

3-18-1988

Developmental and Radiobiologic Characteristics of Canine Multinucleated, Osteoclast-Like Cells Generated in Vitro from Canine Bone Marrow

T. M. Seed
Argonne National Laboratory

L. V. Kaspar
Argonne National Laboratory

F. Domann
Argonne National Laboratory

G. K. Niiro
Argonne National Laboratory

D. A. LeBuis
Argonne National Laboratory

Follow this and additional works at: <https://digitalcommons.usu.edu/microscopy>

 Part of the [Biology Commons](#)

Recommended Citation

Seed, T. M.; Kaspar, L. V.; Domann, F.; Niiro, G. K.; and LeBuis, D. A. (1988) "Developmental and Radiobiologic Characteristics of Canine Multinucleated, Osteoclast-Like Cells Generated in Vitro from Canine Bone Marrow," *Scanning Microscopy*. Vol. 2 : No. 3 , Article 36.

Available at: <https://digitalcommons.usu.edu/microscopy/vol2/iss3/36>

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Scanning Microscopy by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.



DEVELOPMENTAL AND RADIOBIOLOGIC CHARACTERISTICS OF CANINE MULTINUCLEATED,
OSTEOCLAST-LIKE CELLS GENERATED IN VITRO FROM CANINE BONE MARROW

T. M. Seed*, L. V. Kaspar, F. Domann,† G. K. Niro, and D. A. LeBuis

Radiation Hematology Group
Biological, Environmental, and Medical Research Division
†Division of Educational Programs, Student Research Participant
Argonne National Laboratory
Argonne, Illinois 60439-4833 (USA)

(Received for publication October 10, 1987, and in revised form March 18, 1988)

Abstract

We report here our initial observations on the growth and morphology, and developmental radiosensitivity of giant, multinucleated, osteoclast-like cells (MN-OS) generated through *in vitro* cultivation of hematopoietic progenitor-enriched canine bone marrow samples. Maximum cell densities of 5.5×10^3 to 6.5×10^3 MN-OS per cm^2 of growth area were achieved following 10 to 14 days of culture at 37°C. Acute gamma irradiation of the initial marrow inocula resulted in significant, dose-dependent perturbations of MN-OS formation, growth, and development. Attempts to estimate radiosensitivity of MN-OS progenitors from canine marrow yielded a range of D_0 values from a low of 212 cGy measured at six days of culture to higher values of 405 to 542 cGy following 10 to 22 days of culture. At the intermediate times of culture (10 to 14 days), the radiation-induced responses were clearly biphasic, reflecting either (a) the presence of multiple subpopulations of MN-OS progenitors with varying degrees of radiosensitivity or (b) the inherent biphasic nature of MN-OS development involving early progenitor cell proliferation followed by maturation and subsequent fusion. Morphologically, MN-OS generated from irradiated marrow inocula appeared only marginally altered, with alterations expressed largely in a biphasic, dose-dependent fashion in terms of smaller cell size, reduced number of nuclei, increased expression of both surface microprojections, and a unique set of crystalloid cytoplasmic inclusions. Functionally, MN-OS appeared to be impaired by irradiation of marrow progenitors, as evidenced by failure to initiate resorptive attachments to devitalized bone spicules *in vitro*.

KEY WORDS: Bone marrow, *in vitro* culture, monocytes, osteoclasts, radiosensitivity.

Introduction

The collection of basic information concerning the biological nature and function of osteoclasts, i.e., those giant, multinucleated cells that reside at the bone surface with primary bone resorptive functions and associated secondary hematopoietic interactions, has been hampered by lack of appropriate *in vitro* culture methods needed to facilitate functional analyses at the cellular and molecular levels (Barnes, 1987; Chambers, 1978; Gothlin and Ericsson, 1976; Hall, 1975; Patt and Maloney, 1972). Over the last several years, progress has been made in developing such culture methods for osteoclasts derived from various species, including those of primates (Burger et al., 1982; Ibbotson et al., 1984; Roodman et al., 1985a; Suda et al., 1983; Testa et al., 1981). To date, however, work with canine osteoclasts has not been reported.

In our previous attempts to establish *in vitro* long-term "Dexter-type" canine hematopoietic cultures (Coulombel et al., 1983; Dexter et al., 1977a,b; Gartner and Kaplan, 1980; Greenberg et al., 1981; Greenberger et al., 1979; Toogood et al., 1980), we observed, under a select set of culture conditions, the formation of giant, multinucleated, osteoclast-like cells (MN-OS). These cells appeared to be formed by the fusion of monocytic progenitors one to two weeks following plating and had many morphological, cytological, and ultrastructural properties of normal canine osteoclasts *in vivo*.

The aims of this paper are (a) to report our observations concerning the growth characteristics of these giant MN-OS under our newly established *in vitro* methods; (b) to characterize these cells both morphologically and ultrastructurally; and finally (c) to assess sensitivity of MN-OS progenitors to ionizing radiation.

Materials and Methods

Collection and Processing of Initial Bone Marrow Cell Inoculum

Bone marrow samples were collected and processed according to methods previously described (Seed et al., 1982). In brief, marrow

*Address for correspondence:
Thomas M. Seed,
Argonne National Laboratory
Argonne, IL 60439-4833 (USA)
Phone No.: (312) 972-3865

specimens were obtained from normal, healthy beagle dogs via needle aspiration of either humeri or iliac crest sites. The specimens (8-10 mL) were collected in 25-mL syringes containing 4 mL EDTA-saline solution (1.1% EDTA in 0.9% saline), diluted to 50 mL with Hank's BSS-serum wash [Hank's balanced salts solution (BSS) with 10% horse serum, 50 µg/mL streptomycin, and 50 U/mL penicillin] and centrifuged at 1000 x g for 10 min. The cell pellets were resuspended in Hank's BSS-serum wash solution, layered on a Ficol-Hypaque density gradient (1.077 g/L), and centrifuged at 400 x g for 30 min at 4°C. Following centrifugation, the light buoyant density marrow cells at the gradient interface were collected, washed once by centrifugation, and resuspended to 10⁷ cells/mL in a nutrient-complete, "long-term" hematopoietic culture medium (briefly described below). In all preparative stages, the cells were maintained on ice (<10°C).

Giant, Multinucleated, Osteoclast-Like Cell Cultures

Aliquots (1 mL) of the progenitor-enriched marrow cells were resuspended to a concentration of 10⁶/mL in a McCoy's 5A-based nutrient media, originally described by Greenberg et al. (1981) for the purpose of long-term culture of murine hematopoietic elements (Dexter et al., 1977a). In contrast to Greenberg's original media formulation, the media used in this study lacked the 10⁻⁶ M hydrocortisone additive. Similar to the observations of Suda's et al. (1983), our preliminary cell culture work suggested that hydrocortisone had a strong inhibitory activity on MN-OS formation (unpublished observation). In addition to its basal ingredients, the McCoy's 5A medium was supplemented with additional amino acids (2 mM L-glutamine, 16 µg/L L-asparagine, 8 µg/L L-serine, 0.8% essential (50X)- and 0.4% nonessential (100X) amino acids), 1 mM sodium pyruvate, antibiotics (0.1 g/L streptomycin, 10⁵ U/L penicillin), 2.2 g/L sodium bicarbonate, 25% heat-inactivated (56°C, 30 min) sera (12.5% horse serum, MA Bioproducts, Walkerville, MD, plus 12.5% fetal calf serum, Calbiochem, LaJolla, CA).

Following resuspension in nutrient medium, the cells were plated in tissue culture slide chambers having 1.6 mL volumes and 1.8 cm² surface growth areas (Lab-Tek, Miles Scientific). The cultures were incubated at 37°C in 5% CO₂ and >95% relative humidity for periods ranging from 1 to 28 days. Cultures were replenished biweekly with 50% fresh media. Cells/cultures were harvested at predetermined intervals dictated by the protocols of individual experiments.

Cell Growth

In order to develop growth curves, replicate cultures were set up and maintained as described above. Following specific periods of culture (0, 2, 6, 10, 14, 18, 22 days), cultures were sacrificed and processed for light microscopy by an initial saline wash followed by methanol fixation and Giemsa staining. The stained preparations were analyzed in terms of numbers of both mononuclear and multinuclear cells per unit of growth area. In general, 10

to 20 representative and defined microscopic fields per sample were counted (each field equals 1.96 x 10⁻³ cm² or 0.11% of the total growth surface, representing 1.1 to 2.2% of the total growth surface per sample analyzed), and cell yields were recorded.

