily reflect the functional capabilities of the cells. Therefore, we also examined microbial killing by irradiated monocytes. Experiments carried out to assess postirradiation monocyte and monocyte-derived macrophage killing of *L. monocytogenes* did not show a major defect in total extent of microbial killing, but did demonstrate a small, but significant, decrease in the rate of killing. Thus, our data show that several important parameters of the human monocyte are damaged by radiation in doses of 2,500 or 5,000 rads—the doses used to prevent graft-versus-host disease in leukocyte transfusion recipients. The notion that monocytes are radiation insensitive cells should be discarded, and as monocytes may be functionally very important phagocytes delivered in leukocyte transfusion,<sup>4</sup> irradiation of transfusions before administration should not be considered innocuous.

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