Radiosensitivity Testing

Two-milliliter aliquots of the progenitor-enriched marrow cell suspensions (10⁷/mL) were placed into test tubes and gamma irradiated at 25 cGy/min to total doses of 200, 400, 600, 800, and 1000 cGy with a ⁶⁰Co irradiator (Atomic Energy of Canada, Ltd.). In selected experiments, additional aliquots of cell specimens were irradiated to 100 cGy as well. All irradiations were carried out at <10°C. Control samples (0 cGy) were handled similarly, but sham-irradiated. Following irradiation, the samples were resuspended in fresh nutrient medium and plated in culture vessels as described above. At selected times following initial plating (0, 2, 6, 10, 14, 18, 22 days), replicate cultures were sacrificed and processed for light microscopy and analyzed as previously described (for mononuclear/multinuclear cell yields per unit growth surface). Mononuclear and multinuclear cell yields were plotted against time of culture.

In attempting to estimate standard parameters of radiation sensitivity (D_0 , D_q , and n) for the generation of these giant MN-OS (from mononuclear progenitors), the numbers of MN-OS per field in the control samples (0 cGy) were determined at selected times of culture and equated to 100% survival. For each radiation dose group (i.e., 200-1000 cGy), the number of MN-OS generated (surviving) was determined and used to construct standard dose-response curves via linear regression/break point analyses. The response parameters of lethality rate (D_0), sublethal capacity (D_q), and subcellular target number (n) were derived from these analyses.

Electron Microscopy

For both transmission and scanning electron microscopic examination, replicate cultures were set up in the manner described above, except that the multichambered tissue culture vessels were backed with EM-embeddable, Permanox plastic slides (Lab-Tek, Miles Scientific). For transmission electron microscopy (TEM), 10 to 14 day-old cultures were initially fixed for 2-3 h at room temperature by adding 1 mL of a buffered glutaraldehyde fixative (1.25% glutaraldehyde, 2% sucrose, 0.05 M phosphate buffer, pH 7.2) to each of the culture vessels. The specimens were rinsed in buffer, post-fixed in 1% osmium buffered with 0.1 M phosphate buffer (pH 7.2) for 45 min, and washed again in buffer. Subsequently, the specimens were dehydrated in ascending concentrations of ethanol (30, 50, 70, 95, 100% ethanol), infiltrated initially with 1:1 Epon/ethanol, and finally embedded in 100% Epon (after several exchanges in 100% Epon) (Seed et al., 1977, 1979). Following curing at 60°C for 48 h, the specimens were freed from the backing plastic slides by a quick dip in liquid nitrogen. The specimens were then trimmed and reembedded for both cross-sectional and horizontal ("on-face") sectioning. In order to ensure

Canine Multinucleated Osteoclast-Like Cells, *In Vitro*

a representative sampling and viewing of the MN-OS in culture, multiple blocks of each specimen were cut, with multiple sections prepared and examined by a Siemens 101 TEM.

For scanning electron microscopy (SEM), replicate cultures were initially fixed in glutaraldehyde and washed in buffer as described above for TEM. The specimens were dehydrated in ascending concentrations of ethanol, then Freon-113, both at concentrations of 30, 50, 70, 95, and 100% for 5, 5, 5, 5, and 60 min, respectively. The specimens were then critical point dried by using a liquified Freon-13 system (Bomar Critical Point Dryer). Once critical point dried, the specimens were mounted on aluminium studs, borders were painted with silver paint and uniformly coated with gold by sputter coating (Denton Sputter Coater). Samples were examined with both Cambridge Stereoscan (Mark IIA) and JEOL 840 electron microscopes.

Functional Testing

In order to test the functional capacities of MN-OS derived from both irradiated and nonirradiated marrow preparations, devitalized bone spicules were initially incorporated into the culture vessels at the time of plating. Following 10 to 14 days of culture, the bone spicules were removed, fixed, and processed for both TEM and SEM, then subsequently analyzed by methods described above in terms of both (a) the morphology of MN-OS bone interactions and (b) estimates of numbers of adherent cells per unit of bone spicule surface area.

Results

Growth and Radiosensitivity

In control, nonirradiated cultures, the adherent mononuclear cell populations showed a biphasic increase in size: 6 to 14 days following plating, cell counts increased by approximately fourfold and subsequently increased by sixfold after 18 to 22 days of culture (Fig. 1). With increasing radiation dose to the initial marrow cell inocula, the extent of this proliferative response was sharply curtailed in a dose-dependent manner. At the highest dose (1000 cGy), the increase in mononuclear cell count was limited to twofold.

Giant MN-OS within control cultures (derived from nonirradiated marrow inocula) were rarely seen after two days of culture, but they rapidly increased in number reaching a maximum at 10 days (Fig. 2). Subsequently, MN-OS numbers declined and stabilized at approximately 60% of the peak value after 18 to 22 days of culture. Irradiating the initial marrow cell inocula with increasing doses of gamma rays not only reduced the overall number of MN-OS in a dose-dependent manner, but prolonged the time required to achieve these maximum levels (i.e., extended the "lag" period).

On the basis of the MN-OS growth curves shown in Fig. 2 and the cell counts at particular time points for the various radiation dose groups (0, 200, 400, 600, 800, 1000 cGy), dose-response curves for MN-OS progenitor survival were generated, and standard parameters of

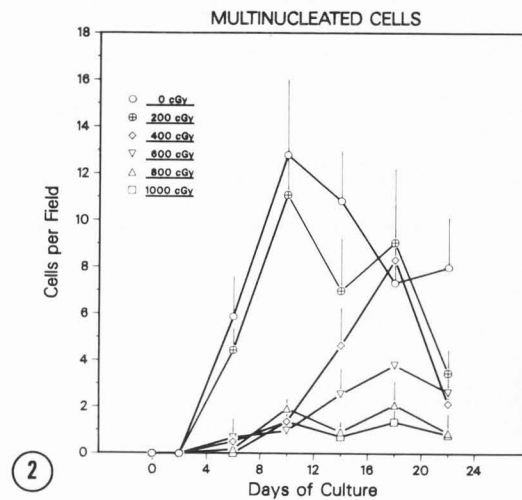
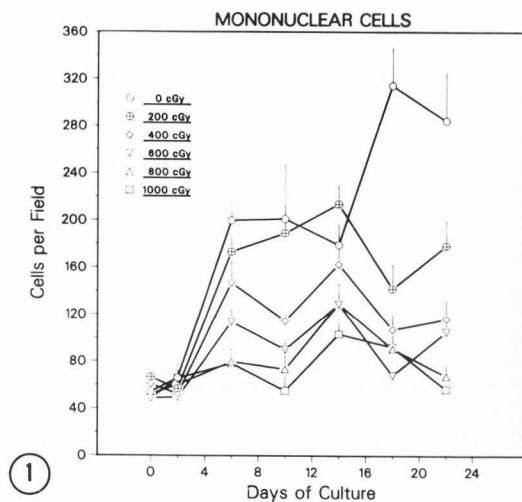


Fig. 1. A representative experimental series ($n=4$) of cultured progenitor-enriched marrow cells showing the change in the average number of adherent mononuclear marrow cells per microscopic field with time of culture and as a consequence of irradiating marrow cell inocula. Error bars represent the standard errors of the means.

Fig. 2. The same series ($n=4$) of cultured progenitor-enriched marrow cells as shown in Fig. 1, except showing the change in number of giant, multinucleated osteoclast-like cells as a function of the time-of-culture and radiation dose to marrow cell inocula prior to plating. Error bars represent standard errors of the means.

radiosensitivity were calculated. Beyond six days of culture, the generation of MN-OS was such that the apparent radiosensitivity of MN-OS progenitors irradiated just prior to the time of seeding (0 day) appeared to markedly decrease. With the use of linear-regression analysis, and the assumption of population homogeneity, the

calculated D_0 values increased from a low of 212 cGy at six days to markedly higher values ranging from 405-542 cGy following 10 to 22 days of culture. In contrast to the linear nature of the MN-OS survival response measured early in the culture period (six day), survival responses measured at latter culture periods commonly appeared biphasic. Such biphasic survival responses were most notable at 10 days of culture, but still evident at 14 days (Fig. 3). This suggested either (a) the presence of at least two responding subpopulations of MN-OS progenitors or (b) the presence of at least two cellular processes involved in MN-OS generation from mononuclear elements. Regardless of either possibility, the first subpopulation/process is clearly more radiosensitive than the second. When assessed at 14 days, the more sensitive subpopulation/process had estimated D_0 and D_q values of 230 cGy and 90 cGy, respectively, whereas the second, more resistant subpopulation process, had higher D_0 and D_q values of 575 cGy and 178 cGy, respectively.

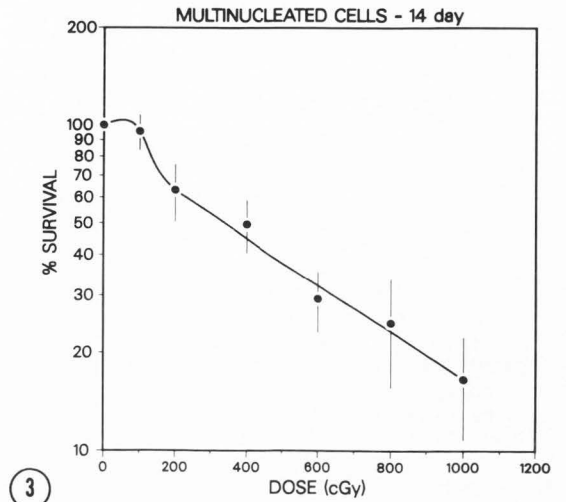
In addition to the effect of irradiation on MN-OS progenitors, in terms of the absolute MN-OS number generated, dose-dependent maturational changes in both cell size and the degree of nucleation were observed (Figs. 4 and 5). With both parameters (size and nucleation), the decline was more rapid at lower doses than at higher doses, with the transition occurring at about 400 cGy. Analyses of size vs. nucleation revealed that while both parameters were increasingly restricted with increasing radiation dose, nucleation was affected to a greater extent than cell size (Figs. 6 A and B).

Morphology and Ultrastructure

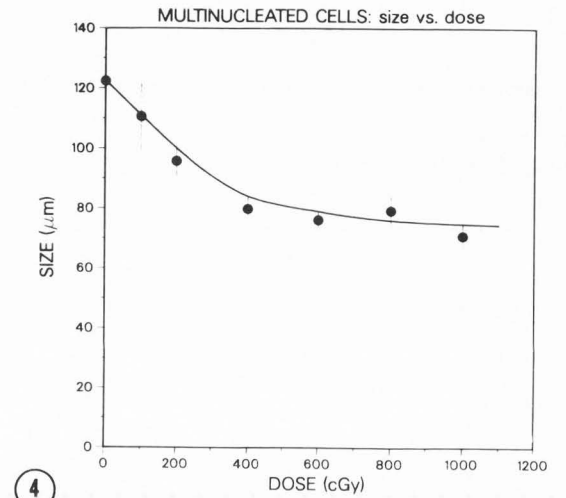
By light microscopy, representative MN-OS (generated from nonirradiated marrow progenitors) appeared as large, flat cells with broad cytoplasmic fringes and scalloped borders (Figs. 7 and 8). In contrast to the smaller, mononuclear (monocytoid) cells with diameters of ~ 15 μ m, these cells had an average diameter of $122 \pm 55 \mu$ m and an average number of 29 ± 28 nuclei per cell. Both the degree of nucleation and the nuclear position were variable features. However, smaller cells with a smaller number of more centrally located nuclei (6-15) were predominant.

A number of characteristic hallmarks of canine osteoclasts *in vivo* were routinely observed by TEM, including ruffled borders and localized peripheral surface areas with many microvilli, periplasmalemmal "clear" zones, vesiculated cytoplasmic regions, and numerous large mitochondria having well-defined cristae (Figs. 10-12). By SEM, the low-lying nature of these cells was highlighted (Figs. 9 and 13). The scalloped borders, as shown by light microscopy, were revealed by SEM as short, broad ridged ruffles, mixed at times with longer, more slender microvilli (Fig. 14).

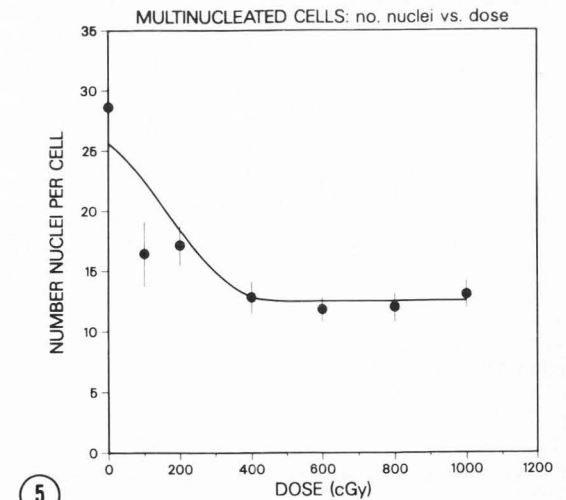
With increasing irradiation of the initial marrow inocula, MN-OS exhibited with increased frequency the following morphological alterations: reduction in mean cell size (diameters); reduction in mean number of nuclei per cell, along with an increased peripheralization of



3



4



5

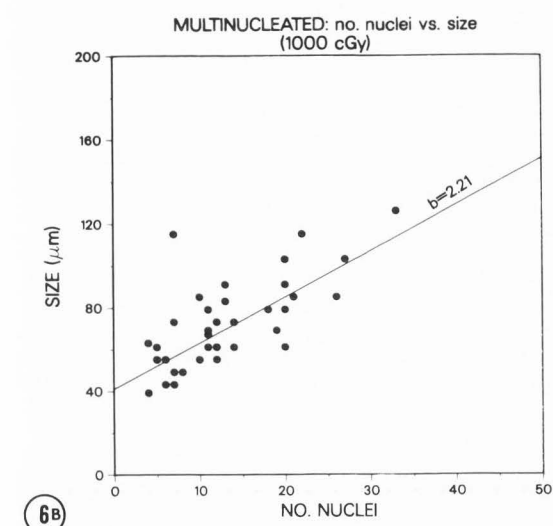
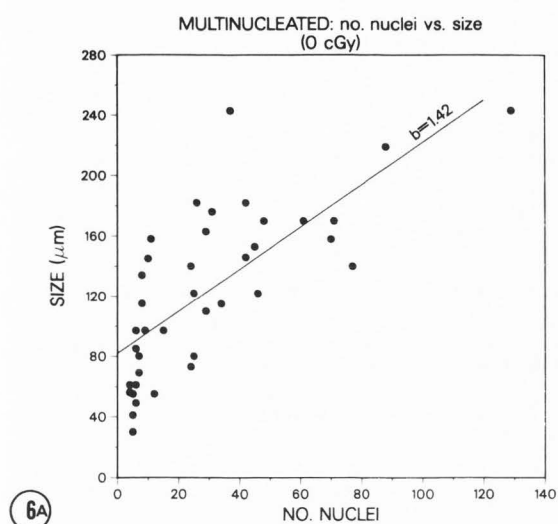
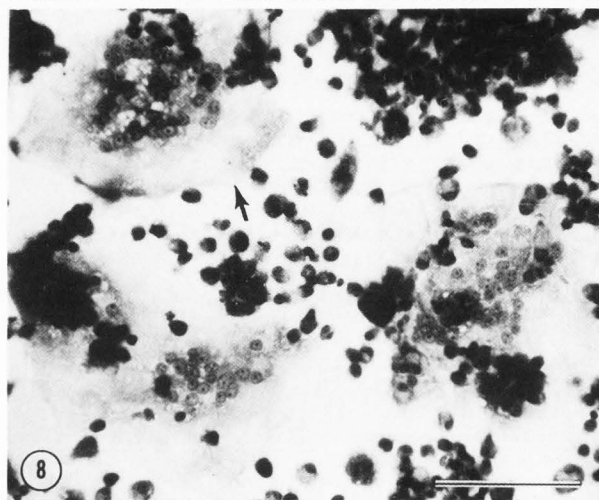
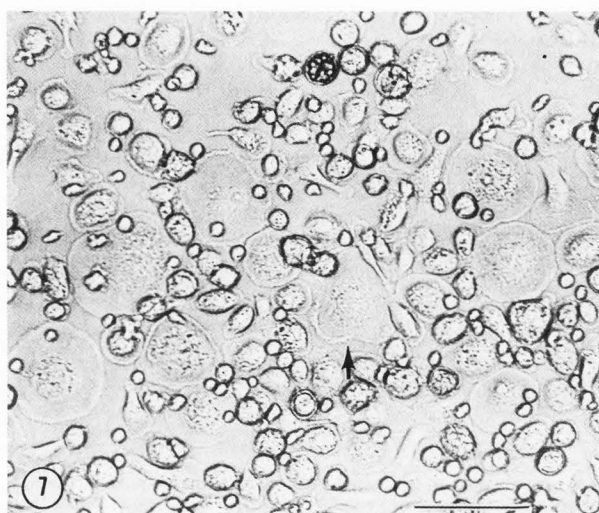


Fig. 3. The relationship of radiation dose and survival response for MN-OS progenitors assayed at 14 days of culture.

Fig. 4. Change in cell-size of the giant, multinucleated osteoclast-like cells following 14 days of culture as a function of radiation dose delivered to marrow cell inocula prior to plating. Error bars represent the standard errors of the means.

Fig. 5. Change in number of nuclei per cell assayed at 14 days of culture as a function of radiation dose delivered to marrow cell inocula prior to plating. Error bars represent the standard errors of the means.

Figs. 6 A and B. Relationship between number of nuclei and cell-size in 14-day-old cultures, seeded initially with (A) nonirradiated marrow cells or (B) acutely irradiated marrow cells (1000 cGy).



Figs. 7-9. Representative low-power micrographs of giant, multinucleated cells (arrows) of 14-day-old cultures generated from control, nonirradiated marrow cells and viewed by phase-contrast microscopy (Fig. 7), bright-field microscopy (Fig. 8), and SEM (Fig. 9). Line markers equal 100 μm , 100 μm , and 10 μm , respectively.

nuclei (Figs. 15 and 16); increased peripheral surface ruffling and microvillus expression (Figs. 17 and 19); and increased coalescence of large, membrane-bound crystalloid inclusions (Fig. 18). By comparison, the latter inclusions, although occasionally observed, were much smaller and fewer in number in MN-OS generated from nonirradiated progenitors.

Functionality

The capacity of MN-OS to adhere to devitalized bone spicules and, in turn, to show signs of bone resorption was tested. As is shown in Figs. 20 and 21, relatively large numbers of MN-OS from nonirradiated preparations readily adhered to the bone spicules, and many appeared to be in the processes of bone resorption (Fig. 22). In contrast, when the initial bone marrow inocula were irradiated, the resulting MN-OS (following 14 days of culture) were impaired, in a dose-dependent manner, in their capacity both to adhere and to initiate morphological signs of resorption. At the highest dose level tested, 1000 cGy, MN-OS adherence was virtually eliminated (Fig. 23).

Discussion

Two major conclusions can be drawn from the data presented. First, under a select set of in vitro culture conditions, subpopulations of bone marrow-derived monocytes from canines have the potential to proliferate and fuse, forming giant, multinucleated cells that share many common characteristics with osteoclasts in vivo. Second, the developmental processes involved in the formation of these giant, multinucleated cells are sensitive to the effects of ionizing gamma radiation.

Relative to the first conclusion, other workers have reported similar culture procedures, developmental patterns and morphological and functional features of giant MN-OS, derived from mononuclear marrow cells of a variety of species, including primates (Burger et al., 1982; Ibbotson et al., 1984; Roodman et al., 1985a,b; Scheven et al., 1986; Suda et al., 1983; Testa et al., 1981; Thesingh and Burger, 1983). We believe, however, that our observations for canine cells represent the first such report in this species.

Our initial observations of MN-OS generation in vitro were clearly fortuitous; they were based on a primary intent not to culture and analyze MN-OS, but rather to develop continuous, trilineal hematopoietic cell cultures for canine tissue. As a result, the culture described here is an amalgam of previously reported procedures used by workers attempting principally to culture human hematopoietic elements. For example, we modified basic "Dexter culture" protocol by incorporating a mix of sera at high concentrations (12.5% horse serum, plus 12.5% fetal calf serum), by not incorporating hydrocortisone into the medium, by refeeding the cultures every four days rather than weekly, and by incubating the cultures at 37°C rather than at 33°C (Coulombel et al., 1983; Dexter et al., 1977a; Gartner and Kaplan, 1980; Greenberg et al., 1981; Greenberger et al., 1979; Suda et al., 1983).

Figs. 10-14. Electron micrographs of representative giant, multinucleated cells generated in vitro from nonirradiated marrow progenitors following 14 days in culture. Fig. 10 shows the characteristic vesiculated cytoplasm (arrow) and ruffled borders. Fig. 11 shows a multinucleated cell with characteristically high surface densities of localized microvilli (arrow). Fig. 12 is a higher-power micrograph of a portion of a giant, multinucleated cell's cytoplasm, showing the details of the organelle-rich and organelle-poor regions. Note the presence of numerous large, elongated mitochondria (M) and the "clear zones" along the cell's periphery (arrows). Figs. 13 and 14 are SEM views showing typical surface features, both at low and high magnification, of representative giant, multinucleated cells. Line markers represent 5 µm, 5 µm, 2 µm, 10 µm, and 10 µm, respectively.

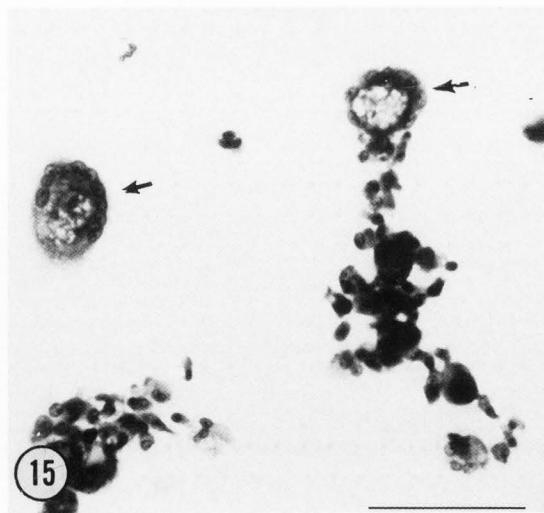
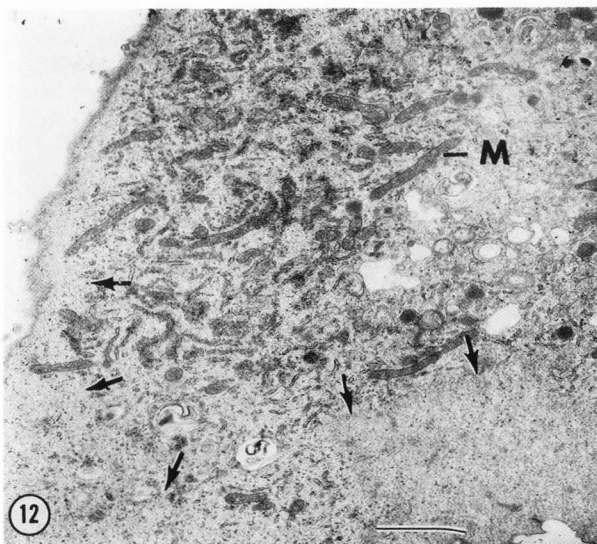
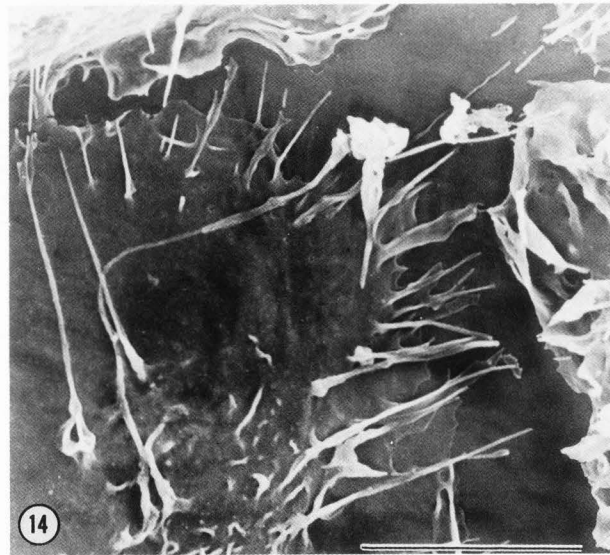
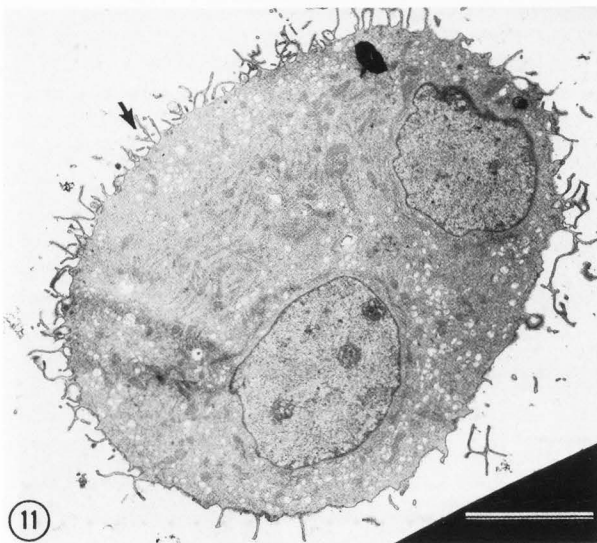
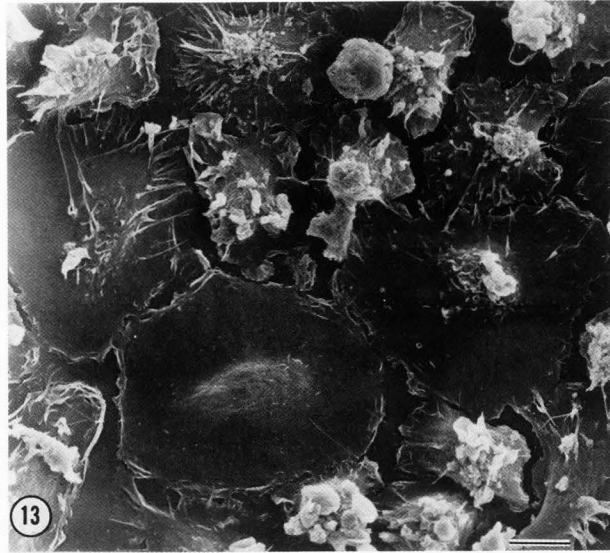
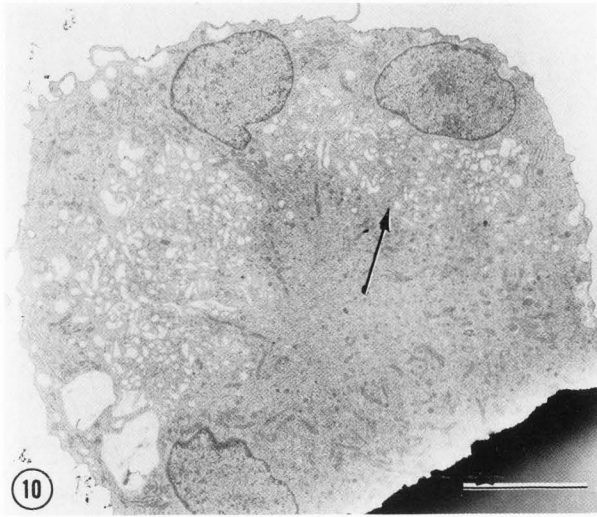
Fig. 15. Representative light micrograph (bright field, Giemsa-stained preparations) shows multinucleated osteoclast-like cells, generated in vitro from acutely irradiated (1000 cGy) inocula following 14 days of culture. Note the smaller size of cells (relative to those multinucleated cells generated from nonirradiated inocula), along with the marked peripheralization of nuclei (arrows). Line marker represents 100 µm.

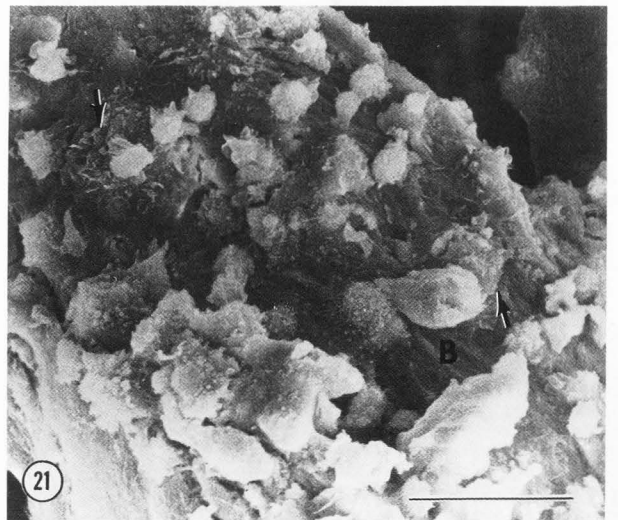
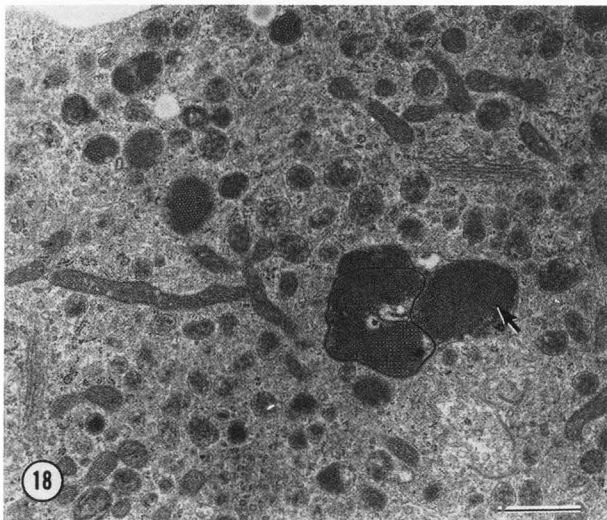
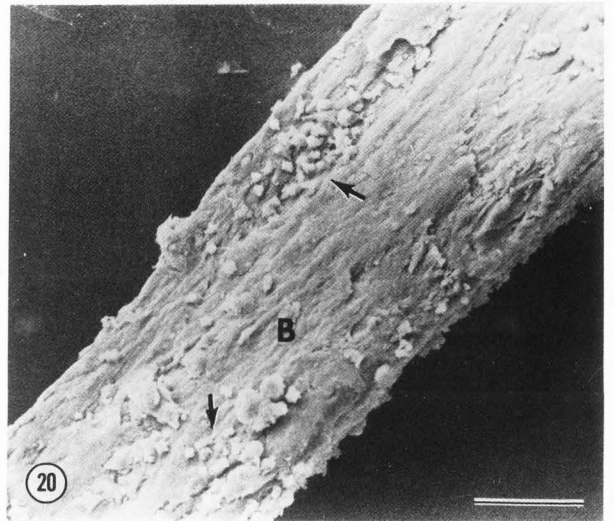
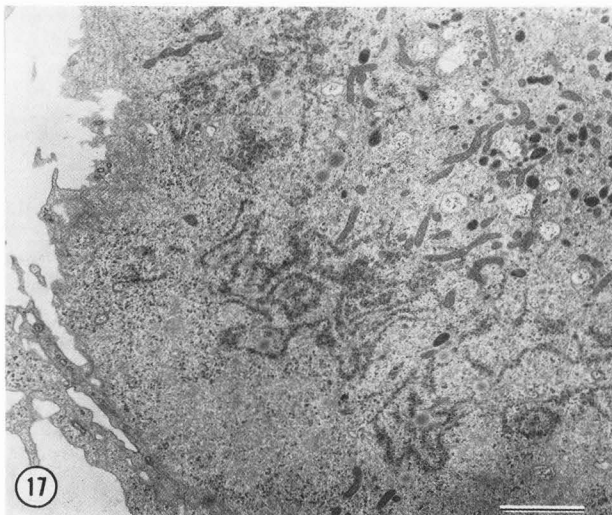
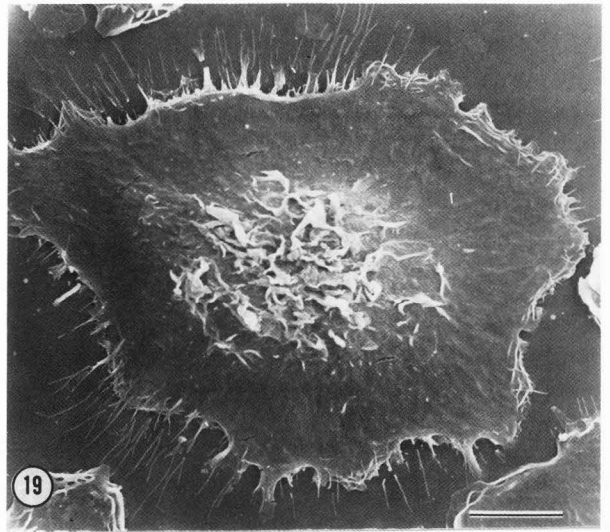
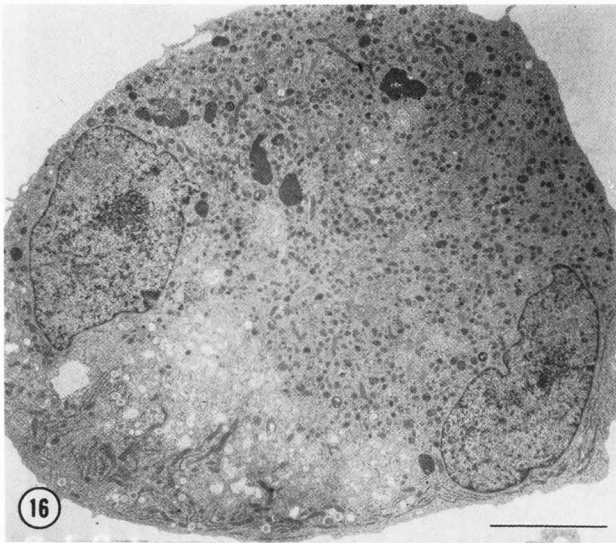
In preliminary experiments, all of these modifications enhanced the rate of MN-OS development in vitro; accordingly, they were routinely applied during the course of the experiments presented here (preliminary culture data are not shown).

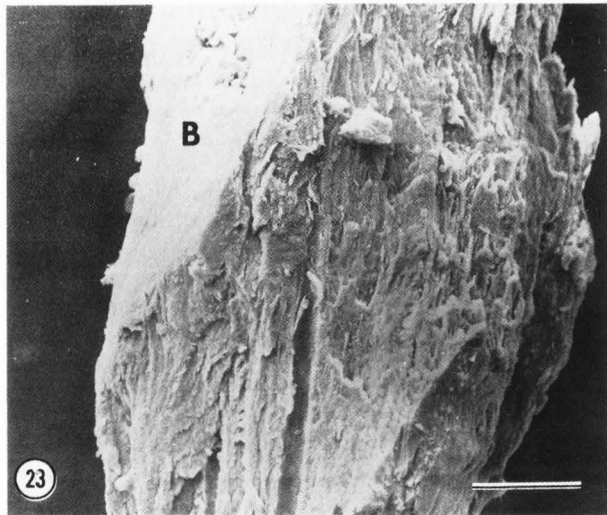
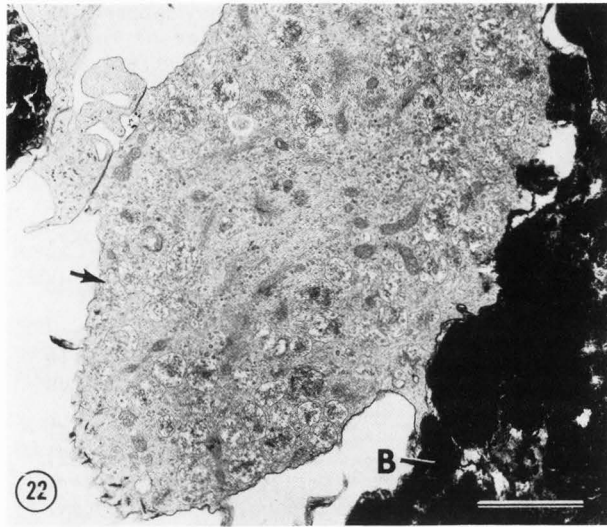
Developmentally, the canine MN-OS exhibit similar patterns to MN-OS of other species. Several groups (Ibbotson et al., 1984; Suda et al., 1983; Testa et al., 1981) working with feline marrow and standard "Dexter-type" hematopoietic culture conditions described the MN-OS growth curve in terms of an initial lag period of several days, followed by a progressive increase in numbers that reached maximum levels after 2-3 weeks of culture and a subsequent small decline in numbers. These MN-OS, generated in vitro, had sufficient numbers of morphological and cytochemical characteristics in common with osteoclasts occurring naturally in vivo to allow them to be classified as such (Ibbotson et al., 1984; Suda et al., 1983; Testa et al., 1981).

The recent immunocytochemical work of Oursler et al. (1985) has clearly demonstrated by using monoclonal antibodies, the antigenic relationship (and presumably the developmental relationship) between a unique subclass of monocytes, the giant, multinucleated cells with osteoclastic features, and normal osteoclasts found in situ. With the exception of the latter immunocytochemical characterizations, virtually all of the other osteoclast-specific features have been observed here for canine giant

Canine Multinucleated Osteoclast-Like Cells, In Vitro







Figs. 16-19. Electron micrographs of representative giant, multinucleated cells generated from surviving marrow cells 14 days following the initial plating of acutely irradiated marrow (600-1000 cGy). Figs. 16 and 17 show typical peripheralization of cell nuclei, along with marked regional differences in cytoplasmic features (e.g., organelle-rich vs. organelle-poor areas, vesiculated area, etc.). Uniquely large crystalloid, cytoplasmic inclusions are a prominent feature of these multinucleated cells from irradiated inocula (arrow, Fig. 18). Fig. 19 is an SEM view showing characteristically increased surface ruffling and microvillus expression. Line markers for Figs. 16-19 represent 5 μ m, 2 μ m, 1 μ m, and 10 μ m, respectively.

Figs. 20-23. Electron micrographs of devitalized bone spicules (B) and adherent giant, multinucleated cells (arrows) generated either from nonirradiated marrow progenitors (Figs. 20-22) or from acutely irradiated (1000 cGy) marrow progenitors (Fig. 23). Line markers represent 100 μ m, 50 μ m, 2 μ m, and 100 μ m, respectively.

multinucleated cells in culture. The more important of these features include (a) a general set of morphological descriptors (Chambers, 1978) (including large cell size, multiple round-to-oval nuclei that are generally clustered centrally along with cytoplasmic organelles, and a clear peripheral zone of cytoplasm that is bounded by scalloped or ruffled borders), (b) the presence of appropriate cytochemical features (Ibbotson et al., 1984; Minkin, 1982; Roodman et al., 1985a), most importantly, a strong, tartrate-resistant acid phosphatase activity following vitamin D3 induction (data not shown), (c) a collection of rather unique and identifying ultrastructural features (Ibbotson et al., 1984; Suda et al., 1983; Testa et al., 1981) including a high density of peripheral, surface-associated microvilli and the presence of large numbers of large, elongated, and often branched mitochondria within organelle-rich areas of the cell's cytoplasm, and (d) characteristic functional properties associated with bone resorption (adherence to devitalized bone spicules *in vitro*). Despite the latter, however, the "acid test" for osteoclastic activity, namely, the quantitative measure of resorptive capacity, remains to be tested (Boyde et al., 1985; Jones et al., 1986a, b).

The second major conclusion drawn from our observations is that MN-OS generation and development is sensitive to the effects of irradiation. Radiation-induced disturbances in MN-OS development take a variety of forms, including both time- and dose-dependent reductions in cell number, cell size, and the degree of nucleation. Our results showed that with increasing time of culture, radiosensitivity of the MN-OS progenitors apparently decreased; e.g., D_0 values (based on the assumption of dose-dependent, linear responses) increased from 212 cGy at the earliest sampling period (six days) to well over 500 cGy at the latest sampling period (22 days). These values are considerably higher than those reported by Scheven and colleagues, who employed a rather novel *in vitro* assay involving cocultivation of both irradiated and nonirradiated MN-OS progenitors with "periosteal membrane-stripped", 17-day-old metatarsal bones, with subsequent sampling and analysis of MN-OS development *in situ* (Burger et al., 1982; Scheven et al., 1987). With this latter technique, D_0 values for MN-OS progenitors derived from various murine tissue sources ranged from 94 cGy for MN-OS from 17-day-old fetal calvarium periosteal, to 101 cGy for adult bone marrow, to 169 cGy for fetal liver (15 day) (Scheven et al., 1987).

Clearly, the radiosensitivity of MN-OS progenitors is variable, dependent on both the nature of the source of MN-OS progenitors and on the culture assay conditions. In this regard, interesting dose response relationships were noted for the canine MN-OS progenitors assayed during the middle period of culture, 10 to 14 days following initial plating. The plotted survival response curves appeared to be biphasic. The presence of such biphasic responses would tend to indicate that the target cell population was mixed, composed of at least two subpopulations, each with distinct

radiosensitivities (Alper, 1979). The more sensitive of the two had an estimated D_0 value of 230 cGy, while the D_0 value of the more resistant subpopulation was 575 cGy.

Despite the suggestion that the expression of biphasic dose responses by irradiated MN-OS progenitors was due to the presence of multiple progenitor subpopulations, equally plausible, alternative mechanisms could account for such responses. For example, the biphasic nature of the dose response curve might have nothing to do with mixed populations, but rather might reflect radiation-induced disturbances of the two fundamental processes involved in MN-OS development. The first process, inherently more radiosensitive than the second, would involve progenitor cell proliferation essential for production of fusible mononuclear cells. The second, more resistant process, would involve the fusion of matured daughters of the mononuclear MN-OS progenitors. Accordingly, the apparent shift in radiosensitivity of MN-OS progenitors with time of culture might be explained in terms of the radiation-induced alterations of these two processes and their temporal patterns of expression. For example, when radiation-induced responses were assayed early (e.g., six days), the apparently increased sensitivity of MN-OS progenitors would be attributed to the radiation dose-dependent temporal delay in production of potentially fusible MN-OS progenitors, essential for MN-OS development. Conversely, the apparently marked decrease in radiosensitivity of MN-OS progenitors assayed during a late period of culture (22 days) would be attributed to a time-dependent renewal in proliferative activity following the initial radiation-induced lag of surviving MN-OS progenitors. This time-dependent renewal would yield increased numbers of potentially fusible mononuclear progenitor cells for generation of MN-OS.

In conclusion, MN-OS production appears to be highly dependent on two interactive, time-dependent processes. The first is a proliferative response that yields, under the constraints of time and radiation dose, an expanded number of potentially fusible mononuclear cells. The second is a fusion response, limited by numbers of potentially fusible progenitors and again by radiation dose. The two processes are linked by a time-dependent maturational process in which immature, nonfusible, proliferative progenitors mature into fusible, nonproliferative elements. This conclusion is implicit in the above-suggested mechanisms of radiation-induced disturbances in MN-OS development.

Acknowledgments

The authors acknowledge the assistance of David Tolle, Susan Cullen, William Keenan, and Craig Wardrip for their hematological and clinical services; Carol Fox for computer graphic services; Gordon Holmblad for assisting in irradiating marrow specimens; and Karen Haugen and Maria D'Arpa for editorial and secretarial assistance, respectively.

We gratefully acknowledge the support of the U.S. Department of Energy, Office of Health and Environmental Research, under contract No. W-31-109-ENG-38.

References

- Alper T (1979) Algebraic expressions for survival curves. In Cellular Radiobiology, Cambridge University Press, Cambridge, 44-46.
- Barnes BM (1987) Close encounters with an osteoclast. *Science* 236, 914-916.
- Boyde A, Ali N, Jones SJ (1985) Optical and scanning electron microscopy in the single osteoclast resorption assay. *Scanning Electron Microsc.* 1985; III:1259-1271.
- Burger EH, van der Meer JWM, van de Gevel JS, Gribnau JC, Thesingh CW, van Furth R (1982) *In vitro* formation of osteoclasts from long-term culture of bone marrow mononuclear phagocytes. *J. Exp. Med.* 156, 1604-1614.
- Chambers TJ (1978) Multinucleate giant cells. *J. Pathol.* 126, 125-148.
- Coulombel L, Eaves AC, Eaves CJ (1983) Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. *Blood* 62, 291-297.
- Dexter TM, Allen TD, Lajtha LG (1977a) Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. *J. Cell Physiol.* 91, 335-344.
- Dexter TM, Moore MAS, Sheridan PC (1977b) Maintenance of hemopoietic stem cells and production of differentiated progeny in allogeneic and semiallogeneic bone marrow chimeras *in vitro*. *J. Exp. Med.* 145, 1612-1616.
- Gartner S, Kaplan H (1980) Long-term culture of human bone marrow cells. *Proc. Nat. Acad. Sci. USA* 77, 4756-4759.
- Gothlin G, Ericsson JLE (1976) The osteoclast: Review of ultrastructure, origin, and structure-function relationship. In *Clinical Orthopaedics and Related Research*, Urist M (ed), JB Lippincott Co., Philadelphia, PA, 201-231.
- Greenberg HM, Newburger PE, Parker LM, Novak T, Greenberger JS (1981) Human granulocytes generated in continuous bone marrow culture are physiologically normal. *Blood* 58, 724-732.
- Greenberger JS, Skakeeny M, Parker LM (1979) *In vitro* proliferation of hemopoietic stem cells in long-term marrow cultures: Principles in mouse applied to man. *Exp. Hematol.* 7(Suppl. No. 5), 135-148.
- Hall B (1975) The origin and fate of osteoclasts. *Anat. Rec.* 183, 1-12.
- Ibbotson KJ, Roodman GD, McManus LM, Mundy GR (1984) Identification and characterization of osteoclast-like cells and their progenitors in cultures of feline marrow mononuclear cells. *J. Cell Biol.* 99, 471-480.
- Jones SJ, Boyde A, Ali NN (1986a) The interface of cells and their matrices in mineralized tissues: A review. *Scanning Electron Microsc.* 1986, IV:1555-1569.

Jones SJ, Boyde A, Ali NN, Maconnachie E (1986b) Variation in the sizes of resorption lacunae made *in vitro*. Scanning Electron Microsc. 1986, IV:1571-1580.

Minkin C (1982) Bone acid phosphatase: Tartrate-resistant acid phosphatase as a marker of osteoclast function. Calcif. Tissue Int. 34, 285-290.

Oursler MJ, Bell LV, Clevinger B, Osdoby P (1985) Identification of osteoclast-specific monoclonal antibodies. J. Cell Biol. 100, 1592-1600.

Patt HM, Maloney MA (1972) Bone formation and resorption as a requirement for marrow development. Proc. Soc. Exp. Biol. Med. 140, 205-207.

Roodman GD, Ibbotson KJ, MacDonald BR, Kuehl TJ, Mundy GR (1985a) 1,25-dihydroxyvitamin D3 causes formation of multinucleated cells with several osteoclast characteristics in cultures of primate marrow. Proc. Natl. Acad. Sci. USA 82, 8213-8217.

Roodman GD, MacDonald BR, Osdoby P (1985b) Demonstration of osteoclast-like cells in long-term human and baboon marrow cultures. Exp. Hematol. 13, 415.

Scheven BAA, Visser JWM, Nijweide PJ (1986) *In vitro* osteoclast generation from different bone marrow fractions including a highly enriched haematopoietic stem cell population. Nature 321, 79-81.

Scheven BAA, Wassenaar AM, Kawilarang, de Hass E, Nijweide PJ (1987) Comparison of direct and indirect radiation effects on osteoclast formation from progenitor cells derived from different hemopoietic sources. Rad. Res. 111, 107-118.

Seed TM, Tolle DV, Fritz TE, Devine RL, Poole CM, Norris WP (1977) Irradiation-induced erythroleukemia and myelogenous leukemia in the beagle dog: Hematology and ultrastructure. Blood 50, 1061-1079.

Seed TM, Chubb GT, Ulrich RG, Wright BJ, Kaspar LV (1979) Preparation and observation by SEM of hemopoietic cells cloned in soft agar. Scanning Electron Microsc. 1979, III:379-410.

Seed TM, Kaspar LV, Tolle DV, Fritz TE (1982) Hemopathologic predisposition and survival time under continuous gamma irradiation: Responses mediated by altered radiosensitivity of hemopoietic progenitors. Exp. Hematol. 10 (Suppl. No. 12), 232-248.

Suda T, Testa NG, Allen TD, Onions D, Jarrett O (1983) Effect of hydrocortisone on osteoclasts generated in cat bone marrow cultures. Calcif. Tissue Int. 35, 82-86.

Testa NG, Allen TD, Lajtha LG, Onions D, Jarrett O (1981) Generation of osteoclasts *in vitro*. J. Cell Sci. 47, 127-137.

Thesingh CW, Burger EH (1983) The role of mesenchyme in embryonic long bones as early deposition site for osteoclast progenitor cells. Dev. Biol. 95, 429-438.

Toogood IRG, Dexter TM, Allen TD, Suda T, Lajtha LG (1980) The development of a liquid culture system for the growth of human bone marrow. Leukemia Res. 4, 449-461.

Discussion with Reviewers

J. D. Szekely: Have you done any characterization of the cytoplasmic inclusions shown in Fig. 18? Were they ever seen in nonirradiated cells? Have they been reported in normal osteoclasts isolated from normal bone marrow?

Authors: In terms of characterizing these crystalloid inclusions, we have not gone beyond the TEM work done here. Regarding their prevalence, the larger of these inclusions appear restricted to the multinucleated osteoclast-like cells derived from irradiated progenitors. A similar but smaller sized inclusion has been observed occasionally seen in both MN-OS derived from nonirradiated marrow progenitors *in vitro* as well as from naturally occurring osteoclasts *in vivo*.

J. D. Szekely: Could you give more detail on how the survival curve parameters were calculated on the multicomponent survival curves such as the one shown in Fig. 3? How did these 14-day values (D_0 , D_q , n) compare to those calculated at other postirradiation times?

Authors: As noted in the Methods section, the numbers of osteoclast-like cells were manually counted by light microscopy per field in the various control and irradiated specimens at various times of culture (e.g., 0, 200-1000 cGy specimens at 6, 10, 14, 18, 22 days). Cell counts within the control specimens (0 cGy specimens) were equated to 100% survival and were used to estimate percent survival values for the irradiated specimens. For each culture time point, the percent survival values were plugged into a specially designed biphasic-response model computer program, which has the capacity to analyze response data either in terms of simple linear responses or multiphasic responses. Lethality rates (D_0) were estimated from the slopes of the response curves as $1/e \cdot D_q$ values, or quasi-threshold values for lethality responses, were estimated from the linear regression/breakpoint analyses (a) for the more radiosensitive subpopulation, by the radiation dose required to reduce survival below 100% and (b) for the more radioresistant subpopulation, the radiation dose required for expression of initial lethality events (i.e., the radiation dose at the "breakpoint" in the survival curves). N values or quasi-target number were estimated by the survival values at the Y -intercept following back extrapolation. It should, perhaps, be pointed out that such values for the more radioresistant subpopulation are at best rough estimates and are intended to serve only as points for comparison. They are based on a very limited number of data points, and the extended dose-response relationships need to be performed in order to fully verify these values.

Dose-response parameters (D_0 , D_q , n) for the two presumptive subpopulations of osteoclast progenitors were estimated as follows:

Mononuclear marrow progenitors
of osteoclast-like cells
Dose-Response Parameters^a

Days	Subpopulation #1			Subpopulation #2		
	D ₀	D _q	n	D ₀	D _q	n
6	212	42	1.22	-	-	-
10	147	228	4.69	587	288	1.09
14	230	90	1.47	575	178	0.93
18	458	347	2.12	707	1183	0.86

^a Parameter values given in cGy.

W. Chelack: In the work presented in this paper, the irradiations appear to have been done in growth media with high serum levels which is known to have large effects on D₀. Were irradiation experiments done HBSS or PBS for comparison? pH effects on D₀ are also well documented. Was pH controlled during irradiation?

Authors: All irradiations were carried out under the standard conditions as specified in the Methods section. Specific experiments designed to assess the effects of varying serum concentrations on the observed radiosensitivity of the MN-OS progenitor cell population were not carried out. Although such serum effects in modulating radiosensitivity of MN-OS progenitors would be of interest, it is doubtful that they significantly alter the overall developmental response patterns as noted here in this study. As to the question of pH, the pH of the suspending solutions were adjusted prior to irradiation (pH 7.2) and were maintained both during and following irradiation.

W. Chelack: Was culture of MN-OS attempted with conditioned media or growth factors at much lower cell numbers, e.g., 10³ - 10⁴/dish to see if colonies of these elements would grow? This would make radiation survival curves easier to interpret.

Authors: We agree. However, we did not attempt to supplement the MN-OS cultures with growth factors or conditioned media. The suggestion to specifically develop "clonal assays" for mononuclear MN-OS progenitors is a good one that will be pursued.

W. Chelack: Is the biphasic nature of the radiation survival curve due to differentiation, i.e., do MN-OS arise from common hematopoietic osteoclast stem cells and differentiate to either osteoclasts or blood elements?

Authors: We are working under the assumption that there are two fundamental processes involved in osteoclast formation: (a) clonal proliferation of a mononuclear osteoclast progenitor and (b) fusion of matured mononuclear cells into multinucleated giant cells. Therefore, in terms of the question asked - Yes, we do believe that the biphasic nature of the MN-OS survival curve rests, in part, with differentiative (maturational) processes.

M. Tavassoli: The most interesting finding is the biphasic survival curve of these cells, indicating a mixture of two populations. Alternatively, the inherent biphasic nature of development of these cells may be the explanation for this phenomenon. Do the authors have any comments on these observations?

Authors: If the question here is "Which alternative do we favor?", we would have to say the latter. We offer several reasons for saying this: first, other than the biphasic dose-response curves themselves, we have no other supporting evidence for the existence of multiple subpopulations of MN-OS progenitors; and secondly, the alternative explanation is decidedly more attractive by virtue of the substantial variation in the apparent radiosensitivity of MN-OS progenitors when time-of-culture is considered. If multiple subpopulations of MN-OS progenitors truly exist, then the dose-response relationships should remain constant over the time course. As they do not, other processes have to be brought into consideration (e.g., developmental processes).

M. Tavassoli: How do the authors intend to further explore these two alternative explanations for survival data?

Authors: If the biphasic dose-response curves are due to the interactions of two distinct developmental processes, then, by "splitting" the radiation dose over the time course of osteoclast cell development, we should be able to effectively separate the two developmental processes (i.e., the initial clonal proliferative response from the secondary maturation and fusion step). Alternatively, a second, more direct approach might be to conduct a rigorous series of cell fractionations of MN-OS progenitor-enriched marrow preparations in an attempt to isolate distinct subpopulations with varying radiosensitivities.

Z. Somosy: What is the authors' evidence for the homogeneity of their model cell culture?

Authors: The homogeneity of the osteoclast progenitor cell population clearly remains an open question. In evaluating the data stemming from the irradiation of freshly isolated, marrow-derived mononuclear progenitors, we attempted to "fit" the survival data with both simple linear and bilinear responses. In the case of the former, "homogeneity" was presumed. Other than the biphasic nature of the dose-response curves determined at sampling times > 6 days, we have no strong evidence to argue for or against homogeneity.

Z. Somosy: What is the probable role of the membrane bound crystalloid cytoplasmic inclusions? Why do they accumulate after irradiation?

T. D. Allen: Would the authors like to speculate on the origin of the crystalloid inclusions observed after irradiation? Are the multinucleated cells still phagocytic or do the inclusions arise as a result of radiation damage directly within the cell?

Authors: At this point, we really do not know the nature of these curious crystalloid inclusions nor do we know the role they play in the radiation-elicited responses. These inclusions appear larger and more numerous in the MN-OS cultures seeded with heavily irradiated MN-OS marrow progenitors (e.g., 1000 cGy). It is possible that these inclusions represent coalesced primary lysosomal vacuoles containing crystalline arrays of degraded cellular material damaged as a consequence of the irradiation insult.

T. D. Allen: In the radiation sensitive formation of these multinucleate cells, would you like to speculate on the cytoplasmic elements which may become damaged by radiation with respect to subsequent fusion, as nuclear phenomena are unlikely to be involved. Have the authors data, for instance, to indicate whether or not putative fusion becomes limited by reduced mobility as a result of irradiation?

Authors: Concerning the nature of the radiosensitive subcellular target(s) responsible for the process of cell fusion, we speculate that the "target" is not a single physical entity, but, rather, lies in the inherent biophysical nature of the lipid bilayer, in particular is its fluidity and mobility of embedded proteins islands. As the latter are critical in the process of cell fusion, it is reasonable to assume that restricted cell fusion, as a dose-dependent function of ionizing radiation exposure, is a consequence of such alterations in bilayer fluidity and the subsequent restricted lateral mobility of cell surface, charge-bearing transmembrane proteins within the bilayer.

As to the question relating to cell mobility as a function of radiation dose, we have not made such determinations, although, clearly, it would be of interest, especially in terms of limiting cell fusions.

T. D. Allen: In our own cultures from cats, there were often a few fibroblastic cells visible in the culture--does this occur with these cultures?

Authors: Fibroblastic cell growth in these cultures was rarely seen.